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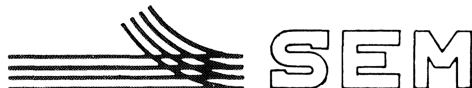

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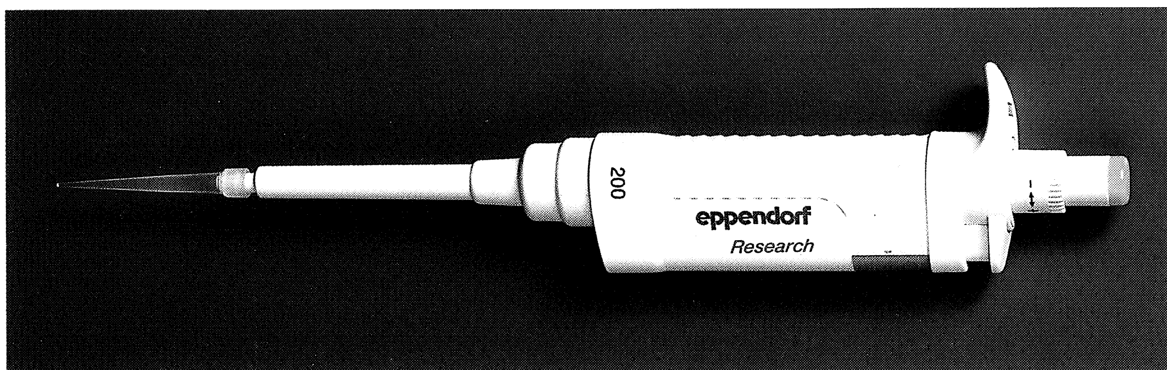

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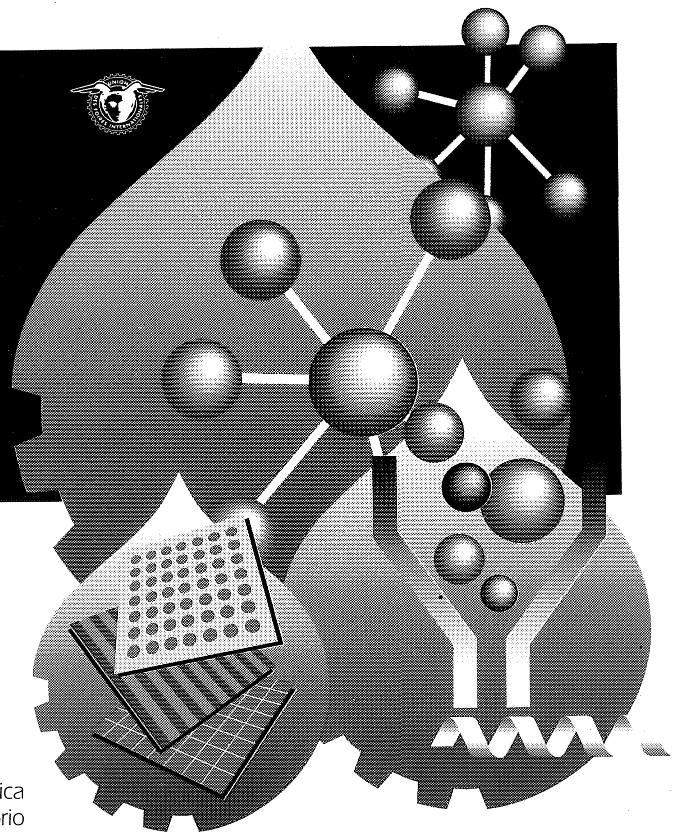

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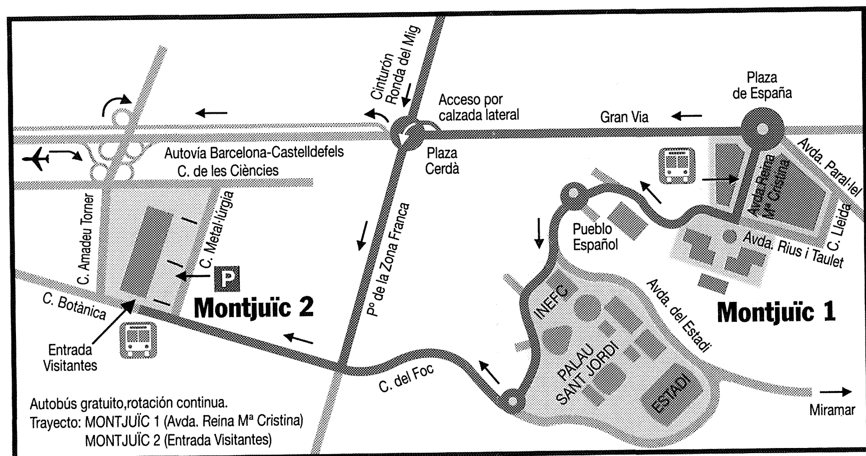

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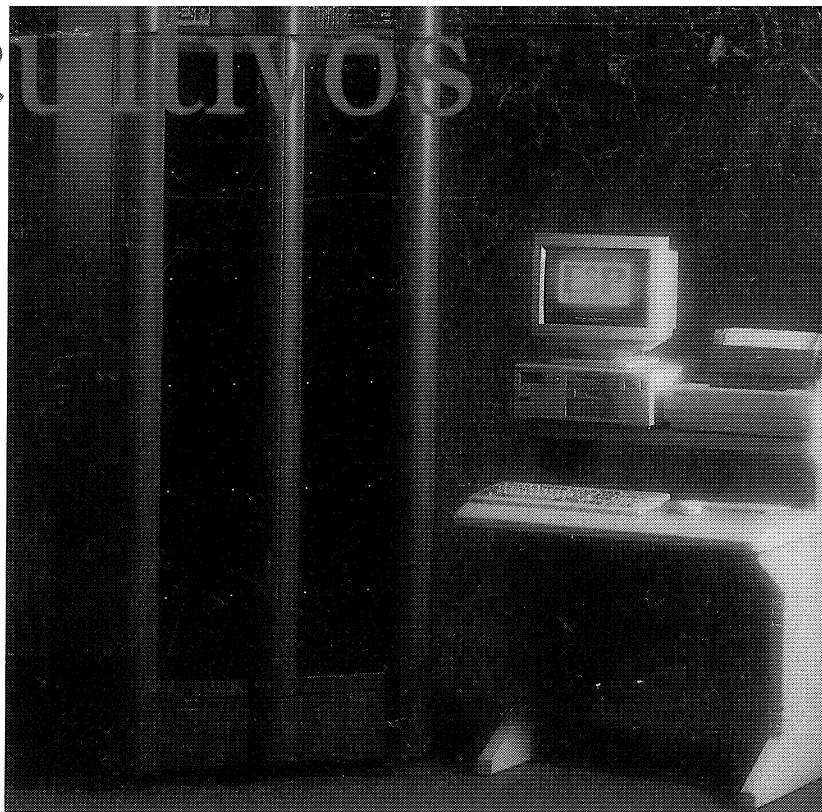
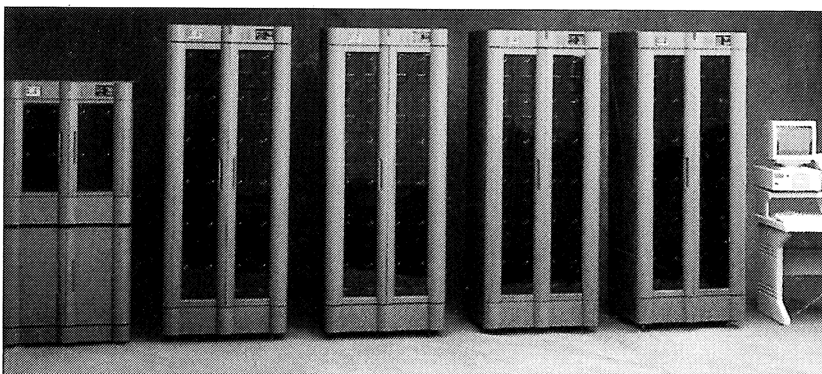


LA NUEVA TECNOLOGIA CONDUCE A UNA MAYOR RAPIDEZ

ESP ofrece lectura contínua, no invasiva, de los hemocultivos con automatización total y monitorización tanto de consumo como de producción de cualquier gas.



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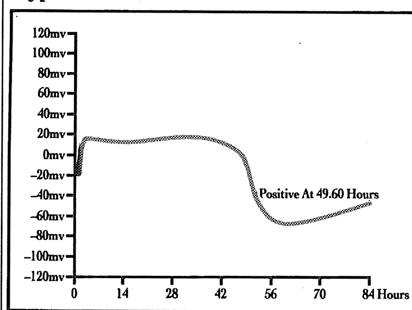

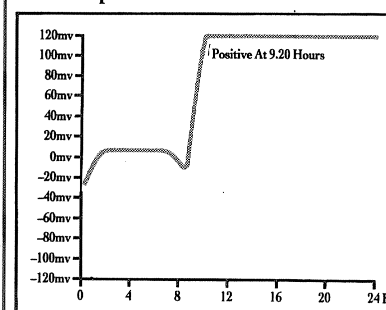

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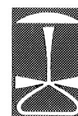

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MICROBIOLOGÍA SEM **12** (1996), 163–166

Editorial*

La ciencia en América Latina

La población de los países de América Latina, con un total de 450 millones de personas, supera la población de los Estados Unidos o de la Unión Europea. Sin embargo, la contribución de los autores latinoamericanos a la publicación científica mundial es del 1,4%, mientras que los autores de la Unión Europea representan el 27,7% y los de Estados Unidos el 35,8% del total mundial (*World Science Report 1993*, UNESCO).

La producción científica de América Latina es también baja si tomamos como referencia el producto interior bruto (PIB): el PIB del conjunto de países de América Latina es de 715 mil millones de dólares. La Unión Europea y Estados Unidos, que contribuyen con 20 y 26 veces más publicaciones, tienen un PIB sólo 6,8 y 7,5 veces mayores, respectivamente, que el de aquellos países.

Aun así, la desproporción en la producción científica de América Latina no debe atribuirse a una menor capacidad intelectual o de temperamento, sino a razones económicas. La fracción del PIB invertida en Investigación y Desarrollo (I+D) en América Latina es del 0,45% de promedio, mientras que en la Unión Europea y Estados Unidos alcanza el 2,0% y el 2,9%, respectivamente. (España invierte en I+D algo más del 0,90% de su PIB, cifra que está muy por debajo del promedio de la Unión Europea, pero que es el doble de la de América Latina. Los únicos países de la Unión Europea que invierten un menor porcentaje de su PIB que España son: Irlanda, Grecia y Portugal; Italia, que es el país con la proporción más baja de los restantes, invierte el 1,6%, casi el doble que España.)

**Microbiología SEM* prosigue en este editorial el análisis de la situación actual de la investigación científica en América Latina. En el número anterior presentamos la visión de Moselio Schaechter (San Diego State University) y Cristián Orrego (San Francisco State University) sobre la investigación en microbiología en diversos países latinoamericanos. En el próximo número ofreceremos el comentario de Harlyn O. Halvorson (University of Massachusetts, en Darmouth) sobre algunos aspectos del desarrollo actual de la microbiología en Cuba.

La correlación entre la inversión en I+D y la producción científica de un país es casi exacta, como he observado analizando los datos publicados por la UNESCO, los del Institute for Science Information (ISI) de Filadelfia y otros disponibles. El caso de España puede servir de ejemplo. De 1982 a 1992, la inversión española en I+D pasó del 0,48 al 0,90% del PIB. El número de artículos se cuadruplicó durante el mismo período, pasando de 3900 a 14.000, un aumento que se extiende prácticamente a todas las disciplinas científicas. Dicho aumento lo es también en términos relativos. La producción científica española representaba menos del 1% de la mundial en 1984; mientras que en 1992 representó el 2%. Con respecto a la producción científica de Estados Unidos, la española pasa del 2,5 al 5,1% durante ese período; y con respecto a la de Alemania, del 16 al 30%.

No se trata sólo de un aumento numérico, sino también cualitativo. El "impacto" de las publicaciones españolas (es decir, el número de citas por artículo en revistas internacionales) pasa de 3,53 a 4,13 por artículo citado en siete años (del quinquenio 1981–1985 al quinquenio 1988–1992), lo cual supone un aumento del 17%. Durante el mismo período, el impacto de las publicaciones de otros países aumenta un 10% en Italia, 11% en Gran Bretaña y 14% en Francia. Sólo Alemania, entre los países europeos, aumenta más rápidamente que España, el 21% durante ese período.

Análisis como el que acabo de presentar son importantes para convencer a los gobiernos de la necesidad de invertir más en I+D si quieren aumentar la cantidad y calidad de la producción científica de sus países. Pero es posible avanzar un paso más en la justificación de la inversión científica haciendo notar las consecuencias económicas que se derivan de tal inversión.

El presidente Clinton, en el documento que define la política científica de su gobierno (*Science in the National Interest*, agosto 1994), ha destacado que "La tecnología engendra el crecimiento económico: crea nuevas industrias y puestos de trabajo, y mejora nuestro nivel de vida. La ciencia es el motor de la tecnología". Federico Mayor Zaragoza, director general de la UNESCO, ha escrito a su vez que "La ciencia y la tecnología han desempeñado el papel decisivo en el desarrollo económico y social de nuestro siglo" (Prefacio, *World Science Report 1993*). Un ejemplo altamente significativo de las repercusiones económicas de la inversión científica viene de los países recientemente industrializados de Asia, los llamados "cinco tigres" (Corea, Hong Kong, Malasia, Singapur y Taiwán). En dos décadas pasaron de invertir el 0,1% del PIB en I+D a invertir el 1,6%; al mismo tiempo, pasaron de ser países subdesarrollados a convertirse en potencias industriales.

La situación actual de América Latina no puede decirse que sea halagüeña. En las décadas de 1950 y 1960, muchos de los gobiernos latinoamericanos crearon consejos nacionales de ciencia y desarrollo, dedicados a promover y financiar la investigación científica; varios países crearon además ministerios de ciencia y tecnología. Consiguientemente, la inversión científica aumentó en las universidades y aparecieron institutos y centros de investigación creados para satisfacer objetivos específicos. Algunos de estos institutos y centros alcanzaron al poco tiempo un gran nivel, y pudieron ser considerados centros de excelencia.

El ritmo de inversión en la investigación científica siguió acelerándose durante la década de 1970 y principios de la siguiente en países como Argentina, Brasil, Chile, Colombia, México y Venezuela. Este proceso fue facilitado por una rápida expansión económica, financiada en buena parte por préstamos de los bancos mundiales. Sin embargo, hacia mediados de la década de 1980 la expansión cesó, como consecuencia de las dificultades encontradas por los gobiernos para pagar la enorme deuda. El estancamiento continúa.

Podemos citar varios ejemplos. Brasil alcanzó el 1,0% de la inversión del PIB en I+D en 1982; en 1985 había bajado a menos del 0,6%, que fue también la inversión de 1992. En Argentina, la inversión era algo más del 0,4% del PIB en 1980, pero menos del 0,4% en 1993. En México, el máximo se produce en 1981, cuando la inversión fue casi del 0,45% del PIB, mientras que en 1994 permanecía en el 0,4%. Una excepción importante es el caso de Chile: de 1980 a 1982 la inversión era aproximadamente el 0,4%; en 1989 llegó al 0,5%; y en 1993 al 0,8% del PIB.

La situación está probablemente a punto de cambiar, debido por una parte al liderazgo del presidente Clinton y de su gobierno, que están influyendo sobre los gobiernos latinoamericanos para que reconozcan el impacto que la inversión en I+D tiene en la economía y en la educación de un país. Como consecuencia, al menos parcialmente, de la influencia de la American Association for the Advancement of Science (AAAS), el presidente Clinton decidió incluir la cooperación en ciencia y tecnología en el orden del día de la cumbre de presidentes de los países del hemisferio. Esta cumbre fue convocada por Clinton en diciembre de 1994, en Miami, y el objetivo general de la reunión era estimular el desarrollo económico y el intercambio comercial. (El autor de este editorial desempeñó en este caso un papel significativo, como presidente de la AAAS y, debido a tener acceso a los niveles más altos del Gobierno, como miembro del Consejo de Asesores de Ciencia y Tecnología del presidente Clinton.)

Una decisión de la cumbre de presidentes fue que los ministros de ciencia y tecnología de los países del hemisferio se reunieran a intervalos regulares y frecuentes, para fomentar la cooperación en el desarrollo de la ciencia y la tecnología. La primera reunión de ministros fue planeada para celebrarse los días 28 y 29 de marzo de 1996 en Cartagena de Indias (Colombia).

Diversos científicos y organizaciones influyentes están, además, actuando de manera concertada para influir en sus gobiernos. Ejemplos notables son la Asociación Colombiana para el Avance de la Ciencia y su presidente, el físico Eduardo Posada, y el presidente de la Academia de Ciencias de Chile, Jorge Allende, ambos con acceso a los presidentes de sus países respectivos, Samper y Frei. En Brasil, dos ministros, el de ambiente y el de ciencia y tecnología, han tenido carreras científicas distinguidas. En México, la bióloga Julia Carabias es la ministra ("secretaria") de Medio Ambiente y Recursos Naturales, y el ministro de Salud es Juan Ramón de la Fuente, que fue decano de la Facultad de Medicina de la UNAM (Universidad Nacional Autónoma de México) y ha sido recientemente elegido presidente de la academia de ciencias (Academia Mexicana de la Investigación Científica).

No obstante, el progreso científico de los países de América Latina se presenta arduo, en el mejor de los casos. Las dificultades económicas de muchos de los países del hemisferio llevan a los gobiernos a percibir la inversión científica como un lujo, al menos la que va más allá de proveer la educación popular y la de técnicos y expertos. Existen, además, problemas estructurales que dificultan el progreso científico.

Un problema estructural es la ausencia de inversión en I+D del sector privado. La participación del sector privado en la inversión I+D representa un 50% en la Unión Europea, y supera el 60% en Estados Unidos. Sin embargo, en América Latina es prácticamente nula. (La falta de inversión del sector privado es la tónica dominante también en España y en algunos otros países de la Unión Europea, como Irlanda y Grecia.) Otro problema estructural en América Latina es que la I+D se lleva a cabo casi exclusivamente en instituciones estatales. Por el contrario, en Estados Unidos, aunque el gobierno paga más del 50% de los gastos de investigación, sólo el 6% se lleva a cabo en centros públicos. (La situación española es, también en este apecto, semejante a la de América Latina, aunque no de un modo tan acusado.)

El tercer problema estructural concierne al número limitado de individuos preparados para participar en los proyectos de I+D. La proporción de científicos e ingenieros por cada mil habitantes es aproximadamente de 4 en Estados Unidos y de 2 en la Unión Europea, mientras que en América Latina es de 0,5 (en España es de 1,4). En números absolutos, el total de científicos e ingenieros en México es 16.679, mientras que en Estados Unidos es 949.200, más de 50 veces mayor, aunque la población estadounidense sea sólo el cuádruple de la mexicana. La deficiencia en el número de científicos e ingenieros refleja, sin duda, la reducida inversión en I+D, y tiene consecuencias que no se pueden corregir a corto plazo. Aun si los gobiernos de América Latina decidieran aumentar inmediatamente su inversión en I+D, pasarían varios años antes de que se formase a un número proporcional de expertos.

La ciencia es una actividad acumulativa y universal, sin delimitaciones creadas por las fronteras internacionales. El avance científico de un país o de una región contribuye al avance de la ciencia en el mundo entero. Poco después de ser elegido presidente de la AAAS, a principios de 1993, instauré un programa para el avance de "la ciencia en el hemisferio occidental", en el cual están participando activamente representantes científicos de casi todos los países latinoamericanos. La razón para justificar la inversión de Estados Unidos en este programa es su posición geográfica. España tiene también una razón especial para comprometerse en el progreso de la ciencia en América Latina, que se deriva de su historia, lengua y cultura compartidas. Un ejemplo de lo que puede hacerse por America Latina, incluso sin disponer de recursos económicos, es la iniciativa de *Microbiología SEM* y de la Sociedad Española de Microbiología (a la cual felicitamos al cumplirse sus cincuenta años de existencia) de dedicar sus editoriales de este año a la situación de la investigación en aquellos países. Que la investigación científica no goza de buena salud en América Latina es evidente. Para encontrar la solución de cualquier enfermedad, primeramente debe acertarse en el diagnóstico. Estos editoriales son parte de las pruebas analíticas que pueden ayudar a establecer el diágnostico y a determinar el tratamiento más adecuado.

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Prólogo

Tal y como ocurre con la mayoría de las disciplinas científicas, la microbiología se encuentra actualmente inmersa en una rápida evolución. Por su privilegiada situación en las fronteras de la ciencia, allá donde se concentran los investigadores más innovadores y productivos, los conocimentos generados en este campo del saber se han ido acumulando de forma exponencial en los últimos tiempos. Dentro de la microbiología, el área que quizás mayor auge haya experimentado es la que conocemos como "patogénesis microbiana".

El desarrollo, en la década de los 70, de la tecnología del DNA recombinante permitió abordar el estudio de los mecanismos patogénicos de los microorganismos desde un nuevo ángulo, que la perspectiva de los años transcurridos nos permite valorar como revolucionario. Anteriormente, las únicas herramientas disponibles para el análisis molecular de la virulencia eran de tipo bioquímico. Así, durante los años 60 y 70 estas investigaciones "sobrevivían", en el contexto de la microbiología y de la ciencia en general, centrándose en la purificación, caracterización y análisis funcional de una restringida variedad de factores de virulencia, fundamentalmente exotoxinas proteicas, endotoxinas y adhesinas. Con la disponibilidad de nuevas y poderosas herramientas genéticas, durante los años 80 se asistió a un rápido y amplio desarrollo de la investigación sobre virulencia bacteriana. La posibilidad de generar mutantes isogénicos, la aplicación de técnicas de clonación y secuenciación de DNA, y el desarrollo y perfeccionamiento de modelos experimentales in vivo e in vitro para evaluar la virulencia, condujeron a la identificación y caracterización genética de un número cada vez mayor de determinantes bacterianos de patogenicidad[†] (3, 6).*

Los modelos basados en cultivos celulares se revelaron fundamentales para el análisis de la interacción parásito–hospedador, especialmente la que tiene lugar durante la infección por patógenos intracelulares facultativos (p.e. los casos de *Listeria*, *Salmonella*, *Shigella* o *Yersinia*). De estos estudios derivaron dos importantes conceptos en patogenicidad microbiana: uno, que los fenómenos de adhesión e invasión están mediados por interacciones específicas ligando–receptor, lo que implica que los microorganismos patógenos han adaptado, en su co-evolución con el hospedador, estructuras moleculares capaces de subvertir, aprovechándolos en su beneficio, mecanismos fisiológicos de la célula eucariótica que intervienen normalmente en la interacción o comunicación intercelular (p.e. reconocimiento de integrinas por parte de secuencias "RGD" presentes en adhesinas bacterianas); y dos, que estos microorganismos patógenos han sido también capaces de adaptar una serie de estrategias moleculares que les permiten eludir los mecanismos bactericidas del aparato fago-lisosómico de la célula eucariótica (4).

Precisamente ha sido la utilización de los cultivos celulares, y la incorporación al estudio de la interacción microorganismo-célula eucariótica de los extraordinarios avances de la biología celular, lo que ha abierto una nueva perspectiva de enorme potencial en patogénesis microbiana. Del enfoque

[†] A lo largo de los distintos artículos de este número monográfico se utiliza en español el término «patogenicidad», por indicación expresa de los editores invitados. En el *Vocabulario científico y técnico* (véanse las pp. 323–324 de este número) de la Real Academia de Ciencias Exactas, Físicas y Naturales (3ª ed., Espasa Calpe, Madrid, 1996), el término que aparece es «patogeneidad». (N. de la R.)

* Véanse las referencias en el pie de la p. 170.

genético y molecular de los años 80, centrado fundamentalmente en la bacteria y sus genes de virulencia, se ha pasado en los 90 al análisis sistemático de los aspectos celulares de la relación parásito-hospedador. Ello, según algunos, está dando lugar a un nuevo campo científico denominado "microbiología celular" (2). Importante objeto de investigación del mismo son los mecanismos que desencadenan la fagocitosis, desde los receptores eucarióticos estimulados por el microorganismo, hasta los fenómenos celulares que intervienen en la reordenación de los microfilamentos y la génesis del consecuente proceso dinámico de membrana que envuelve e internaliza al agente patógeno, pasando por las vías de transducción de señales implicadas. Otro aspecto que está siendo estudiado activamente es la motilidad bacteriana intracelular como fenómeno básico de virulencia (p.e. en *Listeria*, *Shigella* y *Rickettsia*), en el que desempeña un papel fundamental el citoesqueleto de actina de la célula infectada. Igualmente importantes son las investigaciones sobre los productos bacterianos que interfieren con elementos de la cascada de señales intracelulares, como las toxinas ADP-ribosilantes producidas por una gran variedad de bacterias patógenas (*Bordetella pertussis*, *Clostridium* spp., *Corynebacterium diphteriae*, *Escherichia coli*, *Pseudomonas* spp., *Vibrio cholerae*). Estas toxinas modifican post-traduccionalmente distintas GTPasas que participan en la regulación de importantes procesos celulares (p.e. Rho, Ras, Rab y otras proteínas G), interfiriendo con su función, lo cual tiene como consecuencia una multiplicidad de efectos dependiendo de los sistemas situados bajo su control en los circuitos de transmisión de señales (fosfolipasas, adenilato ciclasa, etc., responsables de la liberación de segundos mensajeros). Otros puntos de interés se centran en los factores microbianos que interfieren con la maduración del compartimento lisosómico y el tráfico vesicular, o los que determinan la muerte celular programada o apoptosis, permitiendo o facilitando la supervivencia del patógeno en los tejidos del hospedador. En esencia, estas investigaciones están introduciendo dos nuevos conceptos en patogénesis microbiana. Por un lado, la interacción que se establece entre el patógeno y las células del hospedador (entre ellas las del sistema inmunitario) se empieza a considerar como un sistema dialógico, en el que ambos elementos intercambian señales cuyo reconocimiento mutuo desencadena la serie de eventos celulares y moleculares que definen el proceso infeccioso. En este contexto, los esfuerzos investigadores no sólo se centran en estudiar la respuesta celular durante la infección, sino también en desentrañar los mecanismos de regulación del microorganismo, responsables de su respuesta adaptativa a los variados y dinámicos ambientes que puede encontrar en los distintos tejidos y compartimentos celulares del hospedador. Por otro lado, el diálogo cruzado que se establece entre la célula eucariótica y el agente infeccioso indica que ambos utilizan un mismo lenguaje molecular, lo cual sólo puede ser resultado de una exquisita adaptación co-evolutiva. De este segundo concepto se deriva una consecuencia de gran importancia práctica, que trasciende más allá del campo de la patogénesis microbiana, puesto que implica que muchos productos bacterianos, al interferir selectivamente con determinados procesos celulares eucarióticos, pueden ser utilizados como reactivos extraordinariamente útiles para la investigación en biología celular (1, 2, 4, 7, 9).

No resulta fácil interpretar en términos moleculares los complejos y multifactoriales procesos biológicos que subyacen en la interacción parásito–hospedador. ¿Por qué, entonces, se invierten cada vez más esfuerzos y recursos en tratar de desvelarlos? El afán de saber, sin duda el primer motor de la investigación, no lo explica todo. La investigación científica es cada vez más costosa desde el punto de vista económico, y requiere de la administración racional de unos recursos que, desgraciadamente, son escasos. El gran desarrollo de las investigaciones en patogenicidad microbiana del que estamos siendo testigos en los últimos años no es fruto de la casualidad ni del capricho de los investigadores; obedece

a una necesidad real de dar respuesta a un serio problema. El advenimiento por sorpresa del sida y su desarrollo imparable a nivel mundial; la constatación de que la malaria, la tuberculosis y otras enfermedades infecciosas son todavía importante causa de morbilidad y mortalidad; la posible importación a nuestro entorno de enfermedades exóticas, altamente letales; o, simplemente, la cada vez mayor difusión de las resistencias frente a los antimicrobianos entre los agentes patógenos, nos sitúan en un contexto en el que las enfermedades transmisibles están recobrando el sentido de grave amenaza que antes tenían. Aunque todavía nos resulta difícil de asimilar, por la falsa sensación de seguridad derivada de los pasados éxitos de la terapia anti-infecciosa, el fantasma de las legendarias pestes que han asolado a nuestra especie durante siglos vuelve a hacerse patente. En cualquier momento puede aparecer un clon multirresistente de un patógeno bacteriano altamente virulento, o emerger una nueva enfermedad infecciosa (en medicina veterinaria tenemos múltiples ejemplos de aparición de nuevas enfermedades víricas) de elevada letalidad que, quizás, y a diferencia del HIV, sea altamente transmisible.

Frente a estas eventualidades tenemos que estar preparados. Y la única forma de estarlo es acumular conocimientos y experiencia acerca de los fenómenos celulares y moleculares que ocurren durante la patogénesis de los procesos infecciosos y la consecuente respuesta inmunitaria que generan los microorganismos al interaccionar con el hospedador. Sólo un profundo conocimento de estos aspectos permitirá diseñar, de forma racional, nuevas estrategias terapéuticas y vacunales frente a las enfermedades transmisibles, y nuevas técnicas para el diagnóstico y estudio de la distribución de las mismas. La era del empirismo, a la que indudablemente debemos todos los progresos hasta ahora alcanzados en la lucha anti-infecciosa (no olvidemos que la vacuna que erradicó la viruela de la faz de laTierra era empírica), está tocando a su fin, tal y como ejemplifica perfectamente la situación planteada por el sida en estos últimos años del siglo que ahora acaba.

Coincidiendo con el centenario de la muerte de Louis Pasteur en 1895, hito que puede servirnos para marcar el siglo de existencia de la microbiología médica y veterinaria (8), el área de la patogénesis microbiana, de la que podemos considerar que el extraordinario investigador francés es padre principal, emerge con fuerza desde la microbiología como nueva disciplina científica que aborda los aspectos que acabamos de discutir. Los contenidos de la microbiología han crecido tanto en los últimos años que algunas áreas tradicionalmente comprendidas en ella, como la inmunología o la virología, se han consolidado como auténticas disciplinas científicas. La patogénesis microbiana es un área de marcado carácter multidisciplinar, que se sitúa en la intersección de la microbiología con disciplinas tales como la inmunología, genética y biología molecular, biología celular, enfermedades infecciosas y biotecnología. Teniendo en cuenta la evolución que actualmente está siguiendo la patogénesis microbiana en el mundo de la ciencia, nos atrevemos a predecir que, en un futuro no muy lejano, podría establecerse como una nueva área de conocimento con identidad propia. Ello es, a nuestro juicio, perfectamente coherente y compatible con la aspiración por parte de la SEM de implantar una licenciatura en microbiología (5), de la que, sin duda, la patogénesis microbiana sería una de las materias más atractivas e importantes.

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* Quiero dedicar esta recopilación de artículos sobre patogenicidad bacteriana al Prof. Guillermo Suárez Fernández, quien me introdujo a la microbiología a través de sus enseñanzas en las aulas de la Facultad de Veterinaria de Madrid, como muestra de afecto y agradecimiento.

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Virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae*

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Summary

The Gram-negative bacterium *Actinobacillus pleuropneumoniae* is the etiologic agent of swine pleuropneumonia, a highly contagious respiratory infection with great economic implications. In recent years, considerable efforts have been invested in the study of its virulence mechanisms. Here we review the current knowledge on the determinants of *A. pleuropneumoniae* pathogenicity, paying particular attention to the capsule, the lypopolysaccharide, the outer membrane proteins, and the RTX exotoxins. The contribution of other factors is also discussed.

Key words: *Actinobacillus pleuropneumoniae*, virulence factors, Apx toxins, lypopolysaccharide, outer membrane proteins

Resumen

La bacteria Gram negativa *Actinobacillus pleuropneumoniae* es el agente causal de la pleuroneumonía porcina, una infección del tracto respiratorio altamente contagiosa, con importantes implicaciones económicas. En los últimos años se ha realizado un considerable esfuerzo para esclarecer los mecanismos de virulencia de este microorganismo. Hemos revisado el estado actual del conocimiento sobre los determinantes de patogenicidad de *A. pleuropneumoniae*, dedicando especial atención a la cápsula, el lipopolisacárido, las proteínas de la membrana externa y las exotoxinas RTX. También se comenta la contribución de otros factores.

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Introduction

Actinobacillus pleuropneumoniae is the causative agent of porcine pleuropneumonia, a highly contagious respiratory disease with major economic implications for the swine industry worldwide. The infections occur as acute outbreaks with high mortality in 24 to 48 h, or as a chronic, persistent disease. The latter form is of special concern because of its high prevalence and because it is insiduous, and manifest only as impairment of growth and increased susceptibility to secondary infections. The disease is characterized by lesions which include severe necrotic and hemorrhagic pneumonia with fibrinous pleural adhesions. *A. pleuropneumoniae* has a strict host specificity for the pig and predominantly infects animals under intensive breeding conditions (including crowding) as a result of its direct mode of transmission (66, 84).

A. pleuropneumoniae is a Gram-negative, facultatively anaerobic, encapsulated coccobacillus of the *Pasteurellaceae* family (55). Two biotypes have been identified: biovar 1 requires nicotinamide adenine dinucleotide for growth, whereas biovar 2 does not (71). There are 12 serotypes described on the basis of antigenic differences in the capsular polysaccharide (39, 67) and O-chains of lypopolysaccharide (76); all the serotypes are capable of causing the same disease, although few are highly prevalent in a given area at a given time (84).

In spite of the intense efforts to develop a vaccine against porcine pleuropneumonia, currently available products are not satisfactory for controlling the disease. This subject thus remains an area of active veterinary research. The major drawbacks of the available vaccines include poor prevention of lung lesions, lack of cross-protection between serotypes, and failure to prevent the carrier state and subsequent spread of the infection (38, 78). Clearly, a better understanding of the pathogenesis of pleuropneumonia is required to develop new, efficient tools for successful control and prevention of the disease. Pathogenesis studies on swine pleuropneumonia can now benefit from the recent development of the transposon mutagenesis procedure described by Tascón et al. (90), and of the insertion-replacement method described by Jansen et al. (51). Both techniques allow the generation of well-characterized mutants affected in the expression of molecules possibly involved in virulence. This is likely to provide valuable new information on their contribution to the disease.

The pathogenesis of *A. pleuropneumoniae* infection is a multifactorial process which is only partially understood. Several bacterial structures and products have been implicated, in addition to endogenous host factors. In this article, we review *A. pleuropneumoniae* factors affecting virulence (summarized in Table 1).

Capsule

A. pleuropneumoniae serotype specificity is determined by repeated negatively-charged oligosaccharide units that make up the bacterium capsular polymer (39). The capsule is also responsible for the characteristic iridiscence of the colonies on a clear medium. The structure and composition of the capsules of the 12 serotypes have been published (70). The immunostabilized capsule material of *A. pleuropneumoniae* is 80 to 230 nm thick depending on serotype (45). It has been suggested that the differences in the amount of capsule contribute to variations in virulence (45).

Purified *A. pleuropneumoniae* capsule has been reported to have no biological activity (22, 23). However, there is evidence suggesting that it is essential for *A. pleuropneumoniae* virulence in

TABLE 1. *Actinobacillus pleuropneumoniae* virulence factors and their known or assumed biological function in pathogenesis

Factor	Function	Ref.
Capsule	Antiphagocytic properties	40
	Protection against host humoral defenses by limitation of antibodies and C9 deposition on the bacteria	99
LPS	Synergistic activity with exotoxins in lung lesion production	93
	Adhesion factor	4, 5, 69
	LPS-specific IgG reduce and reflect C9 deposition on the bacterial surface	99
	Hemoglobin binding	6
OMP	Specific antibodies act as opsonins	92
	Transferrin binding and determination of host specificity	36, 75, 83
Apx toxins	Lung lesion production	74, 82, 91
	Impairment of immune response	87
	Increases inflammatory cytokine production	2
	Hemolytic activity	32, 53
	Antiphagocytic activity	15, 89, 94, 97
	Induction of phagocyte respiratory burst	18, 19, 51
Fimbriae and pili	Adhesion factor	40, 96
Hemmaglutination	Adhesion factor	44
HlyX protein	In vivo gene regulation	62
Proteases	Host protein cleavage	65
SOD	Protection against oxygen-free radicals	58
Urease	Unknown	

vivo. Antibodies against the capsule suppressed mortality in experimental infections, although they did not prevent lung lesions or disease chronification (40, 78). An avirulent serotype 5 strain has been shown to have a fragile, easily removable capsule, in contrast to a fully virulent serotype 5 strain which had a more adherent capsule (52). Moreover, less capsulated mutants of serotypes 1 and 5, obtained by serial passages in vitro, were substantially attenuated in virulence with respect to their parent strains (40, 79). Furthermore, a chemically-induced non-capsulated mutant of serotype 5 was almost unable to cause pleuropneumonia, and did not kill intranasally infected pigs at a dose 1.3 log greater than the LD_{50} of the parent strain (42).

The capsular polysaccharide has antiphagocytic properties (40) and is assumed to be the main bacterial shield against host humoral defenses (40, 80). Normally capsulated *A. pleuropneumoniae* strains are resistant to complement-mediated killing in porcine serum in the presence of specif-

ic antibodies (40, 80, 99); in contrast, capsule-deficient mutants are efficiently killed by normal serum (40, 99). The mechanism by which the capsule provides resistance to this bactericidal activity has been partially elucidated by the finding that the capsule material does not prevent complement activation (95, 99) or C3 (a component of the complement cascade) binding to the microorganism, but limits the amount of antibodies and C9 deposition on the bacterial surface in normal serum (99).

Lipopolysaccharide

The *A. pleuropneumoniae* lipopolysaccharide (LPS) is a complex molecule with three different parts: the lipid A, the core, and the O antigen. O antigen is composed of repeated polysaccharide chains, the amount of which determines the *A. pleuropneumoniae* smooth (serotypes 2, 4, 5, and 7), partially rough (serotypes 1 and 5), or rough (serotypes 3 and 6) LPS profiles (8, 23, 70). Although O-chain composition is serotype specific, their length may differ between strains, leading to distinct patterns within the same serotype (1).

Pure LPS is pirogenic, produces a positive dermal Shwartzman reaction, gelifies *Limulus* amebocites lysates, displays chick embryo toxicity, and induces lymphocyte blastogenesis, all biological activities characteristics of endotoxins (22, 23, 63). Although intratracheal inoculation of LPS induces lung injury in vivo, the response does not include necrosis or hemorrhages (93). It seems that the endotoxic activity acts cojointly with exotoxins to cause the full pattern of lesions typical of pleuropneumonia (93).

More recently, LPS has been shown to contribute to *A. pleuropneumoniae* adhesion to porcine tracheal rings and respiratory tract mucus (4, 5). This was confirmed by the finding that LPS adheres to porcine lung and tracheal frozen sections, a property for which polysaccharides seem to be responsible (69).

In addition to the effects reported for the capsule, LPS-specific IgG are involved in another mechanism of resistance to the bactericidal activity of serum. These antibodies are present in normal or immune serum, and reduce C9 deposition on the bacterial surface and promote the binding of this complement component to nonbactericidal sites, limiting serum-mediated killing (99).

Outer membrane proteins

A. pleuropneumoniae produces several outer membrane proteins (OMP) (73), of which some have been assigned a role in inducing a protective immune response (14, 35). Moreover, antibodies to *A. pleuropneumoniae* OMP have been shown to act as important opsonins in porcine polymorphonuclear leukocyte phagocytosis (92).

A. pleuropneumoniae synthesizes two novel proteins when grown under iron-restricted conditions, and antibodies to them are present in convalescent serum, indicating that these polypeptides are produced in vivo (16, 68). They have been identified as specific receptors for porcine transferrin (75), and the ability of *A. pleuropneumoniae* to grow with transferrin as the sole iron source has been documented (36, 83).

Genetic analysis of the determinants encoding the transferrin receptor proteins has identified two genes arranged in an operon in order *tbpB* (encoding the Tbp2 protein with a deduced molecular mass of 59.8 to 65.5 kDa) and *tbpA* (which encodes the Tbp1 protein with a predicted molecular mass of 102.2 kDa) (34, 37). Putative boxes for the ferrous-ion-binding repressor protein Fur have been found upstream from the *tbp* operon, suggesting that environmental iron controls *tbp*

genes through Fur (37). This organization is similar to that described for the transferrin receptors of *Neisseria meningitidis*, *N. gonorrhoeae* and *Haemophilus influenzae* (37), and the *A. pleuropneumoniae* transferrin binding process is therefore assumed to be similar (88).

The binding specificity for porcine transferrin in *A. pleuropneumoniae* has been used to explain the strict host specificity of this bacterium: its limited capacity to infect animals other than the pig may be because the microorganism is unable to utilize transferrins from other origins as sources of iron (36, 83). Failure in this iron uptake system might reduce the *A. pleuropneumoniae* ability to overcome in vivo iron deprivation, impeding growth in the host tissues and thus leading to a concomitant attenuation of virulence (20).

Exotoxins

A. pleuropneumoniae produces three different exotoxins that belong to the RTX family of pore-forming toxins (31), the best characterized member of which is the *Escherichia coli* α-hemolysin. The RTX toxins are widely distributed among Gram-negative bacteria in which they are now recognized as being relevant virulence factors. Members of this group share structural and functional properties, including the presence of glycine-rich repeats, a particular mode of secretion with a signal sequence at the C-terminus, post-translational activation, and cell toxicity via a pore-forming mechanism. However, the toxins differ in host specificities, target cells and toxic activities (hemolytic, cytotoxic, leukotoxic or adenylate cyclase) depending on the producer bacterial species (12, 100). RTX toxins are encoded by operons that consist of four contiguous genes arranged in the order, *C*, *A*, *B*, and *D*. The *C* gene encodes a product that directs the cytoplasmic conversion by an acylation reaction of the structural toxin encoded by the *A* gene to the active form (43), exported by the products of the *B* and *D* transporter genes (100).

RTX toxins in *A. pleuropneumoniae* are called Apx toxins (for *A. pleuropneumoniae* RTX toxins): two of these toxins, ApxI and ApxII, are hemolytic and cytotoxic, whereas ApxIII possesses cytotoxic but not hemolytic activity (31). A striking characteristic of *A. pleuropneumoniae* is that many strains secrete different combinations of two toxins, and carry truncated *apxI* or *apxII* operons containing only two genes (31). The *apx* genetic profile has been established for the reference strains of the twelve serotypes (shown in Fig. 1) (11, 30, 46, 48, 49), and field strains of each serotype all have the same *apx* genoytype or Apx phenotype (3, 32, 54).

ApxI was first described as the strongly hemolytic toxin of *A. pleuropneumoniae* serotype 1 reference strain, with an apparent molecular mass of 105 kDa (25). ApxI is strongly cytotoxic for alveolar macrophages and neutrophils (53). The toxin is produced by serotypes 1, 5, 9, 10, and 11, in which the *apxI* operon contains the four classic RTX genes. Serotypes 2, 4, 6, 7, 8, and 12, are devoid of *apxIA*, and consequently they do not produce ApxI toxin, but they possess a truncated operon containing *apxIBD*, and the *C*-terminal end of *apxIA*; the *apxI* operon is completely absent from the serotype 3 (Fig. 1) (30, 48). ApxIA has a deduced molecular mass of 110.2 kDa and contains thirteen glycine-rich repeats with binding capacity Ca^{2+} (28), which is required for hemolytic activity and binding to neutrophils (17, 98). ApxI is very similar to the *E. coli* hemolysin HlyA (28).

ApxII is the 105 kDa toxin which was originally characterized from the serotype 2 reference strain (29). ApxII is weakly hemolytic and weakly cytotoxic for alveolar macrophages and neutrophils (53). Using monoclonal antibodies, it has been identified as a 103 kDa protein present in all

Serotypes	*apxI*	*apxII*	*apxIII*	Secreted toxins
1, 5, 9, 11	C A B D	C A		ApxI, ApxII
10	C A B D			ApxI
2, 4, 6, 8	B D	C A	C A B D	ApxII, ApxIII
7, 12	B D	C A		ApxII
3		C A	C A B D	ApxII*, ApxIII

FIG. 1. Schematic representation of the *apx* genetic profiles and consequent Apx toxin phenotypes of *Actinobacillus pleuropneumoniae* (data from 11, 30, 46, 48, 49). (*): serotype 3 secretes only small amounts of ApxII, and most of the toxin is found intracellularly (27, 32). It seems that the *apxIIIBD* transporter genes are only able to export efficiently the ApxII toxin from *Escherichia coli* recombinant strains (61) but not from *A. pleuropneumoniae* (32).

serotypes except serotype 10 (53). The *apxII* operons only contain the activator and structural determinants *apxIICA*, the secretion genes being absent (Fig. 1) (30, 46). The presence of truncated genes that may resemble *apxIIB*, downstream from *apxIIA*, suggests that the secretion genes have probably been lost during evolution (10). ApxII secretion occurs via *trans*-complementation by the *apxIBD* genes products (30, 46, 51, 91). The sequence of the *apxIIA* determinant predicts a 102.5 kDa protein with eight glycine-rich repeats, highly similar to the *Pasteurella haemolytica* leukotoxin LktA (9).

ApxIII has no hemolytic activity but is highly cytotoxic for alveolar macrophages and neutrophils (53, 81). It is encoded by an intact *apxIII* operon in serotypes 2, 3, 4, 6, and 8 (Fig. 1) (11, 49). The deduced ApxIIIA protein possesses thirteen glycine-rich repeats and its calculated molecular mass is 112.8 kDa (47). It is more closely related to ApxIA and (to a lesser extent) ApxIIA, than to other RTX toxins with similar activities (47).

Each of the three Apx toxins is able to induce a positive CAMP phenomenon (synergistic hemolysis) (33, 50). Paradoxically, ApxIII, although non-hemolytic, is not only able to induce this co-hemolytic reaction, but has the strongest effect when expresed in *E. coli* (33). The finding that this reaction is due to Apx toxins (and not to an individual protein previously identified in *A. pleuropneumoniae* [26]), confirms that this co-hemolytic activity is virulence-associated (see below).

A. pleuropneumoniae toxins have long been postulated to be important virulence factors, first suggested by the early reproduction of pleuropneumonia lung lesions with sterile culture supernatants (Rosendal, S. et al., Proc. 6th Int. Pig Vet. Soc. Congr., 1980, p. 221). Because of their cytolytic activity, Apx toxins were thought to be involved in virulence directly by damaging tissues and thereby to cause the hemorrhagic and necrotic lung lesions characteristic of swine pleuropneumonia (41, 93). A membrane-damaging mode of action has been suggested for Apx toxins given the ability of *A. pleuropneumoniae* serotype 1 exotoxins to produce channels in phospholipid bilayers (59). Sterile *A. pleuropneumoniae* culture super-

natants are toxic for thymic and splenic T lymphocytes and induce thymic lesions, thus impairing the host immune response and favoring the development of chronic disease (87). Finally, experimental infection with *A. pleuropneumoniae* serotype 1 causes large increases in the level of interleukin-1, 6, and 8, and a role of the Apx toxins in the inflammatory cytokine-mediated tissue damage is suggested by the failure of heat-killed organisms and a cytotoxin-deficient strain to provoke this response, excluding its endotoxic induction (2).

Transposon-mediated mutation analysis of the *apxI* operon has provided the first unambiguous evidence that Apx toxins are major virulence factors of *A. pleuropneumoniae*. A serotype 1 nonhemolytic mutant (defective for ApxI and ApxII secretion as a result of the *apxIB* gene inactivation) was virtually apathogenic for pigs. In contrast, a weakly hemolytic mutant (obtained by disruption of the *apxIA* gene and which still produced ApxII) retained some virulence and was able to produce the typical lesions and clinical signs of *A. pleuropneumoniae* infection in pigs (91). These experiments show that Apx hemolysins are essential for *A. pleuropneumoniae* pathogenicity, and that both ApxI and ApxII are needed for full virulence (91). This has been later confirmed by genetic complementation (with either the *apxIBD* genes or the entire *apxICABD* determinant) of a chemically-induced hemolysin-negative mutant of serotype 5, deleted for the *apxI* operon (74). In addition, using a chemically-induced mutant of serotype 2 producing only ApxIII (but not ApxII), Rycroft et al. (82) have been able to reproduce acute lung lesions in pigs, showing that this toxin alone is also able to induce direct lung injury. However, their findings are consistent with synergy between the toxins.

Although the importance of Apx toxins in the pathogenesis of swine pleuropneumonia has been established, the exact mechanism by which they exert their role is not completely understood. Being a respiratory pathogen, *A. pleuropneumoniae* has first to overcome innate local defenses in the lung, primarily alveolar macrophages and polymorphonuclear leukocytes. Apx toxins may contribute to invasion by expression of their cytotoxicity to phagocytes (7, 13, 97).

This antiphagocytic activity could be induced extracellularly prior to phagocytosis, or intracellularly after internalization. Evidence for both mechanisms has been presented. Sublytic doses of a crude Apx preparation of a serotype 2 strain (unable to cause cell death) severely impair phagocytosis by alveolar macrophages in vitro (89). Large doses of crude toxins from serotype 2 rapidly inactivate porcine pulmonary macrophages and neutrophils without previous stimulation of these cells (18, 19). In addition, a *A. pleuropneumoniae* serotype 2 strain has been shown to cause immediate degeneration of pig alveolar macrophages, impairing phagocytosis; this toxicity was associated with Apx toxins, since only a hemolysin- and cytotoxin-negative mutant was taken up phagocytically (15). These observations are consistent with *A. pleuropneumoniae* being able to prevent bacterial uptake.

On the other hand, phagocytosis of *A. pleuropneumoniae* has been demonstrated (13, 40, 92, 94), and the bacteria have been found to survive within porcine neutrophils and alveolar macrophages (13, 94). They must therefore have a molecular mechanism to overcome the hostile environment of the phagosome. A role of the Apx toxins in this function is suggested by analogy with *Shigella flexneri*: mutants of this bacterium unable to escape from the endocytic vacuole, recover the ability to break out of this compartment if they produce small amounts of the *E. coli* RTX toxin HlyA (101).

Sterile culture supernatants of a *A. pleuropneumoniae* serotype 2 strain stimulate the oxi-

dative activity of porcine pulmonary alveolar macrophages (18), a phenomenon which has been reproduced in porcine neutrophils using recombinant ApxII and ApxIII toxins (19). Using serotype 1 knockout mutants devoid of ApxI and/or ApxII, it has been demonstrated that the oxidative burst and killing provoked by *A. pleuropneumoniae* in porcine neutrophils is due to Apx toxins (51). Extensive production of oxygen radicals induces degenerative changes in the surrounding cells (hyaluronic acid degradation, protein denaturation, DNA damage, and membrane lipid peroxidation), ultimately leading to lung injury and necrosis (24). This may explain the role of Apx toxins in the development of lung lesions.

Summarizing, all the above evidence indicates that each of the Apx toxins is directly able to induce lung injury, and that they may contribute to invasion by exerting antyphagocytic properties. All *A. pleuropneumoniae* strains secrete at least one Apx toxin, and thus this interpretation is fully compatible with the observation that the disease caused by all strains is indistinguishable (66; Nielsen, R. 1982. Doctoral Thesis, Royal Veterinary and Agricultural University of Copenhagen, Copenhagen), despite the differences found in virulence between serotypes and even within the same serotype (52, 56, 64, 77).

There appears to be a correlation between the presence of ApxI and virulence. ApxI-expressing serotypes (1, 5, 9, 10, 11) are frequently involved in high mortality pleuropneumonia outbreaks (21, 32, 56, 72). This may be due either to differences in the ApxI cytotoxic/cytolytic activity or production, to the presence of other unknown factors in these strains. The recent finding that the lipid A region of *A. pleuropneumoniae* lypopolysaccharides binds pig hemoglobin (6) suggests another possible role of Apx toxins in the pathogenesis of swine pleuropneumonia. *A. pleuropneumoniae* can utilize this compound as an iron source (16, 20, 68), thus strains producing the strongly hemolytic toxin ApxI, alone or in combination with ApxII, may have a substantial advantage in vivo because of their ability to acquire iron from lysed erythrocytes (6). Interestingly, serotype 3, the only serotype which does not secrete significant amounts of any hemolytic Apx toxin (27), is generally less virulent (3, 32, 64, 77).

Other factors

Fimbriae and pili. Inzana et al. (40) have identified pili-like structures in bacteria found within phagosomes after internalization by polymorphonuclear leukocytes. More recently, peritrichous fimbriae have been described in 45% of field isolates grown on blood agar (but not on media without blood), and fimbriae are lost after subsequent culture passages (96). These putative adherence factors may contribute to the strict host specificity of *A. pleuropneumoniae* (41).

Hemagglutination. Agglutination of erythrocytes has been described for some *A. pleuropneumoniae* isolates, and on the basis of blood origin, seven serotype-unrelated hemmaglutination patterns have been described. Fimbriae or hydrophobic interactions do not seem to be involved in this adhesion property (44).

HlyX protein. The *A. pleuropneumoniae hlyX* (*cfp*) gene confers a strong hemolytic and CAMP-like positive phenotype in *E. coli* strains (26, 60). Because all the *A. pleuropneumoniae* serotypes carry *hlyX* homologs, and convalescent pig sera neutralized this hemolytic activity, a role in virulence has been postulated (60). Since HlyX does not share any similarity with any known cytotoxin but is highly homologous to the FNR transcriptional regulator, it has been suggested that HlyX may induce a cryptic hemolysin in *E. coli* rather than directly mediating the activity (62).

FNR has been shown to activate the transcription of several genes under anoxic conditions (86), and HlyX expression is regulated by oxygen (85). It has been speculated that HlyX may play a role in pathoghenesis by regulating gene expression under in vivo anoxic situations, for instance those found in fibrinous or necrotic lesions (62).

Proteases. The presence of secreted proteolytic activities degrading porcine IgA and gelatin, and to a lesser extent porcine, human and bovine hemoglobin, has been described in *A. pleuropneumoniae*. Proteases may be involved in lung injury due to host protein cleavage. EDTA inhibition of protein degradation and subsequent calcium reactivation experiments suggest that metalloproteases are responsible for these activities (65).

Superoxide dismutase. *A. pleuropneumoniae* is one of the growing number of bacteria shown to produce a [Cu,Zn]-superoxide dismutase (SOD) (58). The N-terminal leader peptide in the deduced protein suggests an extra-cytosolic location of the enzyme, which may protect against environmental superoxide rather than endogenously released radicals generated during aerobic metabolism (58). Since *A. pleuropneumoniae* promotes a respiratory burst in phagocytic cells (18, 19), SOD may enable bacteria to survive whitin the phagosome by eliminating the toxic oxygen-free radicals produced by this phenomenon.

Several commensal bacteria of the respiratory tract produce SOD, which suggests that the enzyme may be involved in bacterial survival on the airway mucosal surface (57). Further work is needed to confirm whether SOD indeed contributes to the pathogenesis of swine pleuropneumonia.

Urease. The properties of a transposon-induced urease-negative mutant of *A. pleuropneumoniae* show that the loss of urease activity does not reduce the ability of the microorganism to produce acute disease. However, microbial ureases are key factors in several pathogenic states; it cannot therefore be concluded that the enzyme is not involved in other clinical processes or under different experimental conditions (Tascón, R. I. et al., 1996, in preparation).

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Signal transduction and virulence regulation in *Bordetella pertussis*

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Summary

Bordetella pertussis, the causative agent of whooping cough, coordinately regulates the expression of its virulence factors in response to certain environmental stimuli. This coordinate regulation is accomplished by the *bvg* locus encoding the BvgS and the BvgA proteins, which are members of the two-component family of bacterial signal transducing proteins. The sensor protein BvgS shows an "unorthodox" domain structure, combining the characteristic communication modules both of the two component sensors and response regulators, each of which is indispensable for BvgS function. Although under global control of the BvgAS system, two subsets of virulence factors exemplified by the adhesin FHA and the toxins PTX and CYA exhibit, respectively, a differential mode of expression. This is reflected in a differential kinetics of transcriptional activation in vivo, and the different ability of the various virulence promoters to be expressed in the heterologous organism *Escherichia coli*. Evidence is accumulating that this differential regulation may be due to different affinities of the virulence promoters for the phosphorylated form of BvgA.

Key words: *Bordetella pertussis*, sensor proteins, toxin promoters, transcriptional activation, virulence regulation

Resumen

Bordetella pertusis, el agente causal de la tos ferina, regula coordinadamente la expresión de sus factores de virulencia en respuesta a determinados estímulos ambientales. Esta regulación coordinada

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se lleva a cabo por el locus *bvg* , que codifica las proteínas BvgS y BvgA, pertenecientes a la familia de sistemas bi-componentes de transducción de señales. La proteína sensora BvgS muestra un dominio estructural "no ortodoxo", combinando los módulos de comunicación característicos de los sensores bi-componentes y de los reguladores de respuesta, cada uno de los cuales es indispensable para la función de BvgS. Aunque bajo el control global del sistema BvgAS, dos grupos de factores de virulencia, representados por la adhesina FHA y las toxinas PTX y CYA, muestran, respectivamente, un modo de expresión diferenciado. Este hecho queda reflejado por una cinética diferencial de activación transcripcional in vivo y por una diferente capacidad de los diversos promotores de virulencia para expresarse en el organismo heterólogo *Escherichia coli*. Nuevos datos demuestran que esta regulación diferencial puede deberse a diferentes afinidades de los promotores de virulencia por la forma fosforilada de BvgA.

The genus *Bordetella*

The genus *Bordetella* comprises five species, four or which cause infections of the upper respiratory tract in different host organisms. *Bordetella pertussis*, an obligate human pathogen, is the etiological agent of the serious whooping cough disease which predominantly affects infants. Annually more than 50 million cases leading to 600,000 deaths are reported worldwide. *Bordetella parapertussis*, the closest relative of *B. pertussis*, causes a mild pertussis-like disease in humans. *Bordetella bronchiseptica*, a flagellated species which may have retained the ability of surviving in the environment (39), is an animal pathogen that infects a number of mammalian species, e.g. pigs and dogs. *B. bronchiseptica* infections in humans occur only rarely, affecting immunocompromised persons, and can result in pneumonia occasionally accompanied by bacteremia (59). *Bordetella avium* is a pathogen of poultry. *Bordetella hinzii*, which was only recently recognized as a member of the genus *Bordetella*, is frequently associated with poultry, but does not necessarily cause disease (56). Surprisingly, *B. hinzii* was recently identified as the causative agent of a bacteremia in a patient suffering from aids (15).

Upon infection of a suitable host, *B. pertussis* adheres to ciliated epithelial cells of the respiratory tract, multiplies and produces a variety of highly toxic compounds. Filamentous hemagglutinin (FHA), a protein of 220 kDa, is believed to act as the major adhesin (2). The aminoterminal part of this molecule harbours several domains capable of specific binding of eukaryotic cells including a glycoconjugate binding domain (41), an integrin binding ArgGlyAsp (RGD) motif (42) and a heparin binding region (32). In addition, the 69 kDa outer membrane protein pertactin (PRN), which also contains the RGD motif (28) and the fimbriae (FIM), seems to be involved in adhesion and colonization of the host. Pertussis toxin (PTX), a typical AB toxin which is exclusively produced by *B. pertussis,* is probably the main reason for the systemic effects of the whooping cough disease, such as lymphocytosis, histamine sensitization and increased insulin production (23). In addition, PTX contributes to the adherence to certain epithelial cells and macrophages (44, 57). Another major virulence factor which is involved in the defence against local immune reactions of the host is adenylate cyclase toxin (CYA), a bifunctional protein which carries both the hemolytic and the adenylate cyclase activities. Both PTX and CYA act by deregulating the second messenger equilibrium of the eukaryotic cell: in the case of PTX, this is accomplished by the ADP-ribosylation of regulatory G-proteins, while CYA very efficiently ca-

talyses the direct synthesis of cyclic AMP. Tracheal cytotoxin, a disaccharid-tetrapeptide fragment of the bacterial cell wall which is spontaneously released during the logarithmic growth phase, is responsible for the typical cough paroxysms associated with whooping cough caused by a severe damage of the ciliated epithelial cells lining the respiratory tract (14). The mechanism of action of another *Bordetella* virulence factor, the dermonecrotic toxin which has vasoconstrictive activity and causes hemorrhagic scin necrosis when injected intradermally (60), is not well understood so far.

Coordinate regulation of the *Bordetella* virulence properties is accomplished by the *bvg* locus

The expression of the above mentioned virulence factors is (with the exception of the tracheal cytotoxin) coordinately regulated on the transcriptional level. A first hint pointing to the existence of a master regulatory locus of virulence provided the observation of two phenomena termed phase variation and phenotypic modulation, respectively. Phase variation describes the fact that bordetellae tend to spontaneously lose their virulence properties upon cultivation at a frequency of 10^{-3} to 10^{-6} (29). In 1960, Lacey observed that bordetellae could control expression of their virulence properties in a reversible manner depending on the culture conditions: at low temperature or in the presence of nicotinic acid or sulphate ions the bacteria undergo "phenotypic modulation" to the avirulent phase (27). Recently, a single gene locus responsible for both phenomena was identified by transposon mutagenesis (58). Cloning and sequencing of this *bvg* locus, which was formerly called *vir*, revealed the presence of two regulatory proteins, BvgS and BvgA, which belong to a family of signal transducing proteins, the two component systems (1, 50).

This type of regulatory system is frequently involved in the rapid adaptation of bacteria to changing environmental conditions, e.g. in chemotaxis or osmoregulation (38, 53). Two component systems consists of a sensor protein frequently located in the cell membrane, which is able to perceive environmental stimuli via its N-terminal extracytoplasmic input domain and to transform this information into a cellular signal. The second component is a regulatory protein, which frequently is a transcriptional activator. Signal transduction is accomplished via a phosphorylation cascade making use of highly conserved communication modules: in the presence of the appropriate stimulus the sensor's cytoplasmic transmitter domain which harbours a kinase activity causes the autophosphorylation of a His residue which is highly conserved in all sensor proteins. The phosphate group is subsequently transferred to an Asp residue in the N-terminal receiver domain of the response regulator. Phosphorylation of the receiver domain induces a conformational change which activates the C-terminal output domain of the response regulator and (in case of transcriptional activators) may increase its DNA binding capacity.

Therefore, phenotypic modulation is due to an alteration in the phosphorylation status of the sensor protein BvgS as a result of stimulus perception. In contrast, the analysis of avirulent phase variants revealed mutations, frequently small deletions, in the *bvg* locus which inactivates the BvgAS system (36).

The phosphorylated form of the response regulator BvgA induces the expression of the so called *vir* activated genes (*vag*s), which include the genes encoding the above mentioned virulence factors as well as the *bvg* locus itself. In addition to the BvgA dependent promoters P1 and P3 involved in autoregulation, the *bvg* locus harbours a weak BvgA independent promoter P2, which ensures

the presence of a residual amount of BvgAS proteins in the modulated phase (43, 45). Another BvgA dependent promoter P4 located immediately upstream of *bvgAS* directs the synthesis of a small antisense RNA of unknown function complementary to the 5' untranslated region of the *bvgAS* mRNA (45).

A second class of *bvg* regulated genes, the *vir* repressed genes (*vrgs*), are activated in the modulated phase. Four of the five *vrgs* identified so far in *B. pertussis* contain a 32 bp consensus sequence within their coding region, suggesting the binding of a *bvg* activated repressor molecule (3, 5). Recently a gene locus termed *bvgR* was identified immediately downstream of *bvgAS* which is required for the repression of *vrgs* (33). However, it is not clear, whether the *bvgR* gene product, which is expressed in the *bvg*$^+$ phase, acts directly as a repressor protein or whether it is involved in a regulatory cascade. The role of the *vrgs* in *Bordetella* virulence has not been well understood so far. A *B. pertussis* strain with a transposon insertion in *vrg6* showed decreased persistence in a mouse model of respiratory infection, suggesting that *vrgs* may contribute to virulence (4). However, the physiological stimuli causing the switch from the *bvg*$^+$ to the *bvg*$^-$ phase are still unknown.

Molecular analysis of the BvgAS two-component system

The sensor protein BvgS. BvgS is a 135 kDa transmembrane protein which is composed of a periplasmic input domain as well as several cytoplasmic domains. They are named linker, transmitter, receiver and output domain, according to sequence similarities with two component communication modules (Fig. 1). The linker region of 161 amino acids is located between the input and transmitter domains, is the site of several mutations that render BvgS insensitive to modulating signals and therefore confer a constitutive *bvg*$^+$ phenotype (19, 31, 35). These constitutive mutations in the linker eliminate the need for an intact periplasmic domain, which is essential for the wild type BvgS (35). For that reason, it is believed that the linker is involved in the transmission of signals from the input to the transmitter domain by means of conformational changes. The presence of the additional receiver and output domains, which are the characteristic communication modules of the response regulators, places BvgS into a rather small group of "unorthodox" two component sensor proteins (Fig. 1). Surprisingly, Sln1 and ETR1, the first eukaryotic sensor proteins of the two-component family identified in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*, respectively, also belong to this unorthodox class (12, 37).

The primary sequence of the BvgS receiver is in complete agreement with the consensus, as it contains three highly conserved Asp and a Lys residue which are known to form the active site of phosphorylation in the receiver domains of the response regulators. The output domain of BvgS does not show major sequence similarities. Two studies conduced both in vivo and in vitro have adressed the role of the additional BvgS domains in signal transduction (6, 54). In vivo small deletions or insertions in the receiver or output domain, substitutions of the highly conserved amino acids of the receiver consensus (D979, 980, 1023, K1080) and a clean deletion of both the receiver and the output domain completely abolished BvgS activity, suggesting an absolute requirement for the C-terminal domains in BvgS function. This finding clearly distinguishes BvgS from other unorthodox two component sensor proteins e.g. ArcB from *E. coli* or VirA from *A. tumefaciens*, whose receiver domains are believed to merely exert some negative regulatory effect on the kinase activity, as deletions of the C-terminal domains did not abolish the ability of the sensor proteins to

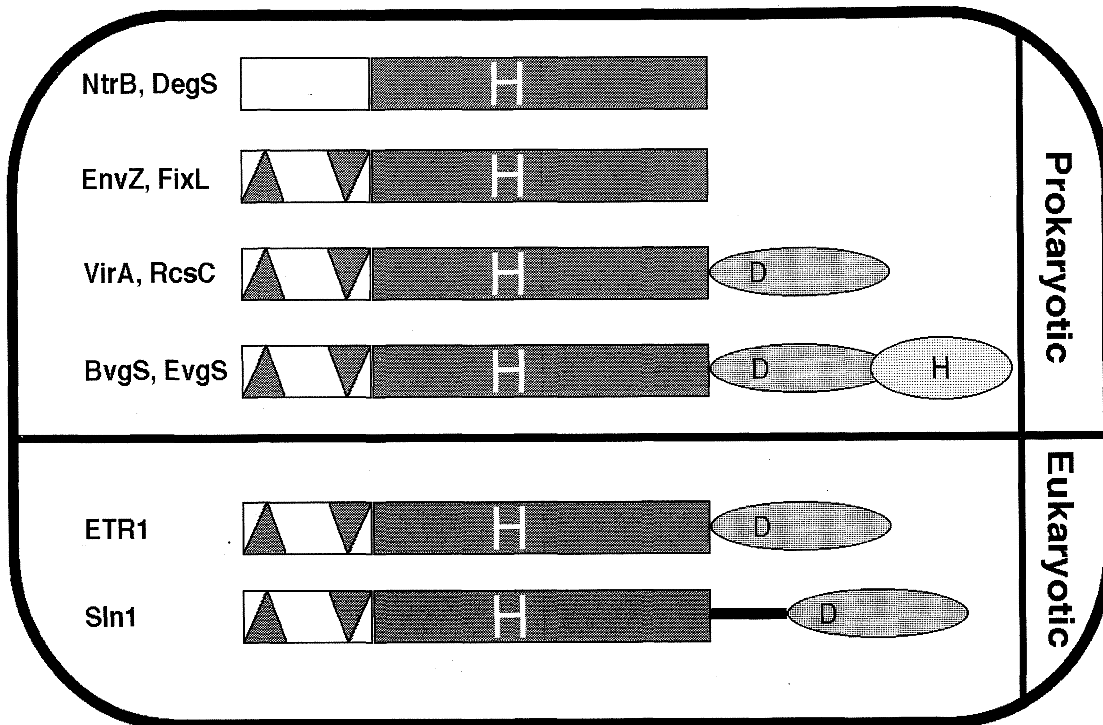

FIG. 1. Domain structure of selected two component sensor proteins. Gray triangles indicate transmembrane spanning regions. Important conserved amino acids are shown and define the signal transduction modules. H, transmitter; D, receiver; the H-domain in the C-terminal part of the BvgS/EvgS sensor proteins represents the output module. For details, see the recent review by Parkinson and Kofoid (38).

activate the corresponding response regulators (11, 26). A detailed in vitro analysis of the BvgAS phosphorelay revealed the uncoupling of BvgS autophosphorylation and phosphotransfer to BvgA in BvgS mutants carrying substitutions of D1023 or K1080 in the receiver: while small C-terminal insertions or deletions abolish BvgS autophosphorylation, the D1023N and K1080R mutants retain their ability to autophosphorylate but are unable to transfer the phosphate group to BvgA (54, 55). Therefore, phosphorylation of the BvgS intrinsic receiver domain, which probably causes a conformational change of BvgS, seems to be a prerequisite for the phosphotransfer to BvgA, possibly by relieving some sterical hindrance at H729, the site of autophosphorylation in the transmitter.

Recently a conserved sequence motif was identified in the output domains of unorthodox sensor proteins of the BvgS class. This sequence motif contains a central His residue which is able to act either as an acceptor or as a donor of phosphate, but does not show kinase activity by itself (24). The direct involvement of this sequence motif in unorthodox signal transduction is still an open question, because substitution of the conserved H717 in the output domain of the *E. coli* sensor ArcB, where this sequence motif was originally identified, did not cause any detectable effect in vivo (24). The substitution of the conserved His residue in the BvgS output domain (H1172) abolished BvgS function in vivo (Beier et al., unpublished). However, this result does not unambiguously argue in favour of a direct requirement for H1172 in BvgS function, because a deleterious

effect of this amino acid substitution on the quite complex tertiary structure of BvgS can not be ruled out. In this context, it should be noted that several mutations located in other regions of the output domain cause the inactivation of BvgS as well (6, 54). Nevertheless, the ability of a truncated BvgS output domain to accept phosphate from wild type BvgS and to transfer it to BvgA has been proven in vitro (55).

Genetic data indicate that BvgS forms dimers or oligomers in its activated state. Recently it was reported that substitutions of the highly conserved amino acids in the BvgS receiver and output domain which inactivate BvgS could be complemented in vivo by the separate expression in trans of a BvgS fragment composed of both the C-terminal domains, suggesting a direct interaction between the mutated and truncated BvgS molecules (6). This assumption is further underlined by the identification of dimerization domains both in the transmitter and the C-terminal domains of BvgS (6). The successful complementation of mutants BvgS proteins by the separate expression of truncated subdomains of BvgS underlines the modular structure of these proteins, and demonstrates that individual modules can retain an autonomous function.

The response regulator BvgA. BvgA is a 23 kDa cytoplasmic protein composed of a N-terminal receiver domain and a C-terminal output domain containing a helix-turn-helix DNA binding motif. According to sequence similarities in the output module, BvgA is grouped into the FixJ subfamily of response regulators (38). BvgA was shown to bind target sequences containing the sequence motif TTTCCTA which is present in the *fha* and *bvgAS* promoter regions as an inverted or direct repeat, respectively (43). The DNA binding capacity of BvgA resides in the output domain requiring its C-terminal 20 amino acids, and is independent of receiver phosphorylation (7). However, the ability of BvgA to bind its target sequences is strongly increased upon phosphorylation of the receiver domain, which is also a prerequisite for BvgA dependent transcriptional activation in vivo (7). From crosslinking experiments, it was deduced that BvgA is able to form dimers in solution (45). Using different experimental approaches the dimerization domain of BvgA was assigned to the output module (6, 7).

Differential regulation of expression of the virulence factors

Although the transcription of most virulence factors of *B. pertussis* is coordinately regulated by the *bvgAS* locus, the regulation of the *ptx* and *cya* operons differs from that of the other factors. Until recently, in contrast to the *bvg* and *fha* promoters, several attempts to show that BvgA is directly involved in the activation of the toxin genes by binding to their promoters were unsuccessful (43). Furthermore, the expression of *ptx* and *cya* is more sensitive to modulating conditions than the expression of *fha* and *bvg* loci (47). Modulation also has a differential effect on the kinetics of the response of the various promoters. When analysing the pattern of transcription of the virulence regulon in response to temperature transition from 25°C to 37°C, Scarlato et al. (46) observed that *ptx* and *cya* transcripts are not detected for hours, whereas *fha*, *fim* and *bvg* transcripts appeared within a few minutes after the temperature shift. Consequently, it was proposed that differential activation of adhesins on the one hand and toxins on the other hand may have in vivo relevance, and might reflect different steps of infections. While immediate expression of the fimbriae or FHA is necessary for a successful adherence to eukaryotic cells, the expression of the toxins may be required in a later stage of infection (40). These results on potentially different activation mechanisms of the toxin promoters as compared to other virulence

promoters are further substantiated by the observation that *bvg* dependent expression and regulation of the *fha* promoter can be reconstituted in *E. coli*, when the *bvg* locus is provided in trans, whereas the *ptx* and *cya* promoters remain silent (34).

In line with these observations, spontaneous *B. pertussis* mutant, BC75 and RPV3, were recently isolated, which showed a strongly reduced transcription of the *ptx* and *cya* loci, while other virulence factors were unaffected (9). BC75 and RPV3 contain mutations within the *rpoA* gene encoding the α subunit of RNA polymerase (10, 52). The α subunit, a 36 kDa protein, has been demonstrated to be directly involved in the activation of various prokaryotic promoters. Several mutants that are deficient in transcription activation from certain promoters indicated that the C-terminal region of the α proteins is involved in direct protein-protein contact with class I transcription factors, which bind upstream of the –35 region of the respective promoters (25). However, BC75 and RPV3 define a new class of α mutants, as they do not harbour mutations in the protein itself, but they contain point mutations within the putative Shine-Dalgarno sequence or even further upstream of the *rpoA* reading frame. These mutations increase the translational efficiency of the *rpoA* gene causing a 2–3 fold overexpression of α (10). Obviously, the excess of the RNA polymerase α subunit leads to a down regulation of the toxin promoters. A similar effect was recently described in *E. coli*, where overproduction of α reduced OmpR dependent activation of porin genes without affecting housekeeping genes (8). Although the mechanism by which overexpressed α causes the lack of transcription of the toxin promoters in *B. pertussis* remains unclear, it is possible that excess of α titrates out transcription factors for the activation of the toxin promoters.

Two more *B. pertussis* mutants were recently described with a similar phenotype as the *rpoA* mutants described above. Both mutants were defective for *ptx* and *cya* expression, but expressed normal amounts of *fha*. The respective mutations were identified in the C-terminal portion of the BvgA response regulator near the putative helix-turn-helix motif (51). It was speculated that the mutations may reduce the ability of BvgA to promote transcription activation without affecting its DNA-binding capacity. A similar effect of mutations in the extreme C-terminal region was suggested for the LuxR protein of *Vibrio fischeri* (13).

To explain these apparent differences in the mechanism by which the two subclasses of promoters within the *bvg* regulon are activated, three models have been proposed:

(i) BvgA is the universal activator of the various virulence factors, but different affinities of this protein for the respective binding sequences in the promoters cause the differential effect of gene activation.

(ii) Specific transcription factors, in addition to BvgA, are involved in the activation of the toxin promoters. These additional factors should be regulated by the *bvg* locus, thus resulting in a cascade of regulatory molecules similar to the situation recently described for the ToxR/ToxT system in *Vibrio cholerae* (17).

(iii) This model combines features of the other two models, and proposes that BvgA may be directly involved in the activation of all virulence promoters, but additional accessory proteins are required in the case of the toxins, which, however, are not specific for the toxin promoters.

Evidence for the various models has been reported in the past years and is summarized briefly in the following. In contrast to earlier results, it was shown recently, by in vitro band shift experiments, that BvgA can directly interact with the toxin promoters, but only after phosphorylation of the purified BvgA protein (Karimova, G., Bellalou, J., Ullmann, A., 1995. 7th European Workshop

Conference on Bacterial Protein Toxins). Obviously, in the former experiments the phosphorylation status of BvgA was not sufficient for the detection of a specific interaction with these promoters. This is the first clear evidence for the involvement of BvgA in the activation of the toxin promoters. However, other investigations suggested the participation of additional factors in the activation of the toxin promoters. The detailed characterization of these promoters by mutation analysis revealed that the relevant cis-acting sequences, which are probably the binding sites for transcription factors, are located far upstream from the RNA polymerase binding site around sequence position –120 to –160 (18, 21). Therefore, they are too far upstream to enable a direct interaction between a potential regulatory protein (e.g. BvgA) and the RNA polymerase.

These upstream activating sequence (UAS) elements do not have enhancer like properties, as their position relative to the RNA polymerase binding site can not be changed. Additional copies of the UAS placed further upstream in front of the regular promoter region caused a decrease in promoter activity (22). This recalls other promoters such as the AraC activated promoters, in which a nucleoprotein complex composed of AraC and other proteins induces DNA looping, which brings AraC and RNA polymerase in close contact, and is necessary for the activation of transcription (30). This may indicate that similar DNA structures may play a role also in the case of the toxin promoters. In line with these results, several reports showed an involvement of the DNA topology in the activation of the virulence promoters.

In the *E. coli* heterologous host, Scarlato et al. (48) showed an increase in *ptx* transcription in the presence of DNA gyrase inhibitors. This effect was reproduced in a in vitro transcription assay, where the addition of DNA gyrase strongly reduced *ptx* transcription, an effect, which could partially be compensated by the action of topoisomerase I. In a study performed in the natural host *B. pertussis*, it was shown that DNA topology indeed plays a role in the control of virulence regulation, because the presence of DNA gyrase inhibitors had a negative effect on the expression of most of the virulence promoters including those of the toxins, whereas one virulence promoter, that of the pertactin *prn* gene, was switched on (20). Interestingly, the presence of DNA gyrase inhibitors had the contrary effect in the natural and the heterologous host, which may indicate that the *ptx* promoter may have a different structure in the heterologous host, indicating that DNA topology may be one reason for lack of expression of the toxin promoters in *E. coli*. It is therefore possible that more general factors such as the histone-line proteins, which locally influence the DNA topology, may participate in the activation of these promoters.

The first histone-like protein of *B. pertussis* has been cloned recently, but so far no interaction of this protein with expression from virulence promoters has been reported (49). More direct evidence for additional factors involved in toxin expression was recently reported by DeShazer et al. (16), who cloned a factor termed Baf which enhanced Bvg dependent expression of *ptx* in *E. coli*. However, the Baf protein does not show any homology to transcription factors, and its mode of action remains unclear.

In conclusion, the observed binding of phosphorylated BvgA to the toxin promoters provides strong evidence for a direct involvement of BvgA in the activation of these promoters. BvgA may indeed be the universal transcription activator of the Bvg activated virulence genes. This assumption is also supported by the failure of many different attempts carried out by various groups to identify additional transcription factors specifically required for the activation of the toxin promoters.

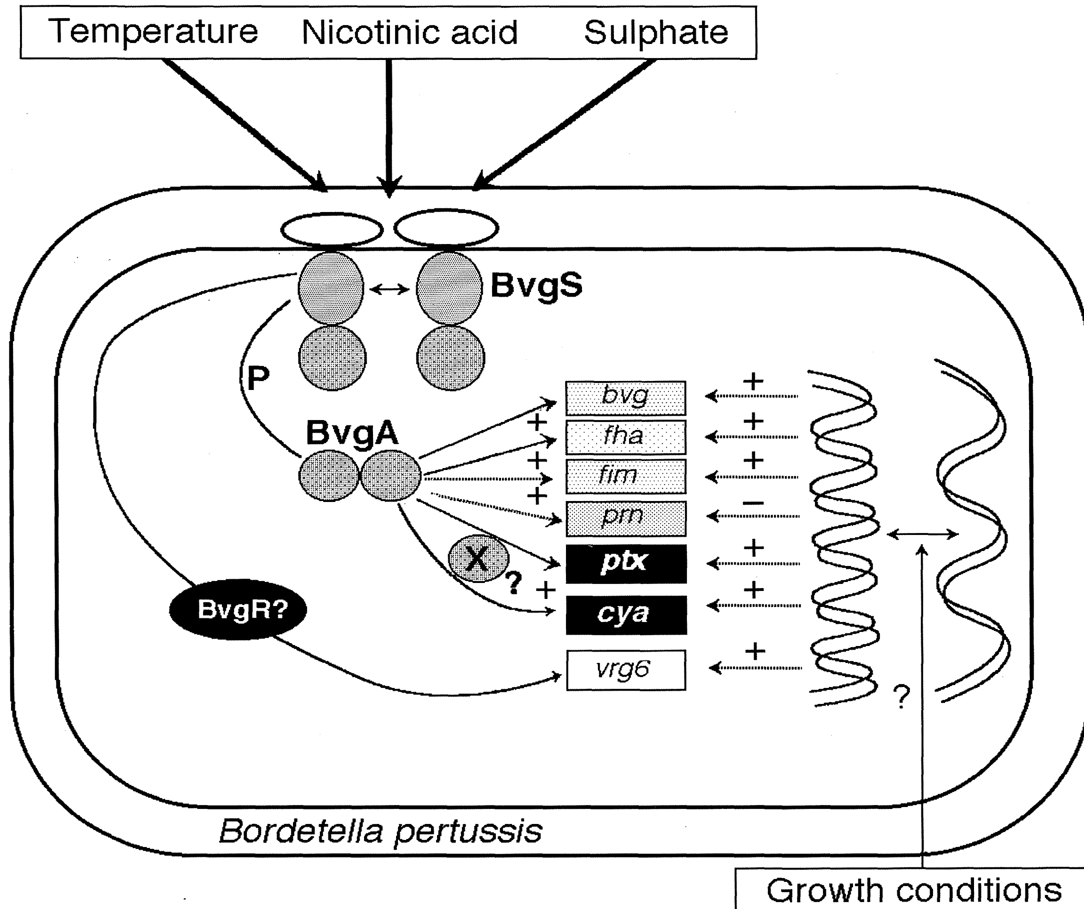

FIG. 2. Model for the differential regulation of virulence factors in *Bordetella pertussis*. The activation of the toxin promoters (*ptx*, *cya*) occurs in a different way than activation of the *fha* and *bvg* promoters. Additional factors "X" such as Baf might be involved in toxin regulation. The activation mechanism of the *prn* and *fim* promoters has not been studied so far. The *vrg6* gene seems to be repressed by the *bvg* activated BvgR repressor protein. The influence of DNA topology on the expression of the various promoters is also shown.

The finding that the phosphorylation status of BvgA is very critical for its binding capacity to the *cya* promoter further suggests that different binding properties of BvgA at the various promoters may be the major reason for the differential effects in transcription described above. In addition, evidence is accumulating that accessory factors such as the Baf protein in the case of the *ptx* promoter or factors involved in DNA topology may also be involved in the transcriptional activation of these promoters (Fig. 2). Therefore, although our understanding of the mechanisms underlying the regulatory phenomena which control virulence in *B. pertussis* has made enormous progress in the past years, we are still far away from understanding their molecular details.

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Interactions between professional phagocytes and *Brucella* spp.

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Summary

Induced pathogenicity in animals and humans differs considerably. This review is devoted to the relations between *Brucella* spp. and professional phagocytes, particularly macrophages and macrophagic cell lines in vitro. Although numerous studies have been reported, the type of ingestion by macrophages, the receptor involved, and the molecular mechanisms, are poorly understood. The ability of most *Brucella* species to actively inhibit their ingestion by neutrophils or macrophages has been proposed as an explanation for the poor rate of in vitro phagocytosis and in vivo alteration of the phagocytic cells.Oxidative burst plays a significant role in the antibacterial processes of phagocytic cells. The effects of whole or fractioned *B. abortus* on the ability of neutrophils to induce an oxidative burst in response to stimulation with opsonized zymosan particles were examined. Besides oxygen-based killing, the phagocytic cells have developed other types of defence, including hydrolytic enzymes and reactive halides. Inside the cell, the bacteria encounter new environmental conditions. Their survival is conditioned by an adaptation to this new situation. Pathogens that have acquired the ability to multiply within macrophages should synthesize products specifically interacting with the host cell defence system. Survival of intracellular pathogens is closely linked to the mechanisms of evasion from cellular defences. Brucellae stay in membrane bound vacuoles called phagosomes, but the exact nature and the maturation pathway of this compartment have not yet been understood. Macrophages play a central role in the evolution of brucellosis; this first interaction between the pathogens and the cell will determine the course of the disease. There are natural differences between brucellae species regarding macrophage response to the bacteria.

Key words: *Brucella*, macrophages, cytokines, phagocytosis, host resistance

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Resumen

La patogenicidad inducida difiere considerablemente en animales y en humanos. Esta revisión está dedicada a las relaciones entre *Brucella* spp. y fagocitos profesionales, particularmente macrófagos y líneas de células macrofágicas in vitro. Aunque se han realizado numerosos estudios, apenas se conoce el tipo de ingestión utilizado por los macrófagos, el receptor implicado o los mecanismos moleculares implicados. La capacidad que tienen la mayoría de las especies de *Brucella* de inhibir su ingestión por neutrófilos o macrófagos ha sido propuesta para explicar la poca frecuencia de fagocitosis in vitro, y las alteraciones de las células fagocíticas in vivo. La súbita respuesta oxidativa tiene un papel destacado en los procesos antibacterianos de las células fagocíticas. Se estudiaron los efectos de la bacteria *B. abortus*, entera o fraccionada, sobre la capacidad de los neutrófilos para inducir una súbita respuesta oxidativa a la estimulación con partículas de zymosan opsonizado. Además de la muerte basada en la respuesta oxidativa, las células han desarrollado otros medios de defensa que incluyen enzimas hidrolíticas y reactivos haluros. En el interior de la célula, la bacteria encuentra nuevas condiciones ambientales; su supervivencia depende de la adaptación a esta nueva situación. Los patógenos que han adquirido la capacidad de multiplicarse en el interior de los macrófagos deberían sintetizar productos que interaccionen específicamente con el sistema de defensa de la célula hospedadora. La supervivencia de los patógenos intracelulares está estrechamente relacionada con los mecanismos de evasión de las defensas celulares. Las brucelas pueden encontrarse en vacuolas limitadas por membranas llamadas fagosomas, pero aún no se comprende bien la naturaleza exacta y el mecanismo de formación de este compartimento. Los macrófagos desempeñan un papel esencial en el desarrollo de la brucelosis; esta primera interacción entre los patógenos y la célula determinará el curso de la enfermedad. Hay diferencias naturales entre las especies de *Brucella* en cuanto a la respuesta de los macrófagos a estas bacterias.

Introduction

Phagocytic cells constitute an effective line of defence against most invading microbial pathogens. In contrast to extracellular parasites, facultative intracellular parasites survive by mechanisms which enable them to evade, inhibit or resist intraleukocytic killing systems. The genus *Brucella* is composed of facultative intracellular bacteria that infect humans and animals. *Brucella* are Gram-negative bacteria. Their LPS and the peptidoglycane layer, however, possess certain distinctive features (25, 26).

The diseases induced in animals and humans differ considerably. Brucellosis in humans is a debilitating disease with diverse pathological manifestations including fever, chills, sweats, weakness, malaises and body ache. Chronic brucellosis may also lead to endocarditis, arthritis, meningitis, pancreatitis, spondylitis, osteoarticular complications and neurological complications. Animal species including cattle, sheep, goat, dog, elk, bison, camel and pig are also infected. The pathology is characterized by abortion due to colonization of the placenta. Mice are not infected in nature. In the laboratory, infected mice show a transitory bacteraemia.

Brucella spp. infect and multiply in professional as well as non-professional phagocytes and localize in macrophages. The bacteria use the host cells as a vehicle to invade the organism. Survival in macrophages may be the basis for the establishment of a chronic infection. Macrophages occupy a central position in the immune system. They are

involved in direct killing and elimination of parasites, they work as antigen presenting cells (APC), and also as modulators of the immune response by secreting a variety of cytokines. They constitute a keystone of the host system responsible for the elimination of parasites. To correctly understand the published results, it should be borne in mind that macrophages from different hosts respond differently to the interaction with *Brucella* spp.

This review is devoted to the relations between *Brucella* spp. and professional phagocytes, particularly macrophages and macrophagic cell lines in vitro. The experiments using either PBMC (Peripheral Blood Mononuclear Cells) or whole animals are reported only if they can enlighten the results obtained in vitro.

Relationship between virulence in vivo and growth within the macrophage

Due to the central position of the macrophage in the invasion process of the host by *Brucella*, the question of a possible relationship between intramacrophagic growth and the in vivo behavior leading to brucellosis is of great importance for the in vitro analysis. Numerous studies have shown that *Brucella* spp. can develop in vitro within macrophages or macrophage-like cell lines. Peritoneal macrophages from guinea pigs were used in early studies to demonstrate a correlation between the virulence of strains of *B. abortus* and their ability to survive in macrophages. Braun et al. (4) found that rough strains of *B. abortus* are ingested by guinea pig macrophages more rapidly than smooth strains but grow more slowly once inside the cell. More recently, it has been shown that *Brucella* species such as *B. suis* and *B. melitensis*, which are able to induce chronic infections in humans, multiply inside human macrophage-like cells (8). On the contrary, the rough species *B. canis* and *B. ovis*, very rarely involved in human brucellosis, are not able to multiply within these human cells (8). Resident peritoneal macrophages from BALB/c mice eliminate intracellular *B. abortus* strain 19 much more effectively than strain 2308. Similarly, it has been shown with bovine macrophages that virulent strains of *B. abortus* have increased survival and growth rates in macrophages, as compared to non-virulent strains or rough mutants (15).

Although the structure of LPS of *Brucella* spp. it appears to be involved is not the only factor responsible for virulence and survival within macrophages (15).

Phagocytosis

Macrophages are phagocytic cells, and their early interaction with pathogens determines the outcome of the infection. The type of phagocytosis, the nature of the receptor, and the activation of the cell are critical variables. In the case of *Brucella*, although numerous studies have been reported, the type of ingestion, the receptor involved, and the molecular mechanisms are poorly understood. The ability of most *Brucella* species to actively inhibit their ingestion by neutrophils or macrophages has been proposed as an explanation for the poor rate of in vitro phagocytosis (7) and in vivo alteration of the phagocytic cells (29). Indeed, non-opsonized *Brucella* spp. are poorly ingested by macrophages or PMN. Opsonization is a prerequisite for significant uptake. Young et al. (35) have shown that virulent as well as attenuated strains are rapidly phagocytized by neutrophils only after opsonization with normal human serum. Studies on the interactions between bovine mammary gland macrophages and smooth or rough strains of *B. abortus* suggest that both strains are readily ingested only if they are opsonized with either complement or specific antibodies (15). Studies using the human

cell line U937 differentiated into macrophage-like cells, reveal a very low rate of ingestion (8) that readily increases after opsonization with IgG (9). Although professional phagocytes ingested non-opsonized *B. suis* very poorly, we recently found that cytochalasine inhibited this function, suggesting the participation of actin polymerization in this process (Köhler, S., Liautard, J. P., unpublished results). The ability of *B. abortus* to alter the phagocytic activity of bovine neutrophils was investigated by determining the effects of whole and fractionated preparations of virulent *B. abortus* on the ingestion of radiolabeled *S. aureus*. No effect has been found (5).

Taken together, these results suggest that *Brucella* does not evade phagocytosis by macrophages or PMNs. However, the natural receptor used for uptake in the absence of opsonizing antibodies has not yet been identified. This receptor and the pathway of entry linked to it may be of great importance for the pathogenicity of the bacterium as opsonized *B. suis* develop only poorly inside human macrophages (9).

Respiratory burst, production of oxygen radicals

Oxidative burst plays a significant role in the antibacterial processes of phagocytic cells. Initial studies of the effects of *Brucella* on the respiratory burst indicated that ingestion did not induce the production of reactive oxygen intermediates (O°)(see references 8, 23, 27). These experiments were performed without opsonization. Bacteria exposed to fresh serum containing anti-*B. abortus* antibody, heat-inactivated anti-*B. abortus* serum, or fresh normal serums were evaluated for their ability to stimulate an oxidative response. Brucellae exposed to fresh antiserum or to heat-inactivated antiserum are capable of inducing the release of significant amounts of O°, comparable to those released following stimulation with opsonized zymosan. In contrast, non-opsonized brucellae or brucellae treated with normal serum failed to induce the oxidative burst (3, 15, 23).

The effects of whole or fractionated *B. abortus* on the ability of neutrophils to induce an oxidative burst in response to stimulation with opsonized zymosan particles were examined. Neither fractions nor whole cells inhibited the production of O° (5). The activity of *Brucella* LPS was tested. Both *B. abortus* intact LPS and lipid-A are very weak activators of nitroblue tetrazolium reduction, but did not inhibit the burst. The comparison with *Salmonella* LPS and lipid-A revealed that at least 100 times more LPS and 1,000 times more lipid-A of *Brucella* are required to induce significant oxidative burst (31). In mice, the reactive nitrogen is involved in the clearing of many parasites. Peritoneal mouse macrophages produce nitrite following IFN-γ activation and *Brucella* infection. However, only a minor role for nitric oxide in anti-*Brucella* activities was evidenced (20).

Non-opsonized *Brucella* spp. or *Brucella* spp. opsonized only by complement, apparently evade the oxidative burst reaction. The bacteria opsonized with antibody induce the production of reactive oxygens.

Inhibition of the degranulation-dependent killing by neutrophils

Besides oxygen-based killing, the phagocytic cells have developed other types of defence, including hydrolytic enzymes and reactive halides. The latter system is primarily found in neutrophils (PMN). By using granule extracts, the MPO–H_2O_2–halide system appears to be effective in killing *Brucella*, if the system is allowed to function normally (23, 32). However, electron microscopy, has shown that degranulation involving both primary and secondary granules is

inhibited by *B. abortus* (32). Degranulation is required for the release of myeloperoxidase (MPO) and to kill *Brucella*. Thus inhibition of degranulation is a major event in virulence that allows *Brucella* to survive the encounter with PMNs. The absence of degranulation is due to an active inhibitory process initiated by the bacterium. Indirect evidence for inhibition of degranulation by *B. abortus* has been obtained from studies of the effects of live or heat-killed bacteria upon the iodination activity of bovine neutrophils (5). The results of these studies indicate that protein iodination by neutrophils is significantly suppressed in the presence of either viable or killed bacteria. Furthermore, iodination activity is also significantly inhibited in the presence of crude supernatants of *B. abortus* (5). After purification, the fraction inhibiting degranulation, contains nucleotide components (5), and 5'-guanosine monophosphate (GMP) and adenine has been identified as active compounds (6). The iodination assay was used to evaluate the ability of neutrophils to bind inorganic iodine to proteins. The degranulation of primary granules that release the MPO enzyme is necessary to catalyze this reaction. Thus GMP and adenine released by brucellae inhibit the degranulation of neutrophils. Further studies (2) have been performed in which transmission electron micrographs of bovine PMNs exposed to *B. abortus* extract have been analyzed morphometrically. Again, these studies indicate that the extract inhibits MPO–H_2O_2–halide activity by specifically inhibiting degranulation of peroxidase-positive PMN granules, this means the primary granules.

These experiments have demonstrated the efficiency of reactive halides in killing brucellae. To avoid these dangerous components, *Brucella* spp. excrete GMP and adenine, which inhibit the degranulation and thus the liberation of MPO enzyme necessary to produce reactive halides. Macrophages have no MPO, and the degranulation during phagocytosis of *Brucella* spp. by macrophages has not yet been investigated.

Bacterial proteins specifically induced inside the phagocytes

Inside the cell, the bacteria encounter new environmental conditions. Their survival is conditioned by an adaptation to this new situation. Pathogens that have acquired the ability to multiply within macrophages should synthesize products specifically interacting with the host cell defence system. They have to sense the environmental conditions of the macrophage and synthesize new proteins to keep off cell attacks. All the bacterial proteins specifically induced inside the cell should be considered as virulence factors. Lin and Ficht (24) studied the protein profile of *B. abortus* induced in response to the cellular environment of J774A.1 murine macrophage-like cells. Prominent changes observed include increased synthesis of *Brucella* proteins with estimated molecular masses of 62, 60, 28, 24 and 17 kDa. The 62 kDa protein has been identified as GroEL by antibody recognition. As the intracellular environment of the phagocytic cell may be to some extent mimicked by certain in vitro stress conditions, the authors have compared the proteins induced inside the macrophage with those induced by acid shock. Proteins of 60 kDa and 24 kDa seem to be induced in both cases, whereas the 28 and 17 kDa proteins have not been observed under any in vitro stress condition. The ability to withstand acidic environments is probably an important factor in the virulence of various intracellular bacteria including *Brucella* spp. (24). Recently, we have studied the role of DnaK in the survival of *B. suis* within macrophages (22). In contrast to the wild type strains, a *dnaK* mutant of *B. suis* cannot

multiply in the macrophage under conditions allowing normal growth in complex liquid medium. This result confirms the central role of this stress protein in the colonisation of the macrophage.

Analysis of *Brucella* spp. proteins specifically induced inside the macrophage and thus involved in virulence has to be dealt with in greater detail.

Maturation of the phagosome

The survival of intracellular pathogens is closely linked to the mechanisms of evasion from cellular defences. Brucellae stay in vacuoles that should be called phagosomes, at least in the macrophage. The biochemical composition of this structure is unknown. In epithelial cells or bovine trophoblasts, it has been claimed that *Brucella* spp. multiply within the reticulum (1, 12). However, neither biochemical nor immunological characterisation of this structure was performed, the results were only based on electron microscopy observations. In macrophages, it is generally admitted that *Brucella* spp. reside in a phagosome (15, 32) although no biochemical characterisation has yet been performed.

Experiments using acridine-orange have suggested that brucellae can alter the normal process of phagosome–lysosome fusion (14, 28). More recent electron microscopy experiments demonstrated that fusion is at least partially inhibited by *B. abortus*. About 50% of the bacteria are positive for phosphatase staining suggesting that a partial fusion occurs (15). These experiments were performed using IgG-opsonized *B. abortus*, and the maturation of the phagosome in the macrophage may be dependent on the route of bacterial ingestion. Indeed, it has been demonstrated that IgG-opsonized *B. suis* have a low rate of development in contrast to non-opsonized bacteria (9).

In search of the molecular mechanisms responsible for the inhibition of phagosome–lysosome fusion, experiments have revealed that a *B. abortus* extract is involved in this phenomenon (14). This property has been found only in virulent strains and is not associated with LPS. No further characterization has been performed (14).

To conclude, brucellae develop rapidly in a phagosome but the exact nature and the maturation pathway of this compartment are not yet understood.

Survival and growth within the macrophage, the role of macrophage activation

Brucella spp. survive and replicate in professional phagocytes. In vitro models have used either peritoneal macrophages, circulating monocytes, mammary bovine macrophages or mammalian cell lines.

Numerous studies using murine macrophages or a macrophage-like cell line (J774A.1) have been published. In mice, a few groups have demonstrated that murine phagocytes support the growth of virulent brucellae. Macrophages have the ability to kill some intracellular brucellae immediately after phagocytosis, but surviving intracellular bacteria multiply (18, 21). However, although IFN-γ enhances the ability of macrophages to kill and to control replication of intracellular *B. abortus* organisms, the cells remain parasitized. This includes resident (21), thioglycollate-elicited (18) and peptone-elicited (20) peritoneal macrophages. In addition, inhibitors of reactive oxygen intermediates partially block the anti-*Brucella* activities exhibited by both untreated and IFN-γ-activated macrophages. In contrast, addition of N^G-monomethyl-L-arginine which blocks the generation of nitric oxide, results only in a minor reduction of macrophagic anti-*Brucella* activity (20). Furthermore, the IFN-γ-induced enhance-

ment of anti-*Brucella* activities of peritoneal macrophages is inhibited by the addition of anti-TNF-α antibodies to the culture, indicating that TNF-α is necessary for full expression of the macrophagic anti-*Brucella* activities (20). However, in the mouse macrophages, direct addition of TNF-α to macrophage cultures does not affect survival and growth of *B. abortus* (18). Killing by IFN-γ-activated macrophages is also inhibited by addition to the cultures of transforming growth factor-β (TGF-β) (20). IL-1α, IL-4, GM-CSF and IL-6 have no consistent effect on the growth of non-virulent *B. abortus* strain 19 in J774A.1 murine cells. On the contrary, IFN-γ and IL-2 treatments result in a significant reduction in the number of intracellular bacteria (18). Participation of iron in mediating brucellicidal activity of IFN-γ-activated macrophages has been established (19). It has been found that iron-loaded macrophages activated with IFN-γ have a strikingly increased capacity to kill intracellular *B. abortus* (19). Increased bactericidal activity in the presence of iron was postulated to result from the ability of iron to catalyse the Haber-Weiss reaction (19).

Survival in bovine phagocytes has also been thoroughly analyzed. Fully virulent, smooth strains of *B. abortus* can grow in cultured bovine blood monocytes (13). Oxygen-dependent mechanisms are responsible for the brucellicidal activity of bovine neutrophils (32, 33). The results are quite similar using mammary gland macrophages (15).

In humans, *B. suis* survives and multiplies well in U937 cells differentiated into macrophages (8). IgG opsonization prior to infection increases the rate of phagocytosis but also reduces the growth rate of *B. suis* (9). However, we were not able to obtain a reduction of the intracellular development of *B. suis* using IFN-γ treatments (Gross and Dornand, unpublished results) under conditions that enhance the killing of *E. coli*. In contrast, TNF-α was found to be very effective in the same model (10).

Cytokines produced by the macrophage during infection

Macrophages are a central component of the immune response. The Th1/Th2 response is at least in part determined by the kind of response of the macrophage during its interaction with the pathogens. The type of response conditions the elimination or the fixation of the bacteria. Of central importance to this balance is the set of cytokines produced by the macrophage during phagocytosis of the bacteria.

Zhan and Cheers (36) have analyzed the abilities of living and of heat-killed *B. abortus* to induce production of TNF-α, IL-1 and IL-6 by in vitro-cultured macrophages. Significant although low quantities of TNF-α are found in the supernatant of naive macrophages infected with live *B. abortus* but not in the supernatant of those phagocyting killed *B. abortus*. Results obtained using peritoneal cells from infected mice reveal a large increase in the expression of TNF-α induced by live brucellae only, and still no TNF-α at all using killed bacteria. On the other hand, IL-1 is not produced by naive macrophages infected with live *B. abortus* and only marginally by the same kind of macrophages in contact with killed brucellae. In contrast, IL-1 is found in the supernatant of macrophages obtained from infected mice challenged with live brucellae. IL-6 is produced in large quantities in all cases described above. These responses always depend on a direct contact between macrophages and bacteria.

Upon infection of the human cell line U937 or blood monocytes by live *B. suis*, no detectable amount of TNF-α can be found in the supernatant (9). Under the same conditions, killed brucellae induce the production of significant amounts of TNF-α. We have recently shown (10) that this phenomenon is due to the production by *Brucella* spp. of a protein factor that inhibits the TNF-α expression by macrophages. Furthermore, this

factor is active only on human macrophages, and is not able to inhibit TNF-α expression by murine macrophages. The inhibition of the TNF-α production is specific because neither IL-1 nor IL-6 are blocked. These results therefore suggest the presence of a species-specific receptor for this inhibitor.

Innate host resistance and macrophage infection

Genetic resistance to infection by *Salmonella*, *Mycobacterium*, or *Leishmania* has been clearly established. In mice, this resistance is monogenetically determined by the so-called gene *bcg/ity/lsh* and is mediated at the level of macrophage function. Resistance to brucellae seems to be more complex and of different origin. In inbred mice, resistance to the attenuated *B. abortus* strain 19 occurs in *bcg/ity/lsh*-susceptible mouse strains C57B1/10 and BALB/c, while susceptibility to brucellosis occurs in the *bcg/ity/lsh*-resistant CBA mice (17). Natural resistance to brucellosis is thought to be determined by the action of several interacting genes (17, 34). It has not been established whether resistance or susceptibility among inbred mouse strains correlate with the ability of their macrophages to regulate intracellular growth of *Brucella* spp. in macrophages.

On the other hand, the natural host resistance in cattle has been analysed. In vaccine trial experiments it has been found that up to 30% of the cows that have not been vaccinated are naturally resistant to *B. abortus* infection (11). Analysis by Price et al. (30) has shown that the mammary macrophages and blood monocyte-derived macrophages of resistant and susceptible cows are significantly different in their ability to control the intracellular survival of *B. abortus*. These results demonstrate that, in cattle, macrophages may play a central role in determining the final outcome of exposure to brucellae. As a possible explanation, Hamon et al. (16) stated that mammary macrophages from naturally resistant cattle are characterized by significantly stronger oxidative burst reactions and higher bacteriostatic activities than macrophages from susceptible cattle.

Natural resistance appears to be a result of the control of the early survival and growth of the bacteria by macrophages, and differences in the ability of macrophages to control replication of *B. abortus* have been determined even before exposure of the animals to the pathogen (30).

Conclusion: host specificity

Macrophages play a central role in the evolution of brucellosis. They are the first warriors, the messengers to the immune system, and the "Trojan horses" that allow the invasion. The first interaction between pathogens and the cell will determine the course of the disease. Due to the Darwinian adaptation of the parasite to the host, a great number of weapons have been developed by the resulting partners. New hosts lead to new forms of adaptation. It is evident that responses of different hosts to the same pathogen will be different. For instance, *B. abortus* spreads in bovine populations but not in human populations. *B. abortus* appears to be not "yet" adapted to the human host. It develops in humans causing a disease but does not spread in the human population, suggesting that humans are probably becoming a new natural host. The same is true for *B. melitensis*. Mice, on the contrary, are not infected in nature and eliminate the bacteria that probably have not developed the weapons necessary to block their defence mechanisms. Thus, there are natural differences between species in macrophage response to brucellae. For example, Th1 response of the mouse results in elimination of brucellae while, in humans, focalized brucello-

sis is associated with a Th2 differentiation. The effect of the difference in the TNF-α production in murine and human macrophages infected by *Brucella* spp. could be part of the explanation.

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Molecular basis of *Brucella* pathogenicity: an update

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Summary

Microorganisms belonging to the genus *Brucella* can infect humans and many species of animals. Virulence of the brucellae is thought to be essentially due to their capacity to survive and replicate within the phagocytic cells. However, many gaps remain in our understanding of this ability of brucellae to elude the bactericidal effects of host phagocytes, and basic questions remain unanswered. Identification of *Brucella* gene products which are related to intracellular survival, as well as those which contribute to the induction of protective immunity, is critical to elucidate the molecular mechanisms of the pathogenesis of the organism. The present article summarizes the current status of the research on gene products and other structural or metabolic factors associated with virulence of the brucellae.

Key words: *Brucella*, intracellular survival,outer membrane proteins, gene products, virulence factors

Resumen

Los microorganismos pertenecientes al género *Brucella* infectan a la especie humana y a diferentes especies de animales. La virulencia de las brucelas se debe fundamentalmente a su capacidad para sobrevivir y multiplicarse en el interior de las células fagocíticas. Sin embargo, nuestro conocimiento actual sobre esta habilidad de *Brucella* para eludir los efectos bactericidas de los fagocitos del huésped es limitado, y aún permanecen por resolver cuestiones básicas. La identificación de los productos de expresión génica implicados en la supervivencia intracelular de las brucelas, o aquéllos que contribuyen a la inducción de una respuesta inmunitaria protectora, es de suma importancia para dilucidar, a nivel molecular, los mecanismos patogénicos del microorganismo. El presente artículo resume el estado actual de la investigación llevada a cabo sobre los productos génicos y otros factores, estructurales o metabólicos, asociados con la virulencia de las brucelas.

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Introduction

Brucella species are non-motile Gram-negative facultative intracellular bacteria that produce brucellosis in a broad range of domesticated and wildlife animals, and humans (73). Members of the genus *Brucella* have not been described to harbor plasmids or produce capsule or exotoxins. Like other intracellular pathogens, their virulence is mainly due to their capability to avoid the killing mechanisms and proliferate within macrophages. The brucellae not only resist killing by neutrophils following phagocytosis (71), but they also replicate inside macrophages (45) and non-professional phagocytes (20). These abilities are thought to be responsible for the establishment of chronic infections in the host. To date, little is known about how the organisms survive intracellularly. In neutrophils, *B. abortus* has been shown to inhibit degranulation (12) and the oxidative burst (48), whereas in macrophages it survives principally by preventing phagolysosomal fusion (30). The mechanisms and virulence factors responsible for this capacity of brucellae to escape the bactericidal effects of host phagocytes are not well understood. Application of molecular genetic techniques should lead to a better understanding of the molecular basis of *Brucella* pathogenesis. In this article we focus on current information on gene products and other bacterial factors associated with virulence of the brucellae.

Structural virulence factors

The role of outer surface components in the virulence of extracellular microorganisms has been well established. However, their implications in the virulence of facultative intracellular parasites are less clear, and it is difficult to extrapolate from one genus to another. In contrast to other Gram-negative pathogens, the outer surface of the brucellae does not express structures such as fimbriae or capsular material. Thus, only two components of the outer membrane have been implicated as potential virulence factors: the lipopolysaccharide and the outer membrane proteins.

Lipopolysaccharide. In general, the lipopolysaccharide (LPS) of Gram-negative bacterial outer membrane contributes to the virulence of the organisms, since strains that lack the O-side chains of LPS, namely rough mutants, are less virulent (88). Relatively minor changes in the LPS carbohydrate structure of organisms that are facultative intracellular parasites may greatly influence the susceptibility of bacteria to the granule contents of lysosomes (69).

For most *Brucella* species, virulence is also associated with the smooth (S) colony morphotype, which contains the full LPS (S-LPS) (50). However, there are notable exceptions to this rule: the naturally occurring rough species *B. canis* and *B. ovis* are fully pathogenic, whereas the smooth *B. neotomae* species is nonpathogenic. *B. abortus*, *B. melitensis* and *B. suis* occur naturally in the smooth phase and contain a homopolymer of perosamine as the O-chain component of their LPS (11). This molecule appears to be an immunodominant antigen, since a major proportion of the protective antibody response of animals or humans infected or immunized with smooth *Brucella* species is directed against this antigen (21, 77). The strong humoral response elicited by the O-chain can cause problems related to the serological diagnosis of brucellosis. In fact, a proportion of the animals immunized with the vaccine strains *B. abortus* B19 or *B. melitensis* Rev-1 maintain agglutinating titers, which makes it difficult to distinguish between immunized and naturally infected animals on serologic examination (63). These antibodies have been shown to be protective, and protective immunity elicited by LPS has been extensively studied in a mouse model (65). Those experiments have shown that immune sera raised against the LPS extracted from smooth cell colo-

nies were very efficient in protection. More specific assays have also proven that monoclonal antibodies directed against the polysaccharide O-chain of LPS can confer protection on mice (54, 61).

From general observations of the biological activities induced by enterobacterial enterotoxins, it has been proposed that the toxin action of *Brucella* LPS mediates most of the pathogenic mechanisms of these bacteria (1, 29). However, by comparing the LPS properties of *Brucella* with those of LPS from other Gram-negative bacteria, it has been demonstrated that most of the biological activities induced by *Brucella* LPS are quantitatively and qualitatively different from those of enterobacterial endotoxin (6, 49, 53, 67, 68). Thus, *Brucella* LPS is not pyrogenic, does not activate the complement system to any significant level, and is a very weak mitogen for murine B cells. Moreover, at least 10 times more *Brucella* LPS is needed for lethality and interferon production than with enterobacterial endotoxin (46). These distinct biological activities induced by *Brucella* LPS are related to the unique structure of the molecule. This has been the reason for some authors to propose that the lower biological activity induced by *Brucella* LPS compared with enterobacterial endotoxin might be one of the factors contributing to the survival of these pathogens in phagocytic cells (67). Other investigators have shown that fully smooth virulent strains of *Brucella* multiply in macrophages at significant higher rates than rough strains (38, 48, 71). However, these comparisons have usually been performed between strains that are separated by many passages or generations, in which additional unnoticed phenotypes may be present.

Progress with respect to the real effect of LPS on virulence of brucellae would be attained by the existence of well-defined lineages of both smooth and their respective rough variants. Unlike spontaneously arising rough mutants or those induced by chemical mutagenesis (92), transposon mutagenesis is a suitable method to generate LPS deficient mutants. By using these methods, and assuming that insertions are located in the genes required for LPS biosynthesis, several authors have been able to isolate brucellae that are deficient in LPS production (66, 81). However, since spontaneous *Brucella* dissociation from the smooth to the rough form often occurs, one has to be cautious to ascertain that transposon insertion is the sole basis of phenotypic change. By comparing smooth virulent strain with avirulent vaccine strains and rough transposon mutants, Price et al. (66) demonstrated that the LPS of the cell wall is not the only factor determining the intracellular survival of *B. abortus*. These and other arguments have induced some authors to suggest that the maintenance of membrane integrity is a much more relevant factor to control the intracellular survival of *Brucella* than the sole presence or absence of S-LPS (67).

Outer membrane proteins. Different groups of proteins have been described in the outer membrane of *Brucella* spp. The major outer membrane proteins (OMP) include: a lipoprotein covalently linked to peptidoglycan (35), group 2 proteins (35–40 kDa) and group 3 proteins (25–30 kDa) (90). Group 2 proteins have been shown to have porin activity (22). It has been proposed, on the basis of amino acid composition (90) and heat-modifiability (31), that group 3 proteins could be the *Brucella* counterparts of *Escherichia coli* OmpA. However, recent studies of cloning and sequencing of the gene coding for the major 25 kDa Omp of *B. abortus* have concluded that these proteins are not of the OmpA family (91). Minor *Brucella* OMPs are group 1 proteins (88–94 kDa) (90), and so are other identified proteins (10–34 kDa) (16). All of these OMPs are exposed on the cell surface of brucellae, as demonstrated by immunological and electron microscope methods (16, 36).

A hypothetical role of bacterial OMPs in virulence can be inferred from their capacity to elicit a protective immune response directed against

these proteins. However, though protective immunity stimulated by OMPs is well known for several Gram-negative bacteria (33, 51), the implication of anti-OMPs antibodies in immunity to brucellosis remains unclear. Monoclonal antibodies to OMPs are less protective against infection of mice with *B. abortus* than monoclonal antibodies to S-LPS (17), even though they can be useful as diagnostic antigens (18). Confer et al. (19) stimulated an antibody response to extractable and chemically modified proteins from *B. abortus* B19 strain, but failed to protect cattle against an experimental challenge with strain 2308.

To prevent residual vaccine-induced antibody titers against the major immunodominant antigen (i.e., LPS) from interfering with the differentiation between sera of vaccinated and infected animals, research has been done to develop new experimental subcellular vaccines, such as cell envelope extracts or OMPs (42, 62). However, the examination of the protective immunity induced by *Brucella* cell envelope proteins is complicated by the presence of amounts of contaminating LPS as a result of the extraction methods. The cloning and expression of *Brucella* genes encoding the cell envelope proteins offer the advantage of obtaining antigens free of contaminating LPS and their evaluation in eliciting protective immune response in animals. The genes for several *Brucella* cell envelope proteins have been cloned, including periplasmic proteins (57) and OMPs (25, 89, 91). Halling et al. (37), by using methods of gene deletion and replacements, demonstrated that periplasmic BCSP 31 protein (31 kDa) was not implicated on invasion, growth or replication of *B. abortus* in the J774 macrophage cell line. Ficht et al. (25) reported the cloning of a region encoding the described group 2 porin proteins from *B. abortus* (*omp*2 locus). These authors demonstrated also that this *omp*2 locus contains two closely related genes, *omp*2a and *omp*2b, which are present in the genomes of all *Brucella* species (26, 27). However, although both genes contain open reading frames encoding proteins which are 96% homologous, expression from only *omp*2b has been demonstrated in laboratory-grown *B. abortus*, and corresponds to the characterized *Brucella* porin. This does not rule out the possibility of expression of *omp*2a in *B. abortus* under specialized conditions. Moreover, the *omp*2a and *omp*2b genes have been expressed in *E. coli*, and their products localized to the outer membrane of the cell, demonstrating the *omp*2a gene product to have a pore-forming activity (56).

Taking these results as a whole, it has been suggested that *Brucella* could adapt to changing environmental conditions by modulating porin gene expression, in a similar way to the well-studied example of environmental adaptation of *E. coli* by the regulation of its major porins, OmpF and OmpC. Accordingly, a change in the environment, such as the intracellular conditions, might stimulate a switch in porin expression and, as a result, the production of larger pores which, in turn, might be related to the ability of this organism to survive in professional phagocytes. Recent studies by Lin and Ficht (55) have revealed that phagocytosis is a stress condition which alters the synthesis of several proteins in *B. abortus*, as it has been described for other intracellular pathogens (2, 9). They observed proteins expressed only under macrophage specific induction or at pH values below 4.0, which suggested that those proteins played an active role in resistance to intracellular destruction within macrophage, which is the most relevant attribute of *Brucella* pathogenicity.

Enzymes and other gene products

Superoxide dismutase. The killing of most extracellular organisms by mononuclear phagocytes depends upon the capacity of these cells to convert oxygen to microbicidal metabolites, including reactive toxic oxygen intermediates such as superoxide anions (O_2^-) and hydrogen peroxide

(H_2O_2). Aerobic bacteria contain several protective enzymes which detoxify active oxygen species: superoxide dismutase (SOD), catalases, glutathione synthetase, and glutathione reductase (7). The contribution of a particular reactive oxygen intermediate-detoxifying enzyme in protection against phagocytic killing mechanisms may vary with the organism and the model system studied. One of these enzymes, SOD, protects cells from the toxic effect of superoxide radicals by converting them into H_2O_2 and oxygen (O_2). SOD has been shown to be protective for both *Nocardia asteroides* (8) and *Shigella flexneri* (28). It has also been suggested to act as a virulence factor in *Mycobacterium tuberculosis* (4). In *B. abortus* two distinct types of SOD, a Cu/Zn SOD and a Mn SOD, have been described (84). As they are present at similar rates in strains of different ranges of virulence, it seems that SOD does not play any role as a virulence factor.

This was confirmed by Latimer et al. (52), who, when comparing the virulence in mice of *B. abortus* 2308 and a Cu/Zn SOD-deficient mutant, did not find relevant differences with regard to splenic infections. Different results were obtained by Tatum et al. (85), who also obtained Cu/Zn SOD mutants of 2308 and B19 *Brucella* strains. They found that growth in HeLa and J774 cells was nearly identical in both the mutant and the parental strain. However, there was a slight difference in the course of infection in BALB/c mice between the parental and the mutant strains. There were consistently lower mean spleen weights and bacterial numbers in mice infected with the SOD mutant than those infected with the wild-type strain. Mice infected with the mutant also had fewer splenic lesions. Those differences were more marked over 10 to 26 days post-infection, which favoured the hypothesis that Cu/Zn SOD could play a role in the first steps of infection, but would not be necessary for the persistence of *B. abortus* in mice. The role of Mn SOD in the pathogenesis of *Brucella* has not yet been studied.

Catalase. Catalase also forms part of the antioxidant defense system of bacteria, and it has been suggested to protect *Brucella* from the toxic oxygen intermediates released by macrophages upon phagocytosis. Favouring this hypothesis is the fact that, within a given species, strains of high virulence show high catalase activities and strains of low virulence show low catalase activities (40). The periplasmic localization of *B. abortus* catalase (78) could also imply that its role is to defend the bacterium from exogenous H_2O_2 rather than to remove cytoplasmic peroxide produced by respiration. The most direct evidence implicating catalase as a *Brucella* virulence factor derives from studies by Jiang et al. (41), who demonstrated that the addition of exogenous catalase partially blocked the anti *Brucella* activities exhibited by both non-cytokine-treated and interferon-gamma-activated macrophages. However, the addition of exogenous catalase to macrophage cultures prior to phagocytosis does not really mimic the conditions encountered in situ throughout the infectious process of the host.

To study the contribution of catalase as a virulence factor during both the process of intracellular growth of *B. abortus*, and that of infection in vivo, we used the Tn*5* transposon to generate a catalase-negative mutant from the strain 2308. This mutant, named FJS1, shows a much greater sensitivity to H_2O_2 in a disk sensitivity assay than its parental strain. It was assayed for intracellular survival in the macrophage-like cell line J774, and for persistence in BALB/c mice spleens. Over the first hours of infection in vitro, FJS1 numbers were consistently one log lower than those of strain 2308. This was probably due to its increased sensitivity to H_2O_2. After this bactericidal phase, FJS1 recovers faster than 2308, reaching similar, or even greater numbers at 48 hours of infection. On the other hand, both strains show only small differences in their ability to colonize the spleen of infected mice until the second week of infection, which corresponds to the maximum of macrophage activation.

After this period, although FJS1 shows a significant reduction in splenic bacterial numbers with respect to 2308, both strains persist at least for 18 weeks. These results suggest that even though catalase might play a role in the survival of *B. abortus* within activated macrophages, this is not enough to produce an intracellular clearance of brucellae.

RecA. RecA is involved in several functions of DNA metabolism, including the mediation of recombination between homologous DNA fragments, recombination-repair of damaged DNA, and the initiation of the SOS response. RecA-mediated genetic rearrangements can potentially either increase the virulence of a pathogen or enable the pathogen to evade host defences, specially in an environment causing DNA damage, like that within macrophages during the oxidative burst. For example, in *Vibrio cholerae*, the amplification of the cholerae toxin gene requires RecA-mediated DNA duplication at sites of repetitive DNA sequences (34). In *Neisseria gonorrhoeae*, the mechanism for pilus phase and antigenic variation involves RecA-mediated homologous recombination (32). *Salmonella typhimurium* mutants in the recombination gene *recA* are attenuated in mice and sensitive to killing by macrophage in vitro (10). In *E. coli*, RecA is even more important than catalase and SOD to protect the bacterium against H_2O_2-induced DNA damage (14). Besides inducing single-strand DNA breaks, H_2O_2 may also impair the mechanisms of repairing of DNA, which are dependent on a *recA*$^+$ function. H_2O_2 is one of the toxic oxygen intermediates produced by macrophages during the oxidative burst, so *B. abortus* exposure to it is likely to occur. To determine if RecA plays a role in the virulence of *B. abortus*, Tatum et al. (86) cloned the *recA* gene, and constructed a *B. abortus recA* mutant by gene replacement. When administered intraperitoneally to BALB/c mice, numbers of bacteria per spleen were consistently lower in animals infected with the *recA* mutant than in those infected with the parental strain. This reduced survival of the *B. abortus recA* mutant in vivo may be attributed, in part, to increased sensitivity to oxidative mutagenesis. This indicates that RecA-mediated recombinational repair might play a major role in allowing the organism to survive in an environment causing DNA damage. However, the persistence of both the *recA* mutant and the parental strain in mice through 100 days post-infection, indicates that RecA is not crucial for the persistence of *B. abortus* in mice.

purE. There is a relationship between auxotrophy and intracellular survival or growth of several facultative intracellular pathogens. Auxotrophic mutations in essential metabolic pathways generate attenuated derivative strains with reduced virulence (3, 39), and these mutants are useful to develop genetically defined, live, attenuated vaccine strains of bacterial pathogens. In order to produce an attenuated and genetically defined mutant of *B. melitensis* for use as vaccine, Drazek et al. (23) cloned and sequenced the *purEK* operon of *B. melitensis* 16M and then produced a recombinant *B. melitensis purE* mutant. Further characterization of the mutant showed that it requires purines for growth and has a decreased ability to replicate in human monocyte-derived macrophages. Lysosomal enzyme secretion by macrophages is inhibited by adenosine and purine nucleosides convertible to adenosine (70). *B. abortus* expresses nucleotide compounds that inhibit primary granule release and myeloperoxidase-H_2O_2-halide bacterial killing by bovine polymorphonuclear leukocytes (12). This raises the possibility that brucellae inhibit phagosome–lysosome fusion by releasing purines. So, the reduction of the intracellular growth of *B. melitensis purE* mutant in monocyte-derived macrophages might be due to the inability of these mutants to produce excess purines, with the consequent failure to block phagosome–lysosome fusion. Although these results are promising, it would be necessary to investigate the level of attenuation, immunologic

response, and protective capability of this strain in animal models.

HtrA. High-temperature-requirement A protein (HtrA) is a protein implicated in the degradation of oxidatively and otherwise damaged proteins, that functions as a cellular stress response protein. Facultative intracellular pathogens, including *B. abortus*, generally express high levels of stress response proteins when growing in the intracellular environment. It has been hypothesized that stress response proteins play a major role in the physiological adaptation of the bacteria to the hostile environment of the host phagosome. Direct support for this hypothesis is the fact that the *S. typhimurium* HtrA stress response protein is required for virulence. *S. typhimurium* strains carrying a deleted *htrA* gene showed a >10,000-fold increase in the 50% lethal dose in BALB/c mice compared with the parental strain (15). Those mutants show also significant increases in sensitivity to oxidative killing in vitro in comparison to wild-type strains, and fail to replicate in macrophage cultures, which suggests that these strains may be defective in their ability to withstand the oxidative killing pathways of host phagocytes (43). As oxidative killing appears to be the primary mechanism by which infected host phagocytes eliminate intracellular brucellae (13), it was hypothesized that HtrA protein might contribute significantly to the survival of these phagocyted bacteria in phagosomes. *B. abortus htrA* gene was cloned and characterized by Roop et al. (72), and a *htrA* deletion mutant was obtained by Elzer et al. (24) via gene replacement. The *B. abortus htrA*-mutant fails to grow on solid medium at 40°C and possesses increased sensitivity to killing by H_2O_2 and O_2^- in disk sensitivity assays. The evaluation of the mutant in the BALB/c mouse model revealed a significantly lower level of splenic colonization than that obtained with the parental strain.

This result was attributed to a possible defect in the ability to withstand oxidative killing within macrophages. However, the study was only made at one week post-infection, and the authors did not state the behavior of the mutant at longer time periods. In a further study, the same authors (64) generated an *htrA* deletion mutant from the *B. melitensis* 16M strain. When the mutant was evaluated in the BALB/c mouse model, a significant attenuation at one week post infection was observed, similar to that previously described for *B. abortus* (24). However, at late timepoints the same numbers of 16M and the mutant were recovered from the spleens and livers of infected mice. Similar results were obtained by Tatum et al. (87), who cloned and sequenced the *htrA* and *htrA*-like genes from *B. abortus*, finding a number of discrepancies in relation to other published sequences of *B. abortus htrA* gene (72). Gene replacement *B. abortus htrA* and *htrA*-like mutants were constructed, and their survival and growth in BALB/c mice was compared to that of the parental strain 2308. After an initial decrease of splenic levels of *htrA* or *htrA*-like mutants, a recovering by 3 weeks post infection was observed, and after 60 days both were even higher than the parental strain. The basis for this described transient attenuation of brucellae *htrA* mutants in the murine model is currently unknown.

Erythritol

Early in the 1950's, erythritol, a four-carbon polyol, was demonstrated to be the only sugar which supported growth of all *Brucella* strains tested and belonging to the species *B. abortus*, *B. melitensis* and *B. suis* (58). Further studies also demonstrated that erythritol stimulated the growth of *B. abortus*. The ability to catabolize erythritol is almost universal in the genus *Brucella*. The avirulent *B. abortus* vaccine strain B19 has lost the capability to utilize erythritol, and is the only strain of *Brucella* which is inhibited by the presence of erythritol (1 mg/ml) in the medium (44). Since the isolation of erythritol from the placentas

of pregnant cattle by a group of British investigators (79, 80), it has been suggested that the presence of this sugar in bovine placental tissue would be responsible for the selective localization of virulent *B. abortus* in this tissue, where it replicates preferentially in the rough endoplasmic reticulum of chorioallantoic trophoblasts of the placenta (5) and resulting in placentitis, faetal death and abortion. Erythritol is also present in the placenta of sheep, goat and hog, but it is absent in the placenta of humans and rodents in general (80).

Considerable controversy has been raised concerning a possible relationship between erythritol utilization and *Brucella* virulence. Arguments in favour would be the susceptibility to this sugar of avirulent strain B19 and its synthesis in the ungulate tissues where *Brucella* is found in higher concentrations: placenta, seminal vesicles and testis (47). Arguments against are: (i) the preferential proliferation of brucellae which do not oxidize erythritol (*B. ovis*), or are even inhibited by it (B19), in the placenta of rumiants; (ii) although very low concentrations of the sugar are found in the placentas of rodents, *B. abortus* also localize preferentially in these tissues (44); and (iii) erythritol is not present in macrophages. Although Meyer (59, 60) demonstrated that the rate of oxidation of erythritol or growth enhancement by it were unrelated to the virulence of *B. abortus*, *B. melitensis* and *B. suis* for guinea pigs, her work was done by selecting spontaneous avirulent strains, where more than one mutational event may be involved in the reduction of virulence. Being erythritol the only known compound hypothetically implicated in the virulence of *Brucella*, the generation of mutants affected only in erythritol catabolism was imperative for the study of this relationship. Following transposon mutagenesis methods, we isolated a stable mutant of the *B. abortus* virulent strain 2308 that shares the sensitivity to erythritol with the vaccine strain B19 (74, 75). This mutant allowed us to clone a chromosomal fragment involved in erythritol catabolism in *B. abortus* (76). The comparison of sequences of cloned *eri* regions from strains 2308 and B19 identified a deletion in the chromosome of *B. abortus* vaccine strain B19.

The catabolic pathway for erythritol in *B. abortus* had been determined in the 70's (82, 83). Those studies found that B19 was defective in one of the enzimes of the pathway (D-erythrulose-1-P dehydrogenase), and this was thought to be the basis for the sensitivity to erythritol of this vaccine strain. Our studies have corroborated this, since further analysis of the cloned *eri* regions demonstrated that the deletion affects the gene which codifies for the lacking enzyme in B19. Furthermore, plasmids containing the entire *eri* region of 2308 were able to trans-complement the erythritol sensitivity of B19. We have also verified that the deletion occurs only in the vaccine strain, which is easily proven by means of PCR amplification using a pair of flanking oligonucleotides (76). This finding is a fast and reliable diagnostic method to differentiate B19 from all the other members of the genus *Brucella*.

We have revealed the genetic basis for erythritol sensitivity of the vaccine strain B19. But is the observed trait also related to the non-virulent phenotype or is it just a marker of the strain? In order to elucidate this, we have accomplished an allelic exchange in erythritol genes of *B. abortus* vaccine strain B19 by homologous recombination with the cloned *eri* region of virulent strain 2308. Following a gene replacement event we have obtained an isogenic *B. abortus* B19 strain which contains the entire *eri* region of 2308 and, like that virulent strain, is not inhibited by erythritol. Experiments using both macrophage and animal models are currently under way to test the virulence of the *eri*-mutant strains derived from 2308 and B19, in order to elucidate whether the capability to utilize erythritol is related to the virulence in *B. abortus*.

Conclusion

We should not presume that virulence of *Brucella* is a simple phenomenon, given the many manifestations and the broad range of brucellosis. As can be assumed for microbial pathogenesis in general, the ability of the brucellae to survive whithin phagocytic and nonphagocytic cells is likely to be multifactorial. Up to now, no single factor has been demonstrated to be responsible for most of *Brucella* virulence. However, the presence of virulent species that either are rough or do not utilize erythritol, points out the existence of other virulence factors. Finally, one can not undervalue the potential role of the host, which should extend our studies of *Brucella* pathogenesis to other disciplines such as cell biology and immunology.

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Regulation of virulence gene expression in pathogenic *Listeria*

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Summary

Dynamic interactions between host and pathogen are characteristic of infections caused by intracellular bacteria. This has favoured the evolution of highly effective control systems by which these pathogens regulate the expression of different virulence factors during sequential steps of the infection process. In the case of the facultative intracellular bacterium *Listeria monocytogenes*, these steps involve internalization by eukaryotic cells, lysis of the resulting phagosome, replication as well as movement within the host cytoplasm, direct cell-to-cell spread, and subsequent lysis of a double-membrane vacuole when entering neighbouring cells. Virulence factors which are involved in each of these steps have been identified and the expression of these factors is subject to a co-ordinate and differential control exerted by the major listerial virulence regulator PrfA. This protein belongs to the Crp/Fnr-family of transcriptional activators and recognizes specific target sequences in promoter regions of several listerial virulence genes. Differential expression of these genes during sequential steps of the infection seems to be at least partially mediated by different binding affinities of PrfA to its target sequences. Activity of PrfA-dependent genes and of *prfA* itself is under the control of several environmental variables which are used by the pathogen to recognize its transition from the free environment into a eukaryotic host.

Key words: *Listeria* pathogenesis, virulence gene regulation, bacterial virulence, infection cycle, intracellular parasitism

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Resumen

Una infección bacteriana es un proceso dinámico y competitivo en el que se establecen complejas interacciones moleculares entre el patógeno y el hospedador. En este contexto, las bacterias han desarrollado sofisticados sistemas de regulación génica, para adaptar de forma eficiente los niveles de expresión de sus factores de virulencia a las peculiares necesidades de las distintas etapas del proceso infeccioso. En el caso del patógeno intracelular facultativo *Listeria monocytogenes*, estas etapas incluyen procesos tan diversos como: (i) la invasión o internalización por parte de distintos tipos de células diana, (ii) la disrupción de las membranas del fagosoma resultante, (iii) la replicación en el citoplasma celular, (iv) la migración directa hacia células adyacentes utilizando como sistema de propulsión el citoesqueleto de actina de la célula hospedadora, y (v) la lisis del fagosoma de doble membrana que se produce en la célula invadida como consecuencia de dicho mecanismo de paso directo de célula a célula. Ya han sido identificados distintos factores de virulencia de *Listeria* implicados en cada uno de estos procesos. Todos ellos se expresan de forma coordinada, y a la vez diferencial, gracias al control ejercido por el activador transcripcional PrfA, de la familia de proteínas reguladoras Crp/Fnr. La proteína PrfA reconoce secuencias diana en las regiones promotoras de los factores de virulencia situados bajo su control, incluida ella misma, y la expresión diferencial de éstos parece obedecer, al menos en parte, a diferencias de afinidad de PrfA por sus dianas. La expresión de los genes dependientes de PrfA, y de su propio gen codificante *prfA*, está bajo el control de varios factores ambientales, que serían utilizados por *L. monocytogenes* para reconocer su transición desde la vida libre en el medio ambiente a la vida parásita en el hospedador eucariótico.

Introduction

At present, the Gram-positive bacterial genus *Listeria* comprises the six species *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi*. These bacteria are widespread in the environment and have been isolated from many sources including soil, sewage, decaying vegetation, and food. In addition to these natural habitats, *L. monocytogenes* and *L. ivanovii* have tapped the body of mammalian hosts as a source of growth and multiplication, thereby causing severe infections with high fatality rates. *L. ivanovii* is principally an animal pathogen. *L. monocytogenes*, can cause infections in both animals and humans. Pathogenic *Listeria* are able to overcome our intestinal barrier, survive the attacks of our immune system, and grow as well as move within our cells, thus belonging to the group of the "facultative intracellular parasites" which also includes the causative agents of e.g. tuberculosis (*Mycobacterium tuberculosis*), leprosy (*Mycobacterium leprae*), plague (*Yersinia pestis*), leishmaniosis (*Leishmania* spp.), trypanosomiosis (*Trypanosoma* spp.), or malaria (*Plasmodium* spp.). Mostly, immunocompromised individuals such as older people, pregnant women, newborns or transplantation patients, are affected by *L. monocytogenes* infections, showing as major clinical manifestations septicemia, encephalitis, meningitis, abortion, and stillbirth (16, 56).

To successfully establish an infection, pathogenic *Listeria* are equipped with a special set of virulence genes. The expression of these factors is subject to a complex and coordinate environmental control in order to ensure that they are transcriptionally silent when they are not needed, i.e., during life in the free environment, and are switched

on when the bacterium encounters a host. In this article, we briefly discuss the current knowledge on the, so far, only known listerial virulence gene regulator, PrfA, and on environmental parameters affecting virulence gene expression in pathogenic *Listeria*.

Infection cycle and molecular determinants of listerial pathogenicity

Intensive studies during the past years, involving molecular genetic approaches as well as an in vivo infection model in the mouse and tissue culture models, helped to evolve an image of the first processes that occur in an animal host during an infection with *L. monocytogenes* (for reviews see 39, 57). This picture can be summarized as follows (see also Fig. 1). Oral uptake of contaminated food seems to be the major natural route through which the pathogen gains contact with the host. Once inside the gastrointestinal tract, *L. monocytogenes* is able to cross the intestinal barrier via triggering its phagocytosis by normally non-phagocytic epithelial cells (see Fig. 1, steps 1 and 2). Subsequent to the lysis of the resulting phagosomal membrane, which occurs about 30 min after internalization (step 3), *L. monocytogenes* replicates within the eukaryotic cytoplasm and starts to induce the polymerization of host-cell actin on its surface (step 4). The actin filaments are rearranged to form a propulsive tail at one pole of the bacterial cell, providing the driving force by which the bacteria can move through the cytoplasm and reach the surface of the host cell. There they are extruded in a pseudopod-like structure (step 5) (for detailed reviews on actin-based listerial motility see the article in this issue by Chakraborty and refs. 5, 6, 52, 62, 63). After the uptake of the pseudopodium by a neighbouring cell, the *Listeria* disrupt the resulting double-membrane vacuole (step 6), escape into the cytoplasm of the new host

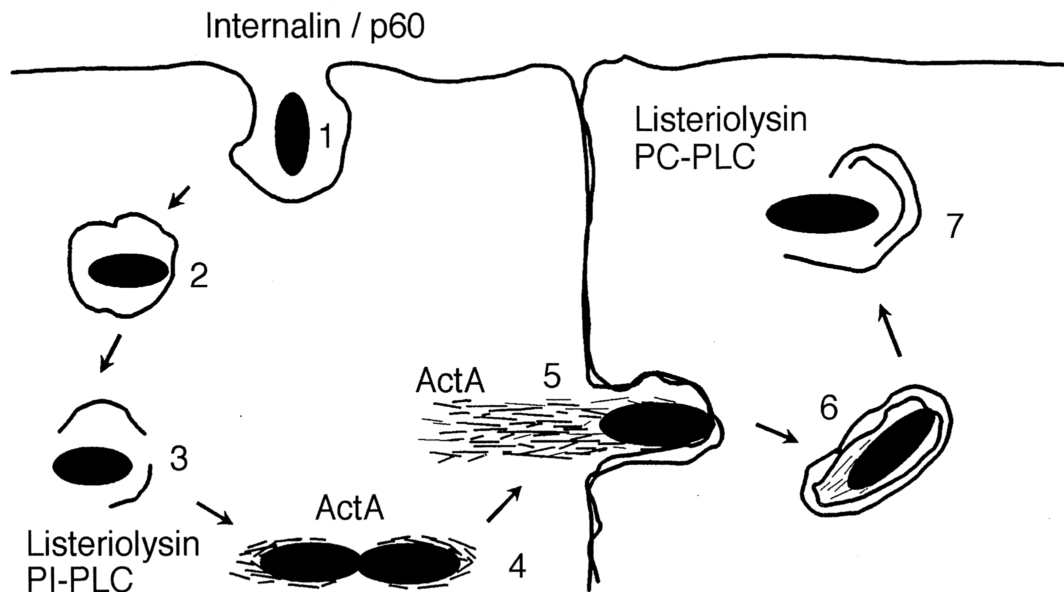

FIG. 1. The infection cycle of *Listeria monocytogenes*. Factors that are required for each step of the process are indicated (adapted from reference 63).

cell (step 7) and repeat the cycle of multiplication and intracellular movement. At present, it is unclear whether a process similar to this "cell-to-cell spreading" is involved in the subsequent uptake of *L. monocytogenes* by resident macrophages (which could be a mechanism to avoid the formation of microbicidal oxygen-derivatives in the phagocytic cell) or whether the bacteria are released into the extracellular lumen before encountering a macrophage. Once inside a macrophage, *Listeria* is able to induce the same cycle of events as in epithelial cells and use these phagocytic cells as means of transport to additional target organs (liver, spleen, brain, placenta) within the infected host.

Several listerial virulence factors involved in the complex phenomena mentioned above have already been identified (for reviews see 37, 39, 53, 57). The genes for six of these factors are located adjacent to each other on the *L. monocytogenes* chromosome, constituting the so-called "virulence gene cluster" (see Fig. 2). The monocistron *hly* encodes a secreted haemolysin (listeriolysin O, LLO) which is essential for the lysis of the phagosomal membrane, thus facilitating the escape of the *Listeria* into the host-cell cytoplasm. The so-called "lecithinase operon" is set up by the genes *mpl*, *actA*, and *plcB*, which encode a zinc-dependent metalloprotease, a protein essential for actin-polymerization in host-cells, and a broad substrate range phospholipase C which is active on phosphatidylcholine or lecithin, respectively. Like *hly*, *actA* is absolutely essential for *L. monocytogenes* virulence. Its gene product, ActA, is a surface protein involved in the nucleation of F-actin during actin tail formation. The *plcB* gene-product (PC-PLC) is involved in the lysis of the double membrane that arises during cell-to-cell spread and requires the *mpl*-encoded metalloprotease to be processed from an inactive proform to the enzymatically active form. Three further reading frames (ORFX, ORFY, ORFZ), located downstream of *plcB*, also form part of the lecithinase operon. The function of these ORFs and their possible role in virulence is, however, presently unknown (64). Finally, the genes *plcA* and *prfA* are expressed as a bicistron. *plcA* encodes a phospholipase C strictly specific for phosphatidylinositol (PI-PLC) that probably acts in concert with listeriolysin O in the lysis of the phagosome. As it will be discussed in detail below, the expression of all genes of the virulence cluster depends on the transcriptional activator protein PrfA, encoded by the autoregulated gene *prfA*.

There is at least one additional locus which is, in part, regulated by PrfA but does not belong to the virulence gene cluster: the *inlAB* locus (13, 20). *inlAB* encodes two homologous proteins (internalin A and B) that, in vitro, mediate in the uptake of *L. monocytogenes* by normally non-phagocytic epithelial cells (13, 20, 44), hepatocytes (14), human endothelial cells (15), and dendritic cells (27). It is assumed that, in vivo, both proteins are crucial for the initial invasion by *L. monocytogenes* of epithelial cells in the intestine, as indicated in Fig. 1. Not all genes with proven or suspected functions in listerial virulence are subject to regulation by PrfA. For example, the 60 kDa protein p60 (encoded by *iap*, for invasion associated protein), which presumably contributes to invasion of *L. monocytogenes* into non-phagocytic host cells (see Fig. 1), is expressed independently of PrfA (35, 36, 66).

Sequences homologous to all genes of the *L. monocytogenes* virulence cluster are also present on the chromosomes of *L. ivanovii* and *L. seeligeri*, but not in the remaining, avirulent and non-haemolytic, *Listeria* species (24, 25, 28, 38, 40). For *L. ivanovii*, this is not surprising because in cultured epithelial cells this species is capable of inducing the same events as *L. monocytogenes* (32) and, under laboratory conditions, it produces even higher activities of virulence factors such as haemolysin or phospholipases (54). Concerning

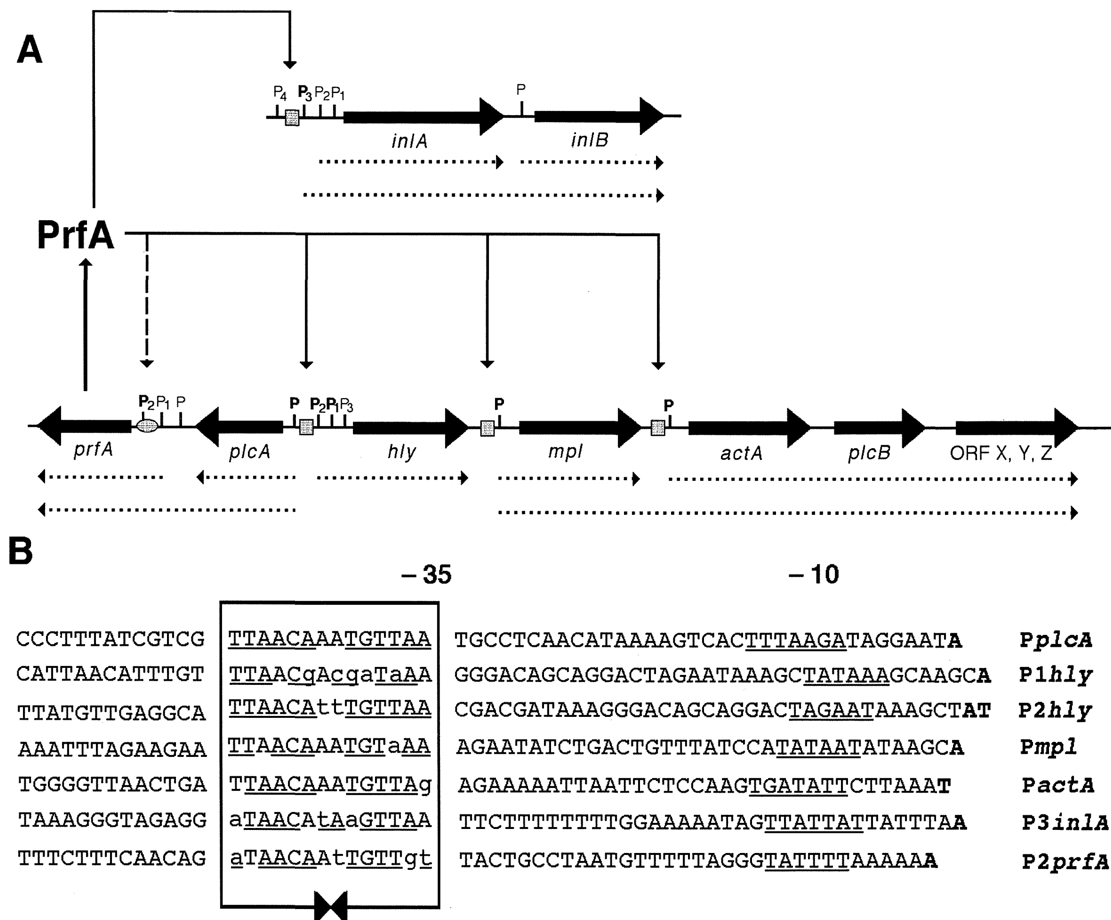

FIG. 2. PrfA regulated listerial virulence genes and their correponding promoter elements. A. Physical and transcriptional organization of the *Listeria monocytogenes* virulence gene cluster and the *inlA/B* locus. Identified mono- and polycistronic transcripts for the virulence cluster (below) and the *inlA/B* locus (above) are shown as dotted line arrows. Promoters (P) which are responsive to activation by PrfA are in bold. The location of "PrfA-boxes" is indicated by grey, square boxes. A grey oval marks the possible PrfA binding site close to promoter P2 of *prfA* which is presumably involved in *prfA* downregulation (19). Thin line arrows pointing from PrfA to promoters indicate activation; a broken line arrow, inhibition of transcription. Genes (thick arrows) and intergenic regions are not represented with their actual proportions. B. Structures of promoters which are activated by PrfA. Transcriptional start points (printed in bold), –10 and –35-motifs are shown. PrfA binding sites are boxed. Arrow heads on the box outline indicate palindromic structure; mismatching nucleotides are printed in lower case; complementary nucleotides are underlined. For the location of the respective promoters see Fig. 2A. The displayed sequences have been taken from references 19, 44, 58 and 64.

L. seeligeri, however, it seems that these bacteria are avirulent because most or all of the virulence genes are constitutively downregulated (24). Therefore, it is also important to examine the characteristics of virulence gene expression in *L. ivanovii* and *L. seeligeri* in order to gain a comprehensive insight into the regulatory elements and features that make up listerial virulence.

Environmental signals affecting virulence gene expression in *Listeria monocytogenes*

Life in the free environment is not less competitive than inside a host. Consequently, facultative intracellular bacteria are forced to turn off the production of their virulence-specific factors within any surroundings where they are not necessary for survival and, therefore, would just result in a waste

of energy. However, these bacteria must also be able to readily recognize the transition from the environment into a potential host, which can be accompanied by the need to dramatically increase virulence gene expression. For these purposes, evolution has equipped them with highly sophisticated signal-sensing and -transduction systems in order to properly control gene expression. Since knowledge on environmental cues that are used by pathogens to discern their entry into the host could be very useful in developing new therapeutics or preventive treatments, investigations on virulence-associated signal-transduction systems of pathogenic bacteria have gained significant interest in recent years.

A list of known signals that affect the production of virulence factors in *L. monocytogenes* is presented in Table 1. Temperature is the first environmental parameter that has been studied with respect to listerial virulence gene regulation. At 37°C (body temperature) *L. monocytogenes* significantly increases the production of all PrfA-regulated virulence factors, and of PrfA itself, whereas at temperatures below 30°C virulence gene expression is coordinately repressed (9, 43). Thermoregulation of virulence gene expression has also been shown to occur in other pathogenic bacteria, e.g. *Vibrio cholerae*, *Bordetella pertussis*, *Shigella* spp., or *Yersinia* spp., emphasizing the importance of temperature as a mammalian host-derived signal used by pathogens to distinguish between life in the free environment and within the host (46). During growth of *L. monocytogenes* within macrophages, enhanced transcription of *plcA* and several other genes which are presumably involved in nucleotide biosynthesis or amino acid transport into the bacterial cell seems to occur (33). Furthermore, an induced synthesis of ActA has been observed when *L. monocytogenes* is replicating within the cytoplasm of infected macrophages (1). These data suggest that there are probably certain cytosolic signals which stimulate the expression of bacterial factors that are specifically required for intracellular growth and movement. Another, recently discovered regulatory mechanism is the repression of *hly* and *plcA* by the disaccharides cellobiose and arbutin, both β-glucosides, when present in the growth medium (50, 51). These sugars are abundant in saprophytic environments which, as already mentioned, are primary habitats for *Listeria*, but are absent from the body fluids of humans and animals. Hence, in addition to elevated temperatures, *L. monocytogenes* could also recognize its transition into a potential host by sensing that it is no longer in a plant-dominated habitat. A role for iron as regulator of virulence gene expression has also been since long established for many bacteria (45). Extracellular host compartments are characterized by low iron concentrations, and when growing under these conditions *L. monocytogenes* produces significantly more haemolysin than during growth in a high iron medium (7).

While for the above mentioned mechanisms a potential in vivo significance is obvious, this has still to be determined for the remaining variables listed in Table 1. It has been reported that addition of activated charcoal to the growth medium results in an increased production of listeriolysin O and PC-PLC by *L. monocytogenes* (21, 22). This "charcoal-response" is characteristic for all *L. monocytogenes* wild-type strains, which typically express only very low levels of virulence factors under laboratory conditions (e.g. growth in rich medium such as BHI), and involves increased transcription of all genes of the virulence cluster including *prfA* (54). Similarly, a shift of *L. monocytogenes* from rich growth medium into MEM, a "minimum essential medium" for culturing eukaryotic cells, with subsequent incubation at 37°C in the presence of 5% CO_2, results in increased transcription of *prfA* and the PrfA-regulated genes

TABLE 1. Environmental signals affecting virulence gene expression in pathogenic *Listeria*

Signal	Effect	Mechanism	References
Physico-chemical signals			
Temperature	Transcriptional activation of the virulence gene cluster at T>30°C	Increased trancription of *prfA* at T>30°C	13, 43
β-glucosides	Inhibition of (at least) *hly* and *plcA* expression in response to cellobiose or arbutin in the culture medium	Unknown	33, 50, 51
Activated charcoal	Transcriptional activation of the virulence gene cluster in charcoal-treated culture medium	Increased transcription of *prfA* (signal molecule unknown)	54
Iron	Enhanced synthesis of Hly[a] under iron deprivation conditions	Unknown	7
Glucose	Increased expression of *prfA* in the presence of 0.5% glucose	Activation of P1*prfA* (inhibition of P2*prfA*)	18
Salt	Stimulated production of Hly[a] in medium with 400 mM KCl or NaCl	Unknown	8, 49
pH	Reduced production of Hly[a] at pH 5/6 compared to pH 7	Unknown	9
Stress conditions			
Heat shock (45–48°C)	Co-induction of Hly[a], ActA, PlcA[b] and several other proteins	Only PrfA-dependent?	59, 60
	Inhibition of *iap* transcription/p60 synthesis	Unknown	60
	Induction of 14 HSP common to pathogenic and non-pathogenic *Listeria* spp. (including DnaK and GroEL)	Unknown	29, 59, 60
Oxidative stress	Inhibition of *iap* transcription/p60 synthesis	Unknown	60
(H_2O_2)	Non-inhibition (or weak induction) of Hly[a] synthesis	Unknown	60
	Induction of 13 stress proteins (including GroEL)	Unknown	29
Nutritional stress (MEM)	Induction of 12 surface-associated, non-HSP proteins as well as PlcA[b], Hly[a], ActA, and PlcB[c]	Only PrfA-dependent?	61
Growth within macrophages	Selective induction of 32 proteins (including HSP and oxidative stress proteins)	Unknown	29
	Preferential expression of various genes including *plcA*, *purH*, *purD*, *pyrE*, and arginine ABC transporter *arpJ*	PrfA-dependent?	33
	Preferential synthesis of ActA	PrfA-dependent?	1

[a, b, c] Also referred to as LLO (listeriolysin O), PI-PLC (phosphatidylinositol-specific phospholipase C), and PC-PLC (phosphatidylcholine-preferring phospholipase C or lecithinase), respectively.
HSP, heat shock protein.

(1, 61). Interestingly, besides the already known virulence factors, five presently unknown proteins are specifically induced in wild-type *L. monocytogenes* but not in a *prfA* mutant during MEM treatment, suggesting that additional listerial genes are subject to regulation by PrfA. Similar results have been found for *L. ivanovii* (40). The molecular basis for both, the charcoal and the MEM effect, is unknown. It seems, however, reasonable to assume that both treatments mimick in some way the environment that is encountered by *Listeria* when entering eukaryotic cells either by providing or depleting one or more factors of potential in vivo relevance.

Stress conditions such as starvation, heat shock, acidic pH, or the presence of reactive oxygen species are known to induce, in pathogenic bacteria, the expression not only of general stress proteins (e.g. the Hsp-family chaperones) but also of virulence genes (46). This also applies to *L. monocytogenes* where heat shock or exposure to hydrogen peroxide have been shown to stimulate the production of listeriolysin (59, 60) and at least 20 additional proteins including the general stress proteins DnaK and GroEL (29). Some intracellular bacteria such as *Salmonella typhimurium* or *Legionella pneumophila* which spend a significant part of their intracellular life cycle within the hostile environment of phagosomes, generally express elevated amounts of stress proteins inside macrophages (2). Interestingly, of 32 proteins that have been shown to be preferentially produced by *L. monocytogenes* after 5 h of growth within macrophages, none corresponded to any factor specifically induced by heat shock or oxidative stress (29). This could be due to the special intracellular life style of *L. monocytogenes*, which involves multiplication outside of microbicidal host cell compartments. On the other hand, it cannot be ruled out that stress proteins are induced by *L. monocytogenes* in early steps of the infection prior to the escape from the phagosome.

Stress resulting from starvation and low pH, both exerted on bacteria while inside phagosomes of activated macrophages, could indeed contribute to an upregulation of *L. monocytogenes* virulence genes. For example, one peak of *prfA* expression during growth in rich culture medium coincides with the onset of stationary phase when nutrients start to become depleted (48). It has also been suggested that nutritional stress could contribute to the enhanced expression of virulence genes in MEM since *L. monocytogenes* is not able to grow within this special medium (61). Addition of glucose to the growth medium was initially observed to cause a repression of *hly* expression in *L. monocytogenes*, but this effect was mainly attributed to the acidification of the growth medium due to glucose metabolism (9). Nevertheless, using buffered culture medium, Freitag et al. (18) detected a direct influence of glucose on *prfA* expression from two promoters that are located between *plcA* and *prfA* (see also below), one of which was stimulated, the other repressed by the sugar.

Up to now, no sensory system has been identified which is involved in the transduction of any of the listed environmetal signals down onto the regulatory level of *prfA* or the genes of the virulence cluster. For some of these parameters, e.g. temperature or iron, the PrfA protein itself could act as a sensor either by conformational changes due to elevated temperatures or by modified activity in the presence or absence of certain metal ions. For the virulence gene repression by β-glucosides or activation by charcoal, however, it is more likely that intracellular and/or membrane-associated factors are participating as a link between the environment and PrfA (and eventually other regulatory proteins). Frequently, factors that are involved in virulence-associated signal transduction mechanisms in pathogenic bacteria belong to the extensive protein family of the so-called "two-

component systems" (26). For example, the PhoP/Q system of *Salmonella typhimurium* or the BvgA/S system of *Bortedella pertussis* (see the article by Beier et al. in this issue) are members of this family and act as main virulence regulators in the respective species. For *L. monocytogenes*, the occurence of such systems has already been shown (Anderson et al., Proc. XII Intern. Symp. Probl. Listeriosis, 1995, p. 204 and same pub. pp. 457–462; Brehm et al., unpublished results; and ref. 65). Whether these identified systems are actually contributing to the regulation of virulence genes in *Listeria*, however, remains to be uncovered.

PrfA, a pleiotropic regulator of virulence gene expression

First assumptions that virulence gene expression in *L. monocytogenes* could be pleiotropically regulated by a *trans*-acting factor were based on two observations: (i) conserved palindromic sequence motifs have been found in the promoter region of *hly* (the first cloned listerial virulence gene) and in those of the neighbouring open reading frames, suggesting that they might serve as recognition sites for a regulatory protein (47); (ii) spontaneous or transposon-induced, non-haemolytic mutants could be isolated which contained an intact but transcriptionally silent *hly* gene, suggesting that a factor necessary for *hly* transcription was not produced in these mutants (23, 41). Subsequently, the gene for this regulator could be identified by genetic analysis of spontaneous, avirulent mutants harbouring deletions upstream of *hly* and displaying a combined deficiency in virulence-associated phenotypes. The affected gene was cloned from the wild type and named *prfA*, for "positive regulatory factor A" (42, 48).

Several lines of evidence substantiate the role of the *prfA*-encoded factor PrfA, a cytoplasmic 27 kDa protein, as a pleiotropic activator of virulence gene expression in *L. monocytogenes*. (i) In transposon-induced or site-specific-generated *prfA* mutants, the expression of all genes of the virulence cluster is abolished or greatly impaired, resulting in complete avirulence (4, 48). When complemented in *trans* by a plasmid-encoded, intact *prfA* gene, these mutants resume to synthesize all virulence factors and, at least partially, regain virulence (42, 48). (ii) After transformation in *Bacillus subtilis* of plasmid constructs carrying *lacZ* fusions under control of the *hly* promoter or promoters of the other transcriptional units of the *Listeria* virulence cluster, reporter gene expression is strongly activated when PrfA is supplied in *trans* (17, 58). (iii) The *prfA* gene of *L. ivanovii* has also been recently characterized; it encodes a protein highly homologous to *L. monocytogenes* PrfA (40). Similar to *L. monocytogenes*, transposon mutants in *L. ivanovii prfA* are unable to produce serveral virulence-associated factors (including ivanolysin O which is homologous to listeriolysin O) (28), indicating that the PrfA proteins of both pathogenic *Listeria* species fulfil similar roles as pleiotropic activators of virulence gene expression.

As indicated in Fig. 2A, several sites within the virulence gene cluster of *L. monocytogenes* are responsive to activation by PrfA. The only monocistronically transcribed gene of the cluster, *hly*, is under control of two PrfA-dependent promoters, located in the intergenic region between *hly* and *plcA* (4, 48). A third promoter, recently identified and located downstream of the two PrfA-dependent ones, drives *hly* expression independent from PrfA but results only in a low-level production of messenger RNA (12). The genes of the lecithinase operon can either be expressed in a single messenger RNA driven by the PrfA-dependent promoter upstream of *mpl*, or in two independent transcripts, one corresponding to *mpl*

and another comprising the remaining ORFs of the operon. In the latter case, *actA* and *plcB* are under the control of a PrfA-dependent promoter which is located in front of *actA* (1, 64).

The expression pattern of the *plcA*/*prfA* operon has been studied in detail (1, 3, 18, 19, 43, 48) and turned out to be rather complex. Under the control of a PrfA-dependent promoter, *plcA* is transcribed either in a monocistronic messenger RNA, terminating in front of *prfA*, or as part of a bicistronic readthrough transcript together with *prfA* (Fig. 2A). In form of this bicistronic messenger RNA, PrfA activates its own expression by means of an autoregulatory, transcriptional amplification loop (48). Full induction of all genes of the virulence cluster depends on the production of this *plcA*/*prfA* transcript (3) whose expression is subject to thermoregulation (43). In addition, *prfA* transcription is also controlled by two other promoters (P1 and P2) which are located in the *plcA*/*prfA* intergenic region and which are not thermoregulated. As already mentioned above, glucose in the growth medium inhibits *prfA* expression via promoter P2 while it stimulates transcription by P1. Furthermore, the bacterial growth phase seems to have an influence on *prfA* transcription mediated by P1, but not by P2. Even when monocistronic *prfA* messenger RNAs from these promoters are produced in large amounts, no significant increase of virulence gene expression can be observed, suggesting that these transcripts are probably not effectively translated. Although the elimination of either P1 or P2 seems to affect virulence gene regulation in vitro, the corresponding mutants behave fully virulent in vivo and in tissue culture models. Mutation of both promoters, however, significantly (100-fold) impaired virulence in vivo. Particularly, the ability to escape from the phagosome is greatly reduced in P1/P2 double mutants whereas no subsequent influence on intracellular growth or cell-to-cell spread could be observed (18, 19). Taken together, these data suggest that an initial low level expression of PrfA via P1 and/or P2 is necessary for *L. monocytogenes* to contrive to escape into the host cytoplasm, while the subsequent steps of the infection process strongly depend on the production of high levels of PrfA protein from the bicistronic *plcA*/*prfA* transcript. Interestingly, PrfA also seems to down-regulate its own synthesis which probably involves direct binding of the regulator protein to recognition sites overlapping P2 (see Fig. 2A). The significance of this additional, negative regulatory mechanism could be the limitation of the autostimulatory loop effect in order to obtain a properly balanced expression of *prfA* within eukaryotic cells.

In contrast to the genes of the virulence cluster, expression of the *inlA*/*B* locus is not strictly dependent on the presence of a functional PrfA protein. Here, several promoters have been identified which contribute to the transcription of *inlA* and *inlB* in either mono- or bicistronic transcripts. While one of these promoters, leading to a bicistronic messenger RNA, is positively regulated by PrfA, the other promoter elements are under control of PrfA-independent mechanisms (13, 44). (See Fig. 2A.)

Structural and functional aspects on PrfA-controlled gene expression

One common feature of all promoters which are positively controlled by PrfA is the presence of a 14-bp palindromic sequence at around position –35 relative to the transcription start point, the so-called "PrfA-box" (10, 11, 13, 47) (Fig. 2B). It has been suggested that PrfA directly binds to these palindromic sequences and evidence supporting this assumption has been obtained (18). However, PrfA-boxes of different promoters are not identi-

cal and there seems to exist a hierarchy among them. The sequence motif of the *hly* and *plcA* promoters is considered to represent the "perfect" PrfA-box and contains no mismatch within the 14-bp palindrome. Mismatches of one (for *mpl* and *actA* promoters) or two (for *inlA/B*) base pairs are present in the remaining PrfA-boxes (Fig. 2B). The same holds true for virulence cluster genes from *L. ivanovii* but, interestingly, all palindromes in this species are two base pairs longer than in corresponding promoter elements of *L. monocytogenes* (40). The relevance of this special feature is presently unknown.

The differential transcription of listerial virulence genes presumably results from different binding affinities of PrfA to its target sequences. It has been shown that the introduction of mismatches into the PrfA-box of the listeriolysin gene negatively affects the expression of *hly-lacZ* fusions in *B. subtilis* when *prfA* was provided in *trans* (18). Furthermore, in similar experiments, PrfA was less able to stimulate the transcription of reporter genes which were fused to *actA* or *mpl* promoter sequences in comparison to *hly* or *plcA* fusions. *inlA-lacZ* constructs did only poorly respond to PrfA activation in these systems (58). Accordingly, after a shift of *L. monocytogenes* into MEM medium, transcriptional activation of *hly*, *plcA*, and *prfA* seems to be induced earlier, i.e., at lower PrfA concentrations, than activation of *actA* and *plcB*, whereas expression of *mpl* could not be stimulated by this treatment (1).

All the evidence mentioned above has led to the currently most favoured model of coordinate and differential, PrfA-mediated regulation of virulence gene expression in *L. monocytogenes* (18, 19). In this model it is assumed that, in an initial step, *prfA* is transcribed by its own two promoters (P1 and P2), leading to the production of limited amounts of PrfA protein. These amounts, however, are sufficient to stimulate expression from promoters with high affinity for PrfA, namely the *plcA* and *hly* promoters, which drive the production of gene products involved in early steps of infection, i.e. in the lysis of the phagosome. Furthermore, via the induction of bicistronic *plcA/prfA* transcripts, increased amounts of PrfA protein are expressed which then also activate transcription from promoters with lower affinity for PrfA. Subsequently, factors involved in later steps of the infection process (ActA, PlcB and Mpl) are produced, and monocistronic *prfA* expression is downregulated. In fact, such mechanism could contribute to the temporal expression of virulence factors during sequential steps of the infection process.

Compared to the extensive information that has been gathered on molecular genetic aspects of PrfA-mediated gene activation, little is known on the biochemical features of these processes and of PrfA itself. One recent finding, however, will surely have a great impact on future experiments with regard to PrfA biochemistry. By computer-based sequence comparisons it has been shown that the PrfA proteins from both *L. monocytogenes* and *L. ivanovii* belong to the well known superfamily of Crp/Fnr-like DNA binding proteins (31, 40). At present, this family comprises mainly transcription factors of Gram-negative bacteria such as Crp, Fnr, LexA, and Fur of *Escherichia coli*, which are involved in catabolite repression, anaerobic growth control, SOS response, and iron regulation, respectively; FixK of *Rhizobium meliloti* and NtcA of *Synechococcus* spp. are regulators of nitrogen metabolism; and *Actinobacillus pleuropneumoniae* HlyX as well as *Xanthomonas campestris* Crp seem to be involved in the regulation of bacterial virulence. The listerial PrfA proteins and an additional factor from *Lactobacillus casei* form the Gram-positive branch of this family (31). Highly conserved amino acid residues, certain β-roll structures, and a C-termi-

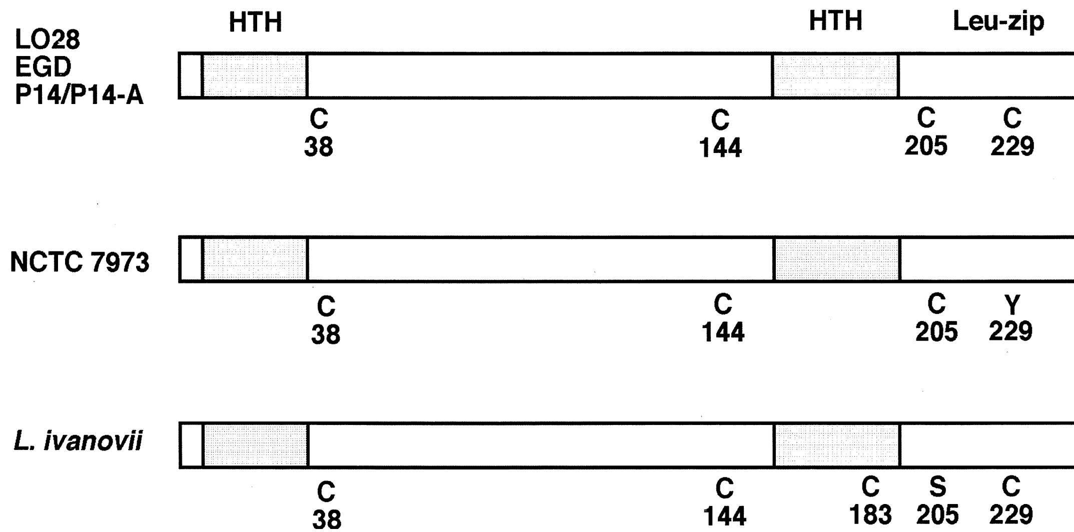

FIG. 3. Schematic primary structure comparison of PrfA from different origins. The *Listeria monocytogenes* strains EGD (weakly haemolytic), NCTC 7973 (hyperhaemolytic), and LO28 (moderately haemolytic) are described in the text. *L. monocytogenes* P14 and P14-A represent recently characterized, isogenic wild-type and hyperhemolytic strains, respectively, which express a PrfA protein identical to LO28 PrfA (see reference 54). PrfA from strain EGD has originally been described as comprising 235 amino acids (42) but a recent re-sequencing of the corresponding gene revealed identical C-termini and protein lenghts (237 amino acids) for PrfA from EGD, NCTC 7973, and LO28 (Bohne et al. and Ripio et al., unpublished results). Except from the differences indicated in the figure, amino acid sequences of all shown *L. monocytogenes* strains are identical. Marked are cysteine (C), serine (S), and tyrosine (Y) residues with the corresponding positions in the proteins. The locations of putative helix-turn-helix (HTH) and leucine zipper (Leu-zip) motifs (see text) are also shown.

nal helix-turn-helix (HTH) motif, all of which are characteristic of Crp/Fnr-like proteins, are also present in PrfA (Fig. 3).

Interestingly, PrfA displays some structural features which distinguish it from the other factors of the family. For example, an additional HTH domain is present at the N-terminus of PrfA, which cannot be found in any other Crp/Fnr-homologue. The C-terminus of PrfA is prolonged and contains a potential leucine zipper motif (Fig. 3). Moreover, PrfA apparently lacks certain metal-binding domains which are typical for members of the Fnr-like subfamily, and the central regions of the protein, which, in the case of Crp, are involved in dimer formation as well as cofactor binding, show less overall homology to the remaining factors. On the other hand, the position of Crp binding sites (palindromic sequences) in catabolite-activated promoters of *E. coli* seems to be in good correspondence with the location of putative PrfA binding sites in front of listerial transcription units. Also DNA bending, which has been suggested to be involved in transcriptional regulation by Crp, could be a mechanism that contributes to downregulation of monocistronic *prfA* expression by the PrfA protein itself (37). At present, it would be premature to draw wide-ranging conclusions on the structure of PrfA from observations which are simply based on sequence similarities. However, the homologies between PrfA and the members of the Crp/Fnr family suggest that this protein could activate gene expression in a homodimeric form and probably also possesses a three dimensional organization similar to that which has already been resolved for *E. coli* Crp. Besides the verification of these assumptions, further experiments are necessary to investigate the significance of the "special equipment" of PrfA such as

the additional HTH domain or the putative leucine zipper motif.

Another finding of potential interest for PrfA biochemistry could be the slight differences observed in the primary structure of PrfA from different *Listeria* species and strains which probably affect its ability to activate target promoters. Under standard laboratory conditions, clinical or field isolates of *L. monocytogenes* characteristically produce very weak activities of virulence factors such as haemolysin or phospholipases (54). However, as already noted above, they are able to dramatically increase virulence factor production when activated by charcoal or MEM treatment (1, 54, 61). With respect to haemolysin and phospholipase production, such strains correspond to the "wild-type" phenotype (or phenotype 1) of *L. monocytogenes*, which is characterized by a weak expression of virulence factors in rich growth media and the ability to respond to charcoal (54). In contrast, some laboratory strains show distinct, variant haemolysin and lecithinase phenotypes, characterized by constitutively high (phenotype 2; e.g., *L. monocytogenes* NCTC 7973) or intermediate (phenotype 3; e.g., *L. monocytogenes* LO28) levels of expression. Such variant strains cannot (phenotype 2), or moderately (phenotype 3) respond to charcoal treatment (54). Since phenotypes 2 and 3 have been exclusively found among strains kept under laboratory conditions for extended periods of time, they most probably represent deregulated spontaneous mutants (54). In Fig. 3, a schematic comparison of primary structures of PrfA from these three different phenotypes is presented. Strains NCTC 7973, LO28 and EGD have been widely used for laboratory investigations on listerial virulence. Interestingly, in PrfA of the type 2 hyperhaemolytic variant NCTC 7973, one of the four cysteine residues which are present in strains expressing very low (e.g. EGD representing the wild-type phenotype 1) or moderate (e.g. LO28 of phenotype 3) levels of virulence factors, is replaced by a tyrosine at position 229. The PrfA protein of *L. ivanovii*, a species which, like NCTC 7973, produces very high activities of haemolysin and phospholipases, is, in general, very similar to its *L. monocytogenes* counterparts (77% identity, 90% similarity) (37), but also shows a cysteine → serine replacement (at position 205) and has an additional cysteine substituting residue 183. At present, it is not known whether any of these cysteine residues plays an important role in the transcriptional activation mechanism of PrfA, nor has the significance of the cysteine → tyrosine replacement for strain NCTC 7973 been worked out in this context. Nevertheless, these structural differences in the PrfA proteins of different *Listeria* strains are remarkable and it has been suggested that they could be the reason for the observed hyperhaemolytic phenotype 2 (1). In addition, slight differences in palindromic PrfA recognition sites or in the positioning of the P1 and P2 promoters, leading to monocistronic *prfA* transcripts, have been detected between different strains which could also contribute to the variant phenotypes mentioned above (1, 18, 43).

Indications for additional regulatory elements involved in virulence gene expression in *Listeria*

In consideration of the significant body of evidence cited above, the role of central virulence regulator in *L. monocytogenes* can be clearly allocated to PrfA. However, several experimental findings also indicate that PrfA is not the sole factor involved in these processes. For instance, although cellobiose in the growth medium does not repress transcription of *prfA* from its own promoters, the transcriptional activation of *hly* and *plcA* is severely impaired in the presence of this

sugar (33). This cannot be explained by the model described before and suggests that additional factors are present which either modify PrfA activity at the post-transcriptional level or which act in a more direct way as repressors of virulence gene expression.

Furthermore, although significant levels of monocistronic *prfA* messenger RNA can be detected in *L. monocytogenes* during growth at 20°C (43) or after transformation with a *prfA*-harbouring multicopy plasmid, this has only a slight activating effect on the expression of the PrfA-regulated virulence genes (48). Ineffective translation of monocistronic *prfA* transcripts has been proposed as a possible explanation (19). However, these transcripts comprise a full-length coding region and the same ribosome binding site that is present in bicistronic transcripts. Thus, certain additional factors could be involved that recognize monocistronic *prfA* messenger RNAs and inhibit translation.

L. seeligeri has been shown to contain a haemolysin gene homologous to *hly* which also contains a perfect PrfA-box in its promotor region (35). The weak expression of this gene in *L. seeligeri* could be due to the lack of a functional PrfA homologue. However, by providing *L. monocytogenes prfA* cloned on a multicopy plasmid, the expression of the *L. seeligeri* haemolysin gene cannot be induced. Therefore, *L. seeligeri* probably does not express a factor which, in *L. monocytogenes*, drives virulence gene expression in concert with PrfA (Kreft, J., unpublished results).

Moreover, since *plcA* is expressed both as a monocistron and together with *prfA* as a bicistron, the putative terminator between these genes has been proposed as a possible target for transcriptional antitermination (48). Such mechanisms has been extensively studied in both Gram-positive and Gram-negative organisms and frequently involve homologous "antiterminator" proteins (34). PrfA, however, shows no similarity to known antiterminators and is most probably not directly involved in processes of transcriptional antitermination. The presence of transcriptional antitermination systems in *L. monocytogenes* as well as their possible role in virulence gene regulation still remains to be determined.

Finally, indications for the presence of additional regulatory elements can also be deduced from recent investigations concerning the two isogenic strains *L. monocytogenes* P14 and P14-A (54). Despite exhibiting the very distinct phenotypes 1 (P14) and 2 (P14-A) with respect to the production of virulence factors in vitro (see above), both strains show identical primary sequences of their PrfA proteins (Fig. 3) and of PrfA-controlled promoters (Ripio, M. T., Vázquez-Boland, J. A., unpublished results; and ref. 54). This strongly suggests that the difference between both strains concerns an additional control mechanism for virulence gene expression which is probably situated in a level higher than PrfA on a regulatory cascade.

Recently, a gene of *L. monocytogenes*, *flaR*, could be isolated and characterized which encodes a histonelike protein that influences DNA topology (55). Changes in DNA supercoiling caused by histonelike proteins such as H-NS of enterobacteria frequently have an effect on virulence gene expression (30). However, when tested for virulence in mice, a transposon-induced mutant in *flaR* displayed similar LD_{50} values as the isogenic wild type. Also invasiveness of *L. monocytogenes* for epithelial cells seems not to be influenced by a mutation in *flaR*, suggesting that this factor is not involved in listerial virulence gene regulation (55). For *L. ivanovii*, however, ongoing investigations on a newly isolated gene, encoding a transcriptional activator protein which is involved in the regulation of at least two virulence factors, have already demonstrated the presence of a virulence control

mechanisms in addition to PrfA (Domínguez-Bernal, G., Kreft, J., Vázquez-Boland, J. A., manuscript in preparation).

In conclusion, significant progress has been made during the past years in characterizing molecular determinants of listerial pathogenesis. Now we are at the threshold of uncovering and understanding the mechanisms which contribute to the complex regulation of these factors. This could lead to gain fundamental insights into host-pathogen interactions, contributing not only to knowledge on bacterial virulence mechanisms but also on how we can get into action against diseases caused by facultative intracellular bacteria. The first steps have been done in the characterization of PrfA and the elaboration of its role as a central regulator of virulence gene expression. However, as there are many open questions concerning additional regulatory elements and signal-transduction mechanisms in *L. monocytogenes* indicate, we are just at the beginning.

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The molecular mechanisms of actin-based intracellular motility by *Listeria monocytogenes*

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Summary

A key virulence trait of bacteria and viruses that multiply in the cytoplasm of the infected cell is their ability to direct movement intracellularly and to spread from cell-to-cell. Intracellular movement is effected by harnessing components of the host microfilament system. This mode of locomotion by intracytoplasmic parasites has recently gained much interest as a model to examine microfilament assembly and function. Of the intracellular bacteria employing association with the host cytoskeleton to effect movement, the Gram-positive pathogen *Listeria monocytogenes* is the most well studied. This review summarizes the current state of the understanding, at the molecular level, of how *L. monocytogenes* subverts the host cell contractile machinery to meet its own need to move and spread within infected host cells.

Key words: *Listeria monocytogenes*, actin-based motility, ActA nucleator, vasodilator stimulated phosphoprotein (VASP), profilin

Resumen

Una característica clave de las bacterias y virus que se multiplican en el citoplasma de las células infectadas es su capacidad de moverse intracelularmente y de expandirse de célula a célula. El movimiento intracelular se produce utilizando los componentes del sistema de microfilamentos del hospedador. Este modo de locomoción de los parásitos intracitoplásmicos tiene actualmente mucho interés, ya que constituye un sistema modelo para examinar el ensamblado y el funcionamiento de los microfilamentos. Entre las bacterias intracelulares que entran en asociación con el citoesqueleto del

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hospedador para llevar a cabo el movimiento, el patógeno Gram positivo *Listeria monocytogenes* es el mejor estudiado. En esta revisión se resume el estado actual del conocimiento, a nivel molecular, del modo en que *L. monocytogenes* altera la maquinaria contráctil de la célula hospedadora para moverse y propagarse en las células hospedadoras infectadas.

Introduction

Intracellular pathogens are responsible for many severe forms of diseases world-wide with devastating morbidity and high mortality rates. Despite their significance in human disease, the understanding of the basis of the intracellular lifestyle of many bacterial and eukaryotic parasites is still in its infancy. Understanding early interactions of parasites with host cell membranes, their subtle interactions with host signal transduction pathways and other cellular processes, as well as defining how virulent gene products modulate and promote intracellular survival of the pathogen, will undoubtedly lead to novel measures for prevention and treatment. Much of the lack of progress in studying intracellular pathogens can be summarily narrowed down to the difficulty in cultivation, lack of appropriate animal and tissue culture models, as well as a dearth of useful genetic systems to examine the pathogen. The facultative intracellular Gram-positive pathogen *Listeria monocytogenes* has emerged as a major paradigm to study many facets of host–pathogen interactions. For many decades *L. monocytogenes* has been a model pathogen to study the generation of cell-mediated immunity in mice (12). More recently, the model character of this bacteria has been extended to include many other basic mammalian cell activities in particular the dynamic nature of the host actin cytoskeleton (24).

L. monocytogenes is a ubiquitous Gram-positive pathogen (1). It is a major food-borne pathogen implicated in severe infections following consumption of *Listeria*-contaminated food products. Although of low incidence, listerial infections have a significant mortality rate and threaten the unborn, newborn, immunocompromised and elderly individuals.

L. monocytogenes usually enters the host in the gut after consumption of listeria-contaminated food (10). The precise locus of bacterial invasion is not known, but during an acute infection, many tissues are infected, demonstrating the ability of these bacteria to invade numerous eukaryotic cells in different tissues. Microscopic studies have demonstrated penetration of *L. monocytogenes* into epithelial cells of both the cornea and the intestine in vivo (26, 27). Tissue culture assays of invasion show that *L. monocytogenes* are capable of invasion of various cell types, including hepatocytes and fibroblasts albeit with widely different rates of efficiency (18). However, once within the intracellular compartment the doubling time of these bacteria is about one hour, regardless of cell type (20). Survival of the internalized bacteria is however dependent on cell-type. Following intravenous infection, *L. monocytogenes* appears to be first ingested and rapidly killed by resident macrophages such as the Kupffer cells of the liver (5).

A primary aspect of *L. monocytogenes* pathogenesis is the ability of these bacteria to escape from a host vacuole following uptake. Release of bacteria into the cytoplasm requires the concerted action of listeriolysin and phospholipases (25). Once inside the host cytoplasm, bacteria start to grow, a process that can last for more than eight hours. The intracellular environment appears to be a rich milieu for bacterial growth, because even auxotrophic mutants are capable of unrestricted growth.

Actin-based bacterial motility: a crucial virulence phenotype

Within the host cytoplasm, bacteria induce the formation of phase-dense actin-rich clouds (34). After a few rounds of bacterial division the actin cloud rearranges into a comet tail, located at one pole of the bacterial surface which remains stationary in the cytosol. Movement is generated by polymerization of F-actin behind the forward-propelling bacteria (29). Bacterial motility ranges from about 0.05 to 1.0 mm/s (6, 32). Intracellular motility permits interaction of the bacteria with the plasma membrane, which can result in the formation of large pseudopodal extensions that are rapidly endocytosed by the neighbouring cells, leaving bacteria now in a two membrane vacuole. The bacteria induce lysis of these vacuolar structures, and are released into the cytosol where a new cycle of infection can ensue (25). Hence, the actin-based motility of *L. monocytogenes* is crucial for its successful lifestyle within the infected host (Fig. 1).

This mode of exploitation of the host cell contractile system by *Listeria* to generate intracellular locomotion provides a simple and reproducible system to study the generation of an actin-based cytoskeleton. Time-lapse video microscopy of *Listeria*-infected cells, microinjected with flourescently labeled actin monomers, shows that

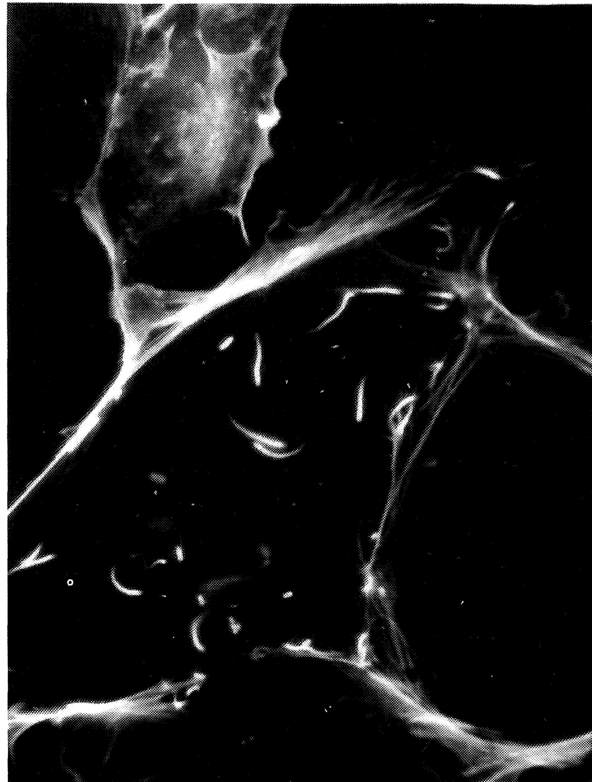

FIG. 1. Actin-based intracellular motility of *Listeria monocytogenes*. Fluorescence microscopy of infected cells which have been stained with fluorochrome-labelled phalloidin to reveal actin filaments. Note the F-actin trails, similar to "comet tails", left behind by moving listerial cells.

the movement of the bacterium is associated with the lengthening of the actin tail (6, 32). Growth of the actin tail takes place strictly at the juncture between the bacteria and the actin tail, where new actin monomers are incorporated into filaments and actin-crosslinking proteins bridge the neighboring filaments (6, 7). These newly formed filaments attach to the cytoskeleton providing the traction whereby the growing filament propels the bacterium in the direction opposite to the tail. During disassembly the actin monomers are released, and the distal regions of the tail become detached from the cytoskeleton. When monomers addition increase, the actin-rich tail lengthens, and the bacteria migrate swiftly. Treatment of cells with cytochalasin D or the C2 toxin of *Clostridium botulinum*, both of which inhibit actin-assembly, rapidly blocks the formation of actin tails halting both intracellular movement and cell-to-cell spread (34). Thus all indicates that the assembly of actin filaments is the force producing process necessary for intracellular motility.

A single bacterial gene *actA* required for host actin-assembly

Characterization of mutants of *L. monocytogenes* unable to induce actin accumulation of actin-filaments led to the identification of the 90 kDa surface-bound ActA polypeptide (8, 13). The *actA* gene predicts a protein of 639 amino acids comprising from its amino- to carboxy-termini, a signal peptide, a highly charged amino-terminal domain, a central region containing four copies of a repeated motif rich in proline residues, a hydrophobic carboxy-terminal end that tethers the protein to the bacterial surface. Immunolocalization of ActA in infected tissue culture cell lines demonstrated that ActA is located on the bacterial surface at the site of actin-filament assembly, but it is not detected in the actin comet tails of motile bacteria (15, 21). Despite its predicted molecular mass of 67 kDa, ActA migrates in SDS–PAGE with an apparent molecular mass of 90 kDa. During intracellular growth of the bacteria, ActA migrates with even higher molecular masses, which are a result of phosphorylation of the protein by cytosolic host protein kinases (2, 17).

To examine the role of ActA in actin assembly and movement in the absence of other listerial factors, we and others have expressed ActA from a eukaryotic vector by transfection of eukaryotic cells (9, 22, 23). When expressed in eukaryotic cells, ActA spontaneously targets to mitochondria, where it induces the assembly of mini actin-based cytoskeletons around these subcellular organelles (22). Thus ActA acts as a nucleator, initiating actin assembly. Using the mitochondrial targeting assay, a systematic study to identify binding sites for host microfilament proteins was performed using a series of N-terminally truncated deletion mutants. This study revealed that there are at least two separable domains on ActA required for interaction with the actin cytoskeleton: an amino-terminal 23 amino-acid region required for actin-filament nucleation and the proline-rich repeat region whose presence significantly enhanced the ability of ActA to recruit F-actin (23). Hence, ActA is the sole listerial factor required to recruit and generate formation of an actin-cytoskeleton.

Although the mitochondrial targeting assay provided evidence for ActA as a nucleator of the actin cytoskeleton, it did not address the question whether ActA is required to generate bacterial motility. This has been examined using two independent approaches. In the first instance, an ActA-LytA fusion protein was expressed and the recombinant protein physically attached to the surface of the unrelated pathogen, *Streptococcus pneumoniae* (30). Addition of these ActA-coated bacteria to cytoplasmic extracts of *Xenopus* eggs, an in vitro system to study bacterial movement, demonstrated that these bacteria can trigger both

actin-assembly and bacterial movement. In an independent study, the ActA polypeptide was expressed in the non-pathogenic species *Listeria* innocua (16). When motilities of these bacteria were examined in *Xenopus* cytoplasmic extracts, actin assembly and movement of these bacteria were observed at rates that were indistinguishable from wild type *L. monocytogenes*. Cumulatively, these data demonstrate that the ActA polypeptide is both necessary and sufficient for the generation of intracellular motility of this bacteria.

Only ActA is required for bacterial motility

As indicated above, bacterial movement results from the polymerization of actin monomers on the bacterial surface which would normally be otherwise sequestered as stable complexes together with thymosinβ4 or profilin. Following assembly on the bacterial surface, actin filaments are released and crosslinked into the tail. Although ActA induces actin nucleation and polarized movement, there is no evidence to suggest that it binds F-actin directly. Thus interest has focused on host factors that directly interact with ActA. When the protein microfilamentous structures recruited by intracellular motile *L. monocytogenes* were examined, numerous host actin-binding proteins, including α-actinin, filamin, fimbrin, villin, and ezrin/radixin were found to be localized within the actin-comet tails (7, 14, 31). Nevertheless, these proteins are associated with even the most distal actin-filaments in the tails suggesting that none of them interact directly with the bacterial surface.

Examination of the motility of *L. monocytogenes* in *Xenopus* cytoplasmic extracts, showed that the actin-sequestering protein profilin was a major component to generate bacterial motility (33). Profilin appeared to be associated with moving bacteria, and profilin-depletion experiments suggested that it was required for bacterial motility. Since profilin is known to bind to polyproline sequences, it was hypothesized that profilin bound to the proline rich repeats of ActA. Later experiments with the in vitro motility assay suggested that profilin was dispensable for bacterial motility (19). The varying results might reflect differences in depletion protocols. Thus, a direct role for profilin could not be inferred from these experiments. Furthermore, purified profilin did not bind to *Listeria* grown outside the host cell, despite the presence of surface-bound ActA, suggesting that another host factor might be required to mediate ActA-profilin interaction.

A host protein directly interacting with ActA

As a prelude to identify host factors that might interact with bacterial ActA we used immunofluoresence analysis in search of known microfilament proteins around intracellularly motile *Listeria*. Our analysis was guided by the following assumptions: firstly, the factor of interest should always be associated with the bacterial surface, since ActA is not present in the actin-comet tails. Secondly, the factor should also be present at the interface between the bacteria and the elongating actin tail. Thirdly, since the proline-rich peptide motif in ActA is highly related to a similar motif in vinculin, a focal adhesion protein, we hypothesized that a potential candidate could also be a protein associated with focal adhesions or vinculin itself. The actin filament associated vasodilator-stimulated phosphoprotein (VASP) was found to fulfill all three requirements for a host protein interacting with ActA (4). VASP was originally characterized as a protein which is phosphorylated in response to cAMP- and cGMP-elevating vasodilators and platelet inhibitors mediated by cAMP- and cGMP-

dependent protein kinases (3, 11). It was later identified as a novel cytoskeletal protein that is particularly concentrated at sites of focal contacts as well as in the peripheral regions protruding lamellae in locomotion or spreading cells (28). In *Listeria*-infected cells, VASP was always associated with the surface of the moving bacteria. At very early times following escape into the host cytoplasm, VASP accumulation on the surface of bacteria even preceeded that of F-actin. At later times, VASP's localization was polar, juxtaposed between one extremity of the bacterial surface and the front of the actin comet tail. A *L. monocytogenes* mutant deleted for the entire *actA* gene did not associate with VASP. Furthermore, purified VASP was found to bind directly to the ActA polypeptide in vitro (4).

Multiple microfilament protein binding sites on ActA

Analysis of truncated and deletion derivatives of ActA in the mitochondrial targeting assay revealed that mutant ActA polypeptides lacking the first 151 amino acids were unable to recruit F-actin. This ability was regained in deletion derivatives starting at amino acid 128, suggesting an essential site for the recruitment of F-actin lies between amino acids 128 and 152. A deletion derivative lacking the proline-rich repeat region was unable to recruit VASP but showed low levels of actin accumulation (23).

In order to examine the relative roles of the N-terminal 23 amino acid region defined above as being required for F-actin accumulation, and the VASP-binding polyproline rich repeats in mediating actin assembly and bacterial movement, we have constructed independent chromosomally in-frame deletion mutants in either region of the *actA* gene. Deleting the 23 amino acids between positions 128 and 152 of the ActA polypeptide completely abrogated the ability of bacteria to recruit F-actin. Such bacteria however stain strongly for the presence of VASP using immunofluoresence. On the other hand, deletion of the polyproline-rich repeat region, lowered the ability of these mutant bacteria to accumulate F-actin. These bacteria were intracellularly motile, but their motility was 10% of that in wild-type bacteria. Furthermore, these bacteria were unable to generate actin-comet tails.

The results and observations presented above allow us to formulate a general model as to how *Listeria* subvert the host cell machinery to meet its own need to move and spread within cells. The elegance of the molecular mimicry is truly remarkable. By producing a single protein, ActA, which contains multiple sites for the binding of host microfilament proteins, *Listeria* causes host-cell actin to assemble in filaments behind the bacterium, thus providing the propulsive force for disseminated spread in pathogenesis. A 23 amino acid region within the N-terminus of ActA is a prerequisite for accumulating F-actin. The cellular factor(s) involved in the nucleation of F-actin remains to be discovered, and could fundamentally contribute to the understanding of the generation of this structure. However, a clearer picture is emerging with respect to the proline-rich binding sites for VASP. VASP has been shown to be the first natural ligand for profilin, a major sequestering protein for monomeric actin (G-actin). Thus, by binding VASP, profilin is concentrated to the surface of bacteria where new actin filaments are being assembled. This high concentration could lead to an exchange of ATP for ADP on actin monomers. Such a mechanism at the bacterium-actin tail juncture would favor the rapid formation of filaments and supports a role for VASP acting as an accelerator of F-actin dynamics.

The study of actin-based motility has contributed to our understanding of a basic cell property, namely that of movement. Results presented above

implicate VASP as a key protein required in the maintenance of dynamic changes in the actin-based cytoskeleton. Clearly eukaryotic ActA analogues must exist, and are important structures involved in cell motility. As we dissect and peel away the host proteins that contribute to the efficient mode of listerial locomotion, we will also learn more of the backup and redundant systems that the eukaryotic cells use to generate motility. The *Listeria* model provides us with a molecular handle to identify the proteins that are directly involved in this regulated behaviour of cells.

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The pathogenesis of infection by *Listeria monocytogenes*

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Summary

Listeria monocytogenes is a Gram-positive bacterium responsible for severe infections in human and a large variety of animal species. It is a facultative intracellular pathogen which invades macrophages and most tissue cells of infected hosts where it can proliferate. The molecular basis of this intracellular parasitism has been to a large extent elucidated. The virulence factors, including internalin, listeriolysin O, phospholipases and a bacterial surface protein, ActA, are encoded by chromosomal genes organised in operons. Following internalisation into host cells, the bacteria escape from the phagosomal compartment and enter the cytoplasm. They then spread from cell to cell by a process involving actin polymerisation. In infected hosts, the bacteria cross the intestinal wall at Peyer's patches to invade the mesenteric lymph nodes and the blood. The main target organ is the liver, where the bacteria multiply inside hepatocytes. Early recruitment of polymorphonuclear cells leads to hepatocyte lysis, and thereby bacterial release. This causes prolonged septicaemia, particularly in immunocompromised hosts, thus exposing the placenta and brain to infection. The prognosis of listeriosis depends on the severity of meningoencephalitis, due to the elective location of foci of infection in the brain stem (rhombencephalitis). Despite bactericidal antibiotic therapy, the overall mortality is still high (25 to 30%).

Key words: *Listeria monocytogenes*, listeriosis, intracellular parasitism, meningoencephalitis, infection processes

Resumen

Listeria monocytogenes es una bacteria Gram positiva causante de graves infecciones en los humanos y en diversas especies animales. Es un patógeno intracelular facultativo que invade los macrófagos y las células de la mayor parte de los tejidos de los hospedadores donde puede proliferar. La base molecular

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de este parasitismo intracelular se conoce bastante bien. Los factores de virulencia, incluyendo la internalina, la listeriolisina O, las fosfolipasas y una proteína de la superficie bacteriana, ActA, están codificados por genes cromosómicos organizados en operones. Después de entrar en las células hospedadoras, las bacterias escapan del compartimento fagosómico y entran en el citoplasma. Posteriormente se propagan de célula a célula mediante un proceso en el que ActA induce la polimerización de actina. En los hospedadores infectados, las bacterias atraviesan la pared intestinal por las placas de Peyer, invadiendo los nódulos linfáticos mesentéricos y la sangre. El principal órgano diana es el hígado, donde las bacterias se multiplican dentro de los hepatocitos. La actuación inmediata de las células polimorfonucleares ocasiona la lisis de los hepatocitos y, por consiguiente, la liberación de las bacterias. Este hecho produce una septicemia prolongada, especialmente en los hospedadores inmunocomprometidos, exponiendo a la infección la placenta y el cerebro. El pronóstico de la listeriosis depende de la gravedad de la meningoencefalitis debido a la "localización selectiva" de los focos de infección en el tronco del encéfalo (romboencefalitis). A pesar de la terapia antibiótica bactericida disponible, la mortalidad global es todavía elevada (25 al 30%).

Introduction

Listeria monocytogenes is a Gram-positive bacterium found widely in the environment where it lives as a saprophyte (46). It is frequently the cause of infection, sometimes severe, both in human and numerous other animal species (46). *L. monocytogenes* is an invasive pathogen that can cross the placental barrier and penetrate the central nervous system (CNS) (34, 50). Most animal species, including almost all mammals, birds, fish and crustaceans, are susceptible to infection (46). This ubiquitous pathogenicity of *L. monocytogenes* suggests the existence of universal molecular mechanisms of virulence.

One of the remarkable features of *L. monocytogenes* is its capacity to survive inside the host cells where bacteria find a sanctuary protected from the immune system (57). It was long believed that macrophages (monocytes, resident macrophages and polymorphonuclear neutrophils) were the major site of *Listeria* multiplication (57). However, it is now clear that polymorphonuclear neutrophils are highly bactericidal against *L. monocytogenes* (23, 24), although monocytes and macrophages are permissive for growth (Fig. 1). *L. monocytogenes* can invade and multiply in numerous cell types in its host, including epithelial cells (enterocytes), fibroblasts, parenchymal cells (hepatocytes) and endothelial cells (36, 48, 65, 73, 74). From the replication sites of infection, *L. monocytogenes* can disseminate through the blood to various target organs including the placenta and the CNS. Listeriosis during pregnancy can be serious and can cause abortion or severe neonatal infections (3, 14, 49, 54, 64, 68, 81, 90). CNS infections were described in sheep as long ago as 1933 by Gill (42), as a circling disease, only a few years after the identification of the bacterium. About two thirds of patients infected by *L. monocytogenes* develop a meningoencephalitis (3, 14, 49, 54, 64, 68, 81, 90), which account for the 25% to 30% overall mortality associated with listeriosis and the high incidence of neurological sequelae. To cause meningoencephalitis, the bacterium must cross the blood-brain barrier. The mechanism by which it does so is unknown. Currently, *L. monocytogenes* is responsible for almost 1% of cases of bacterial meningitis, and less than 0.15% of infant mortality (88). It is nevertheless the third most common cause of neonatal meningitis, after *Escherichia coli* and group B *Streptococcus agalactiae*.

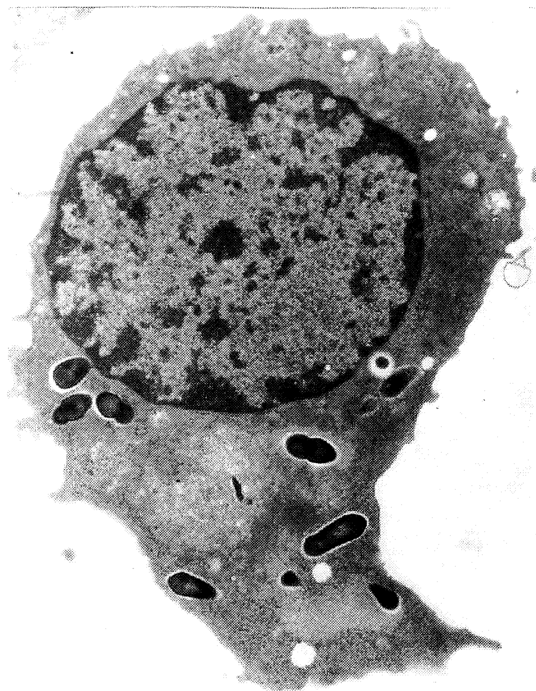


FIG. 1. Growth of *Listeria monocytogenes* inside mouse monocytes (photo by C. de Chastellier and P. Berche, unpublished results).

Intracellular parasitism of *Listeria monocytogenes*

Intracellular parasitism is the crucial mechanism of pathogenicity of *L. monocytogenes*. Work to investigate this phenomenon is valuable not only to elucidate *L. monocytogenes* pathogenicity, but also to elaborate a conceptual approach of the molecular mechanisms of bacterial pathogenicity. Consequently, since 1985 there have been numerous studies addressing the molecular mechanisms by which *L. monocytogenes* penetrates and survives in eukaryotic cells (see review in 82).

Infection of the eukaryotic cells by *L. monocytogenes* implicates a sequence of events, each involving specific virulence factors allowing intracellular parasitism of this pathogen (Fig. 2). First *L. monocytogenes* makes close contact with the host cell via an 80 kDa protein named internalin, encoded by a chromosomal gene *inlA* (37, 38). Internalin is exposed on the bacterial surface and its structural organisation is reminiscent of that of a variety of Gram-positive surface proteins (including streptococci and staphylococci) (31). Internalin interacts with eukaryotic receptors on the host cell surface recently identified as cadherin E, a protein implicated in the cellular tight functions (Mengaud et al., unpublished results). In cells (for example the Caco-2 cell line) cultured in monolayers, this specific interaction induces a phagocytosis-like process (36), involving the formation of pseudopods and rapid internalisation of bacteria. The process is blocked by cytochalasin D, which inhibits cytoskeleton function. Mutants defective for the production of internalin are not able to penetrate eukaryotic cells (37). The gene adjacent to *inlA*, named *inlB*, (37) encodes another bacterial surface protein, which acts in synergy with internalin to facilitate bacterial penetration of certain cell types, for example hepatocytes (33).

This process of internalisation results in the bacteria being enclosed in a phagosome, which is rapidly acidified by proton pumps (28). The environment thus becomes too acid for the bacteria to multiply, and indeed, phagosomes express a variety of microbicidal activities (including fusion with lysosomes and an oxidative burst). However, the bacteria escape from this site by destroying the membrane of the phagosome so that they are released into the cytoplasm. Two virulence factors produced by the bacteria are involved in this step. The first one is listeriolysin O (LLO), a haemolytic exotoxin of 58 kDa (40), encoded by the *hly* gene, which is able to break down the lipid bilayer of the phagosomal membrane at acid pH (58, 91). The second one is phosphatidyl-inositol phospholipase C (PI-PLC), encoded by the *plcA* gene, which may act in synergy with LLO (13, 80). Mutants defec-

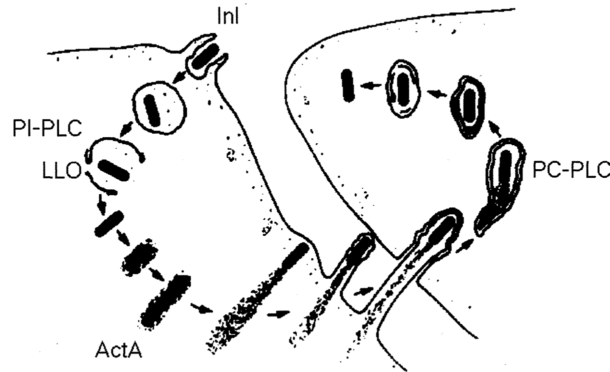


FIG. 2. Steps of the intracellular parasitism of *Listeria monocytogenes* (from 87). (See abbreviations in the text.)

tive in LLO production remain in the phagosomes and are rapidly destroyed (35, 52, 71). The importance of LLO has been confirmed by genetic complementation and directed mutagenesis experiments (20, 61). Released from the phagosome, bacteria can multiply in the host cell cytoplasm. However, escape from the phagosome must be rapid, because if the phagosome fused with lysosomes, the bacteria would be destroyed (28). Thus, intracellular survival of *L. monocytogenes* depends on the outcome of the competition between phagosome–lysosome fusion and escape from the phagosome. In general, most bacteria infecting epithelial type cells are released in the cytoplasma where they multiply (36). In contrast, almost 90% of bacteria are destroyed after phagocytosis by monocytes, the remaining bacteria multiplying in the cytoplasma of these cells (28).

Intracytoplasmic replication of *L. monocytogenes* (like that of the pathogens *Shigella flexneri* and *Rickettsia* spp.) is associated with an unusual phenomenon, actin polymerisation. Actin F polymerises around the bacterial surface, to form actin G. Actin G can be used by various bacteria to move around the cell, and even outside the cell (63, 84) at speeds of up to 1 µm/s (27). Thus, each moving bacterium is associated with a long actin tail (sometimes called the actin comet) forming in the opposite direction of the move of bacteria (84, 86, 87). The bacteria are probably propelled by the assembly of the filaments. Cytochalasin D, which paralyses the cytoskeleton, immobilises intracellular bacteria. A bacterial surface protein of 639 amino acids, called ActA, encoded by the chromosomal gene *actA* is responsible for actin polymerisation (30, 53). However, this protein does not interact directly with actin, but acts through intermediates which lead to the nucleation of actin. These intermediates are host cell proteins, including profilin (21). Intracellular movement of *L. monocytogenes* also leads to the dissemination of the bacteria to adjacent cells. ActA-negative mutants are unable to spread from cell to cell, and unlike virulent strains, do not cause plaques of lysis, on confluent cell cultures, which are evidence for bacterial spreading (53). Furthermore, these mutants are avirulent in animals (53). Thus, this system is essential for virulence allowing bacteria to spread from cell to cell without being exposed to the host immune defence system.

The bacteria are projected into adjacent cells through cytoplasmic protrusions that can reach several micrometers in size. Thus, bacteria that penetrate the adjacent cells are enclosed in a double membrane: the inner layer is the cytoplasmic membrane of the first host cell, and the outer layer

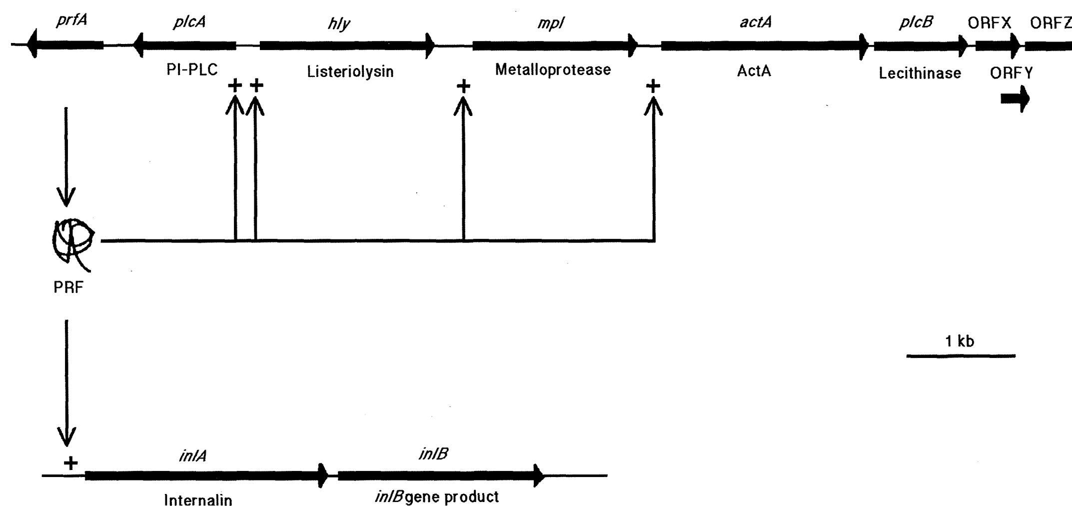


FIG. 3. Genetic organisation of the virulence genes of *Listeria monocytogenes*. (Review in 82.) (See abbreviations in the text.)

that of the new host cell. This vacuole is very different from a phagosome. There is no fusion with lysosomes due to the double membrane and the pH is neutral. The escape from this vacuole to the cytoplasm of the adjacent cell involves the production of a second bacterial phospholipase, phosphatidyl-choline phospholipase (PC-PLC) (41), encoded by the chromosomal *plcB* gene (89).

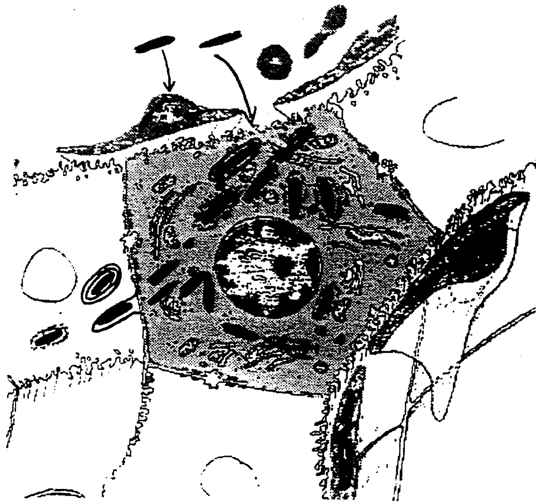
All these virulence genes involved in intracellular survival have been isolated and their sequences has been determined (see review in 82). They all map in a small region of the bacterial chromosome (Fig. 3). The gene *hly* (encoding LLO) is adjacent to the lecithinase operon, which contains the gene *mlp* (60) encoding a zinc metalloprotease implicated in the maturation of the product of *plcB* (PC-PLC) (72, 75), and the genes *actA* and *plcB*. The second operon (the *prfA* operon) is located upstream *hly* and involves both the regulatory gene *prfA* , and *plcA* encoding phosphatidyl-inositol phospholipase C (PI-PLC). Gene*prfA* is an autoregulated activator (59) of the adjacent virulence genes, and also of the more distant *inlA* and *inlB* genes (32).

The steps of the infectious process in vivo

The pathogenesis of listeriosis has been elucidated by anatomopathological analysis and studies in experimental animals. The oral route following consumption of contaminated food is the most frequent mode of transmission in human (34, 79). However, other routes of entry have been suspected but not unambiguously demonstrated (46), including the upper respiratory tract (angina, pharyngitis, flu-like infections).

Passage through the gut. *L. monocytogenes* enters the gut by crossing Peyer's patches (15, 56). Invasion of M cells might occur, allowing spread to enterocytes through the basolateral side of enterocytes, as described in *Shigella flexneri*. In experimentally infected animals, bacteria can be observed within the enterocytes of the intestinal villi (74). They are able to enter in vitro non differentiated enterocytes, without a brush border (36), suggesting that bacteria might invade non differentiated enterocytes in the crypts of Lieberkuhn. It was recently shown that the bacteria can also penetrate differentiated enterocytes

with brush borders (51). The bacteria multiply in the enterocytes and spread from cell to cell, leading to microfoci in the intestine. These microfoci release bacteria into the lamina propria. It is probable that bacteria are then opsonised by the complement (22) and are ingested by monocytes and macrophages.



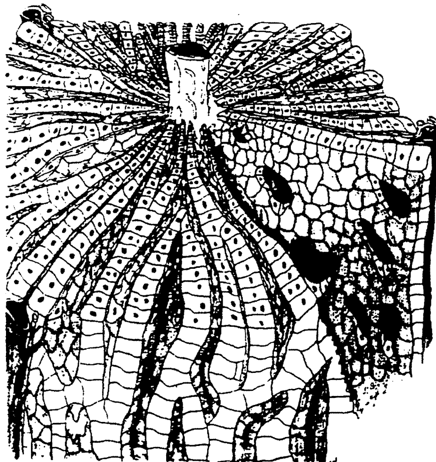


FIG. 4. The hepatocyte as a key-cell for in vivo multiplication of *Listeria monocytogenes*. Up: Infection of hepatocyte by bacteria (a direct invasion into hepatocytes might be possible). Below: Structure of sinusoid capillaries of the liver.

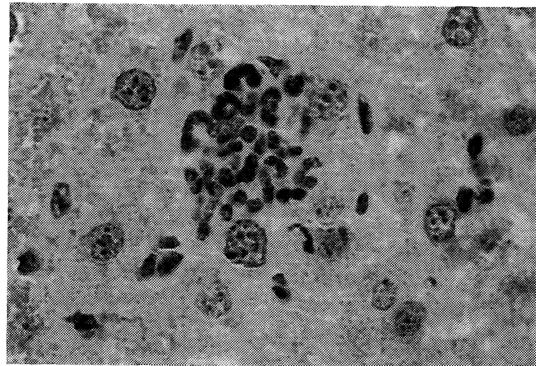
Bacteria reach then the mesenteric lymph nodes and can be disseminated to the blood. Infected monocytes kill around 90% of the ingested bacteria (28), due to rapid fusion of the phagosomes with lysosomes and an oxidative burst. Nevertheless, a small percentage of the bacteria escape from the phagosomes and multiply free in the cytoplasm of the monocytes, which are consequently destroyed. The bacteria are then released into the blood where they are quickly phagocytosed by resident macrophages in the tissues, especially the Kupffer cells in the liver and the resident macrophages in the spleen (57). The fate of the bacteria in Kupffer cells is unclear. Possibly, they multiply and then reach hepatocytes by cell to cell spread. However, they may be killed by the bactericidal activity of Kupffer cells. Indeed, bacteria are destroyed in Kupffer cells, as seen by electron microscopy following experimental infection by intravenous route (39).

Multiplication in hepatocytes, as a key-step in the infectious process. The liver plays an important scavenging role for the elimination of microorganisms from the blood. In addition to *L. monocytogenes* (17, 33, 39, 65, 83, 92), various viruses, bacteria such as *Salmonella typhimurium* and *Francisella tularensis* (17), or parasites (e.g., *Plasmodium falciparum*) can also invade and multiply inside hepatocytes. The sinusoid capillaries of the liver are constituted by a discontinuous endothelium and Kupffer cells, allowing a direct contact between hepatocytes and the blood (Fig. 4). Free *L. monocytogenes* in the blood might directly penetrate hepatocytes. The presence of intrahepatocytic bacteria surrounded by a phagosomal membrane is consistent with this belief (39). Internalin is essential for hepatocyte penetration: internalin-defective mutants are incapable of entering hepatocytes in vivo and are rapidly destroyed by Kupffer cells (39). These mutants express attenuated virulence and only induce a mild inflammatory reaction in liver tis-

sue, although they remain invasive through the gut (39). Electron microscopic observation confirms that virulent *L. monocytogenes* enter hepatocytes, and multiply freely in hepatocyte cytoplasm and spread from cell to cell, due to the polymerisation of actin in vivo (39).

The bacterial multiplication and the subsequent destruction of hepatocytes cause an intense inflammatory reaction. The polymorphonuclear cells appear at the sites of infection as soon as a few hours following infection (57), as illustrated in Fig. 5. The polymorphonuclear cells are cytotoxic for the hepatocytes, and thereby release bacteria from their intracellular sanctuary, allowing the subsequent killing by these cells (16, 18, 26, 77, 78). Cellular immunity also contributes by the involvement of CD8 lymphocytes (2, 6, 25, 44, 62, 67) directed against some of the virulence factors, including LLO (10, 12, 45). These cytotoxic lymphocytes also destroy infected hepatocytes (16, 78), releasing the intracellular bacteria.

The disease is often controlled at this stage in immunocompetent patients, so that the infection may be asymptomatic. Indeed, this is probably the most common outcome, considering the frequency of exposure to *L. monocytogenes* and the rarity of the clinical disease. However, if the inoculum is large, or in pregnant women or immunocompromised patients (patients with AIDS, patients receiving chemotherapy or with neutropenia) or those with liver anomalies (cirrhosis, haemochromatosis), the liver infection is not controlled, and thus large numbers of bacteria are released into the circulation. This allows the dissemination of bacteria to the placenta and to CNS.



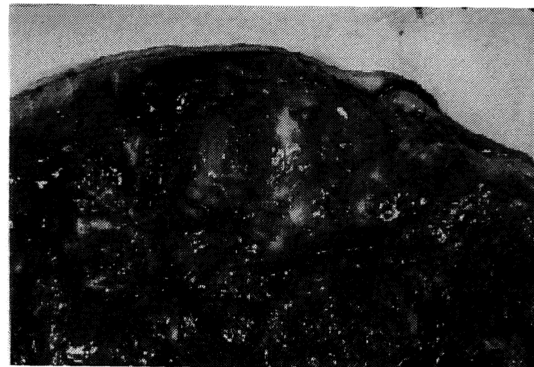


FIG. 5. Top: Hepatic infectious granuloma with polymorphonuclear neutrophils (after 24 h of infection) (Berche, P., unpublished results). Bottom: Multiple abscesses in the placenta of the patient with listeriosis.

Infection of the placenta

L. monocytogenes can infect the foetus via the placenta, most often following maternal septicaemia. Experimental work (9, 47, 55, 76) indicates that the blood-borne bacteria first access the decidua and then progress to the trophoblast and foetal circulation. The bacteria subsequently proliferate in all the foetal tissues. There is a diffuse inflammatory response involving a recruitement of neutrophils in the decidua, but there are no inflammatory cells in the trophoblast despite bacteria being visible in this tissue (76). During infections occuring in pregnancy, granulatomatous foci are observed at the surface of the placenta (Fig. 5). Pregnancy seems to favour bacterial growth in the liver and spleen of animals (9, 47, 55, 76, 83). Nevertheless, except in rare cases the infection causes only minor symptoms in the mother, usually a brief subfebrile episode, either isolated or associated with a flu-like syndrome or angina.

Thus, the maternal listeriosis is often retrospectively suspected by the occurence of severe infection of the foetus. Septicaemia in the first months of pregnancy causes spontaneous abortion, and this aetiology may not be recognised (43). During the last three months of pregnancy, severe infection of the foetus causes death in utero, or premature labour with a severely infected child. The most severe clinical outcome of infection is the classic *granulomatosis infantiseptica* (81, 88) where most organs (particularly spleen, liver, lungs and brain) show miliary lesions associated with skin eruption and rapid death. The severity of the disease is due to the duration of the infection, the infant being usually infected in utero for several days, or even longer. The placenta is covered with infectious foci from which bacteria can be easily isolated (Fig. 5). Nothing is know about the mechanism by which *L. monocytogenes* crosses the placenta, and the genes involved have not been identified. In a small number of cases (<10%), the infant is infected during labour without there being no placental infection. In such cases, the infant develops clinical infection 8 to 60 days after birth. The prognosis is good largely because of the early diagnosis. The pathogenesis of the infection is probably similar to that in adults infected orally.

Infection of the central nervous system

Neuromeningeal listeriosis in human is manifest as a lymphomonocyte or purulent bacterial meningitis. In almost half the cases, the involvement of the meninges is associated with encephalitis (49). This encephalitis is diffuse, with intracerebral abscesses mainly located in the rhombencephalon, where they are multiple, necrotic and coalescent, causing substantial loss of nervous tissue (Fig. 6). More rarely, there is encephalitis without meningitis (11, 46). The occurence of an encephalitis is unusual during bacterial infections, which mostly induce meningitis, in contrast to viruses often producing meningoencephalitis. In addition to *L. monocytogenesis*, only a few bacterial infections are capable to induce meningoencephalitis, including *Mycobacterium tuberculosis* and rare cases of meningoencephalitis due to *Nocardia asteroides*, *Brucella* or *Leptospira*. Various parasites, for example *Toxoplasma gondii*, are also able to cause severe encephalitis, particularly in patients with AIDS.

The rarity of bacterial encephalitis is due to the structure of the cerebral capillaries. The brain is well protected against numerous aggressions, including microorganisms present in the blood, by the cerebral endothelium. The structure of the cerebral capillaries is complex, consisting of endothelial cells surrounded by pericytes, perivascular microglial cells and astrocytes, that project cellular feet onto the basal membrane of endothelial cells.

These endothelial cells are unique. They are closely associated with tight junctions, and there are few pinocytosis vesicles or fenestrations. This largely prevents metabolic exchange with the circulation. The pericytes act as the musculature of the cerebral capillaries. The astrocytes contribute to maintaining the tight junctions and thus the low permeability of the capillaries. The microglial cells function as macrophages, thereby protecting the capillaries from any microorganisms that may be in a position to cross the barrier. However, the Achilles heel of the CNS is the periventricular organs. In particular, the choroid plexus is perfused by capillaries with endothelial cells without tight junctions. They are highly porous like those in the periphery, thus allowing the secretion of the spinal fluid.

There have been few studies on how *L. monocytogenes* crosses the blood-brain barrier. The evidence available suggests that the CNS is infected from the blood. However, the route through which the bacteria penetrate the sub-arachnoidian

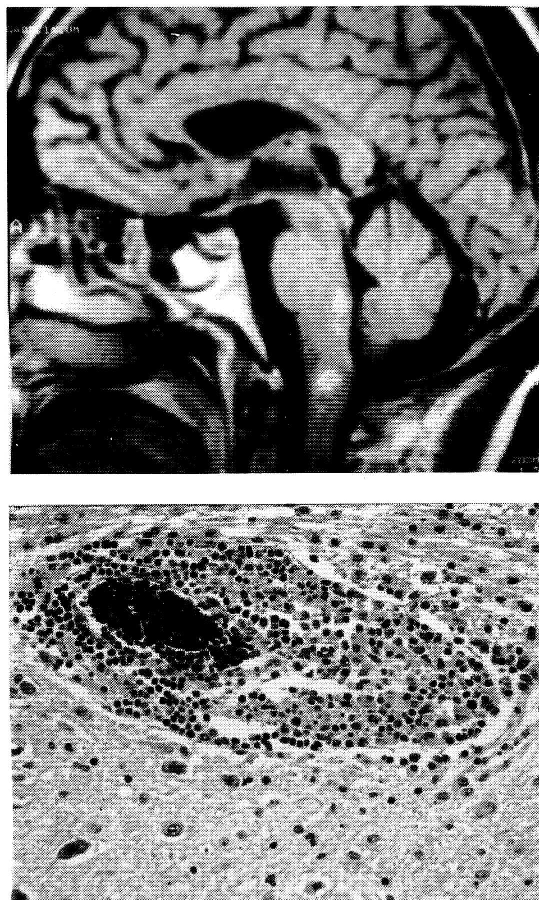


FIG. 6. Top: Rhombencephalitis due to *Listeria monocytogenes* in a patient (Nuclear Magnetic Resonance). Bottom: Perivascular inflammatory granuloma in the cerebral tissue of a patient dead of encephalitis.

space is unclear. Lesions of the choroid plexus are rare in both human and other animals (8, 69, 70), although this is the route taken by most bacteria causing meningitis. Experimental infections with large doses of bacteria given intravenously leading to massive proliferation in the liver show inflammation of the choroid plexus where bacteria can be seen (7). However, this pattern is not usually observed in clinical infections.

The mechanism by which *L. monocytogenes* causes encephalitis is unknown. Anatomopathological data (7, 8, 19, 29, 69, 70) suggest that bacteria cross the endothelium of the capillaries. The encephalitis presents as microabscesses in the grey matter which have characteristic perivascular cellular mantles with destruction of the neurones (Fig. 6). The microabscesses are distributed throughout the brain and are particularly numerous in the cerebral stem causing rhombencephalitis. The meninges might be invaded directly from the choroid plexus or the periventricular organs, or more probably through the adjacent infected cerebral capillaries. The bacteria may diffuse along the Virchow-Robin space which communicates directly with the sub-arachnoidian space in contact with the cephalorachidian fluid.

Nothing is known about the molecular mechanisms by which *L. monocytogenes* crosses the endothelium and invades the CNS. The bacteria may adhere to and directly enter the endothelial cells: *N. asteroides* has been shown by electron microscopy to do so in vivo (5); it is also able to multiply in astrocytes but not in microglial cells (4, 66). Alternatively, *L. monocytogenes* may cross the endothelia by diapedesis, being carried by infected blood monocytes. The endothelium may be permeabilised by inflammatory mediators (TNF or interleukin-1), which could cause the production of receptors on the surface of the endothelial cells and adherence of monocytes loaded with bacteria. The bacteria could then cross the endothelium with the monocyte by diapedesis or by cell to cell spreading from monocytes to endothelial cells. These various possibilities are entirely hypothetical. Indeed, it is possible that the invasion of the CNS involves as yet unsuspected mechanisms completely different from those associated with infection of enterocytes and hepatocytes.

The microabscesses of the brain are mostly constituted of monocytes and polymorphonuclear cells. The lesions are surrounded by numerous microglial cells mobilised by contact with the infectious focus and expressing phagocytic activity.

The foci grow to attain several millimeters in diameter. Most bacteria are associated with phagocytic cells, but some are also found free at the periphery of the infectious foci (7).

Severe bulboprotuberential infection leads to infection along the proximal few millimeters of the cranial nerves, causing for example facial paralysis observed in some cases of neuromeningeal listeriosis. The topology of these lesions of the brainstem has not been clearly described. The simplest explanation is that the rhombencephalon is highly vascularised. The frequency of lesions may thus simply be correlated with the density of capillaries and therefore with the blood supply in the brainstem. The elective location of listeriosis suggests that the CNS may be infected via the nerves, possibly the cranial nerves innervating the upper airways. Although this route has been demonstrated in experimental infection (4), it is probably very rare in natural infections. Whereas systemic and hepatic *L. monocytogenes* infections are usually controlled by the host defences, the cerebral lesions are much less accessible to the immune system. The brain abscesses can therefore grow while the systemic and hepatic infections are controlled.

Listeriosis remains a poorly understood infectious disease

Few pathogenic bacteria have been the subject of as much recent epidemiological, physiopathological and genetic research as *L. monocytogenes*. The intimate molecular mechanisms associated with intracellular parasitism have been partly elucidated within a few years. The route of oral transmission during epidemics has been described. The major steps of the infectious process are better understood. Furthermore, prophylactic measures have reduced the incidence of listeriosis in developed countries. However, there are numerous questions awaiting answers: the duration of incubation; the inoculating dose; whether infections result from single or repeated oral contamination; the true incidence of the disease in the population; the involvement of *L. monocytogenes* in repeated spontaneous abortion; the virulence genes involved in crossing the placenta and the blood-brain barrier; and the regulatory mechanisms of the virulence genes. The therapeutic protocols remain based on a limited clinical experience, because the sporadic nature of *L. monocytogenes* infections does not allow systematic therapeutic trials. Thus, the development of an animal model of rhombencephalitis would be a significant contribution to determining the best antibiotherapy against intracerebral infection.

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Interaction of *Salmonella* with lysosomes of eukaryotic cells

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Summary

Salmonella species are intracellular facultative pathogens which survive within phagocytic cells such as macrophages and proliferate inside vacuoles of epithelial cells. Early reports suggested that the capacity for surviving within macrophages was due to the inhibitory effect on the phagosome–lysosome fusion event induced by intracellular *Salmonella*. However, recent cell biology data, obtained both with phagocytic and epithelial cells, have shown that *Salmonella*-containing phagosomes have large amounts of lysosomal membrane glycoproteins (lgp), major components of the lysosomal membrane. This apparent discrepancy has partly been clarified at least in epithelial cells: the *Salmonella*-containing phagosome fuses with lgp-rich compartments different from the classical mature lysosome, as they do not contain certain lysosomal enzymes and are not connected with the endocytic route. Therefore, *Salmonella* seems to use an alternative strategy not merely based on the inhibition of phagosome–lysosome fusion event. This strategy essentially involves acquisition of only certain lysosomal components to form a specialized phagosomal compartment in which to survive or proliferate intracellularly. These observations have also exemplified the potential use of intracellular bacterial pathogens as biological probes to understand normal biological aspects of the eukaryotic cell. The intracellular lifestyle of *Salmonella* will undoubtedly provide new insights into the process of lysosome biogenesis.

Key words: *Salmonella*, phagosome, lysosome, intracellular proliferation, intracellular trafficking

Resumen

Las especies del género *Salmonella* son patógenos intracelulares facultativos que sobreviven en el interior de células fagocíticas tales como los macrófagos, y proliferan dentro de vacuolas de células

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epiteliales. Estudios iniciales sugirieron que la capacidad para sobrevivir en el interior de los macrófagos se debía al efecto inhibitorio de *Salmonella* sobre el proceso de fusión fagosoma–lisosoma. No obstante, datos recientes de biología celular, obtenidos tanto en células fagocíticas como en células epiteliales, han demostrado que el fagosoma que contiene *Salmonella* posee una elevada cantidad de glicoproteínas de membrana del lisosoma (lgps), componentes mayoritarios de este orgánulo. Esta aparente discrepancia ha sido resuelta, al menos para el caso de células epiteliales: el fagosoma que contiene *Salmonella* se fusiona con compartimentos de la célula huésped ricos en lgps, pero que son distintos del clásico lisosoma maduro o secundario, debido a que carecen de ciertas enzimas lisosomales y a que no están conectados con la ruta endocítica. Por tanto, se puede concluir que *Salmonella* usa una estrategia distinta a la mera inhibición del proceso de fusión del fagosoma con el lisosoma. Esta estrategia consiste esencialmente en la adquisición de ciertos componentes del lisosoma, para formar un fagosoma altamente especializado, en el que puede sobrevivir o proliferar intracelularmente. Todos estos datos sirven de ejemplo para el uso potencial de los patógenos intracelulares como sondas biológicas para la mejor comprensión de los procesos biológicos de la célula eucariota. El estilo de vida intracelular de *Salmonella* suministrará sin duda alguna nuevos datos relativos al proceso de formación del lisosoma.

Introduction

Intracellular parasite and bacterial pathogens have developed diverse strategies to survive and proliferate within the host eukaryotic cell (7, 12). Many of these strategies share a common objective: to prevent the exposure to antimicrobial activities present in the lysosome. Certain intracellular pathogens, such as *Listeria monocytogenes*, *Shigella* species, and *Trypanosoma cruzi*, use the apparently most favorable of these strategies as is the lysis of the phagosomal membrane to gain access to the host cell cytosol (17, 18, 23). Remarkably, all the rest of intracellular pathogens have adapted to live within membrane bound compartments (12). Many studies have been performed to determine whether these types of pathogens prevent fusion of the phagosome with the lysosome. This blockage permits the intraphagosomal environment be free of antibacterial activities present in the lysosome, including: (i) lysosomal enzymes, (ii) low acidic intravacuolar pH, (iii) metabolites originated by the oxygen burst activities including superoxide and hydroxide anions, (iv) nitric oxide, and (v) antimicrobial peptides such as defensins. Globally, the results obtained have shown that a certain group of these pathogens prevents phagosome–lysosome fusion (12). Methods used to monitor this event include the delivery of fluid endocytic tracers to the phagosomal compartment, and fusion of phagosome with lysosomes loaded with fluorescent or electron dense probes prior to the internalization of the pathogen (6, 29). Intracellular pathogens with capacity to block phagosome–lysosome fusion include *Mycobacterium* spp., *Salmonella typhimurium*, *Legionella pneumophila*, *Chlamydia psitacci* and the protist *Toxoplasma gondii* (12). Beside this inhibitory effect on the phagosome–lysosome fusion, some of these pathogens, such as *Mycobacterium* species, *S. typhimurium*, *L. pneumophila* and *T. gondii*, have the capacity of attenuating or blocking the intravacuolar acidification process (12). Therefore, concomitant with the effect on fusion with the lysosome, these pathogens ensure that the phagosomal pH does not reach very acidic values, which certainly can prevent the viability and proliferation within the phagosome. In this report I will present the current data regarding maturation of phagosomes containing *S. typhimurium* in the two types of cells which are target of this pathogen to cause disease: epithelial cells and macrophages (8).

Survival within macrophages and induction of macropinosome formation

The first studies that investigated the interaction of phagosomes containing *Salmonella* with lysosomes concluded that *S. typhimurium* did not block phagosome–lysosome fusion in mouse peritoneal macrophages, since phagosomes containing intracellular bacteria also contained ferritin (Table 1), an electron dense tracer loaded in secondary lysosomes prior to infection (3, 5). The results obtained from these studies suggested that intracellular survival of *S. typhimurium* within macrophages might depend on resistance, and not on evasion of, lysosomal activities (5). This suggestion has been further reconsidered, as more recent studies, performed using similar analysis to the localization of endocytic tracers in bacteria-containing phagosomes, have shown that *S. typhimurium* does block phagosome–lysosome fusion in macrophages (Table 1) (4, 19). The capacity of *S. typhimurium* to block phagosome–lysosome fusion in macrophages requires viable bacteria, but does not depend either on the route of entry into the macrophage, as opsonized bacteria also inhibit the process, or on the lipopolysaccharide, as rough strains are able to inhibit fusion (4).The discrepancy of these later results with the early reports was explained by the fact that long-term culture of macrophages before infection, as was performed in the first studies, seems to enhance phagosome–lysosome fusion levels (4). Recently, a transposon-mediated mutant of *S. typhimurium* has been described that lacks the ability to block phagosome–lysosome fusion (20). Although this mutant had reduced survival in macrophages, it was still virulent in the murine typhoid model (20). This result seems to discard a clear correlation between capacity for blocking phagosome–lysosome fusion and development of virulence.

Another point of debate was added to this crucial issue of phagosome–lysosome fusion when Alpuche-Aranda et al. (1) published an elegant study showing that *S. typhimurium* attenuates the acidification of the phagosome in macrophages. While phagosomes containing dead bacteria reach pH <4.5 at 1 h post-infection, phagosomes containing live *S. typhimurium* maintain a pH >5.0 up to 4 h post-infection. One of the most relevant data cited in the study, albeit not shown, is that phagosomes seem to fuse rapidly with lysosomes, as fluorescent dyes to assay phagosome–lysosome fusion and antibodies to specific lysosomal proteins, labeled phagosomes containing *S. typh-*

TABLE 1. Studies describing interaction of *Salmonella typhimurium* with lysosomes of the host cell[1]

Year	Eukaryotic cell	Technique used	Conclusion of the study	Ref.
1968	Macrophage	TEM	*S. typhimurium* does not block P-L fusion	3
1979	Macrophage	TEM	*S. typhimurium* does not block P-L fusion	5
1990	Macrophage	TEM	*S. typhimurium* inhibits P-L fusion	4, 19
1992	Macrophage	IIF	*S. typhimurium* phagosomes rapidly fuse with lysosomes which do not acidify	1
1993	Epithelial cell	IIF	*S. typhimurium*-containing vacuoles fuses with lgp-containing vacuoles inducing formation of Sifs	14, 15
1995	Epithelial cell	IIF	*S. typhimurium*-containing vacuoles fuse with a specialized type of lysosome	13

[1] Abbreviations: P-L fusion, phagosome–lysosome fusion; TEM, transmission electron microscopy; IIF, indirect immunofluorescence; lgp, lysosomal membrane glycoprotein; Sif, *Salmonella*-induced filamentous lysosome.

imurium very early after phagocytosis (Fig. 1). The main conclusion of this study was that *S. typhimurium* reside in an atypical phagolysosome, which does not undergo rapid acidification. This characteristic could be the basis of the capacity of this pathogen to survive within macrophages.

The development of an 'atypical' phagolysosome containing *S. typhimurium* in macrophages might be related to the fact that phagosomes containing *S. typhimurium* derive from a specialized transient organelle named 'macropinosome', a very enlarged vacuole formed as a result of uptake of large amounts of extracellular fluid (2). Macropinosomes normally appear when cells are stimulated with certain active molecules as growth factors, and are always associated to alterations in the morphology of the plasma membrane, which forms large pseudopod-like extensions, named ruffles, in response to the growth factor-derived stimulus (2, 9, 24). These long membrane extensions permit the engulfment of large amounts of extracellular fluid. Macropinosomes are very transient organelles as they shrink rapidly (1–10 min) after formation. Some active molecules known to induce this type of effects include the epidermal-growth factor (EGF) (9), and the macrophage-colony stimulating factor (MCS-F) (24).

S. typhimurium is also able to induce membrane ruffling and macropinosome formation in both macrophages and epithelial cells (2, 11). It has been shown that, at least in macrophages, these macropinosomes contain internalized bacteria, and are therefore known as spacious phagosomes (SP). In contrast to macropinosomes induced by active molecules, SP induced by *S. typhimurium* shrink much slower (2). Although in macrophages it is currently unknown which percentage of SP containing *S. typhimurium* have lysosomal markers, different observations made in epithelial cells have shown that only a low proportion of macropinosomes (<10%) have lysosomal membrane glycoproteins (García-del Portillo, F., unpublished observations). Therefore, it seems that SP formation and fusion with lysosomal compartments could be unrelated processes, and presence of lysosomal markers in SP depends exclusively on the balance between SP shrinking and fusion with lysosomes dictated by separated signals. Nevertheless, a correlation between SP formation and development of an atypical phagosome cannot be totally discarded, as it has been shown that SP are required for *S. typhimurium* survival within macrophages (2), and this organelle can contribute, for example, to the attenuation of the intraphagosomal acidification process (2).

Proliferation within epithelial cells and formation of *Salmonella*-induced filamentous lysosomes (Sif)

One of the most atractive features of the intracellular lifestyle of *Salmonella* is its capacity to proliferate actively within membrane bound vacuoles in nonphagocytic cells such as epithelial cells (8). This property is essential for this pathogen to develop disease, as it has been shown that intracellular replication defective mutants (rep^-) of *S. typhimurium* are avirulent in the mouse typhoid model (22). In vitro studies have shown that within 4–6 h post-entry, intracellular *S. typhimurium* initiates a proliferation period within the vacuole.

Why does a lag period of a few hours exist before *Salmonella* starts to proliferate? Does *Salmonella* proliferate in a phagosomal compartment which has fused with lysosomes? What is the phagosomal environment that permits *Salmonella* to grow within this compartment? These are some of the open questions which remain not fully answered. Perhaps, one of the most striking results that may provide clues about intracellular proliferation of *Salmonella*, has been the discovery of a unique host-derived structure in infected epithelial cells at the time bacteria initiate growth (14, 15). This structure, named Sif, for *Salmonella*-induced filamentous lysosome, has large amounts of

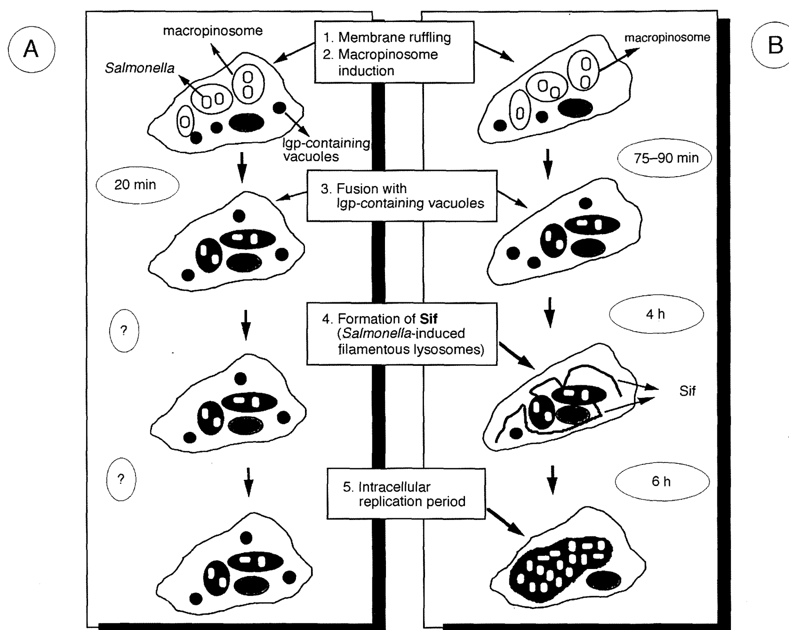

FIG. 1. Interaction of phagosomes containing *Salmonella typhimurium* with lysosomes of (A) macrophages; and (B) epithelial cells. Shown are the main steps recently described regarding fusion of *Salmonella typhimurium*-containing phagosomes with lysosomal compartments containing lgps. Note that this fusion process is more rapid in phagocytic cells, and the Sif (*Salmonella*-induced filaments) are only formed in *Salmonella typhimurium*-infected epithelial cells.

lysosomal membrane glycoproteins (lgp), and connects *Salmonella*-containing vacuoles (SCV) within the same cell forming an intrincate network (14, 15). Sifs are visible in infected epithelial cells from 4 h post-infection, a time at which an increase in viable intracellular bacteria is detected (14). Interestingly, fusion of SCV with compartments containing lysosomal membrane glycoproteins and lysosomal enzymes, such as lysosomal acid phosphatase (LAP), has been completed at 2 h post-infection (Fig. 1) (13). Therefore, intracellular *Salmonella* reside in lysosomal compartments within epithelial cells in which some signal, related to bacterial growth, triggers a further rearrangement of the lysosomal compartments of the host cell to induce Sif formation. Sifs are not present in uninfected epithelial cells and are not induced by other intracellular bacterial pathogens which lack capacity for growing intracellularly, such as *Yersinia* species (14). Additional experimental evidence supports the involvement of Sif in *Salmonella* intracellular replication (14), including: (i) Sifs are not observed when intracellular wild type *Salmonella* is killed with antibiotics; (ii) Sifs are not present in epithelial cells infected with *S. typhimurium rep*⁻ mutants; and (iii) other *Salmonella* serotypes which grow intracellularly, such as *S. typhi*, *S. choleraesuis*, *S. enteritidis*, and *S. dublin*, induce Sifs when they infect epithelial cells. Despite all these pieces of information, the exact function of Sif remains to be defined.

The *S. typhimurium* gene which product is required for Sif formation has been recently isolated and characterized (28). This gene, named *sifA*, is located in an unusual position in the *Salmonella* chromosome, as is inserted into the *pot* operon (*potABCD* in *Escherichia coli*), involved in polyamine transport. In *S. typhimurium*, *sifA* locates between the *potB* and *potC* genes (28). Remarkably, in the flanking regions of the *sifA* gene there are non-enconding sequences (size ~300 bp) containing direct repeated sequences

(14 bp) in their ends, suggesting that *sifA* was probably acquired by *Salmonella* via a transposition event. Interestingly, *sifA* seems to be a *Salmonella*-specific gene, as no homolog has been found by hybridization studies in other enterobacterial pathogens and non-pathogenic bacteria (28).

With the current data, it is obvious that Sifs represent a dramatic redistribution of lysosomes to permit *Salmonella* to grow within SCV. An atractive postulate is that Sifs may function as channels to provide intracellular bacteria with nutrients. Similar filamentous networks connected to phagosomes have been described with analogous function for intracellular eukaryotic parasites such as *Toxoplasma gondii* and *Plasmodium falciparum* (12).

Intracellular trafficking of *Salmonella*-containing vacuoles (SCV) in epithelial cells: the postulate of different types of lysosomes

As mentioned above, fusion of SCV with compartments containing lysosomal markers is completed by 2 h in epithelial cells, preceding Sif formation, which initiates at 4 h post-entry (Fig. 1). A very recent report has shown that fusion of SCV with lysosomal compartments is unsual, as not all the classical lysosomal markers are present in SCV (13). For example, lysosomal membrane glycoproteins and LAP appear in the SCV with the same kinetics and, at a certain time post infection, 2 h, all SCV contain these two lysosomal markers (13). However, other lysosomal markers, such as the soluble enzyme cathepsin D, appear in a low percentage of SCV (~30–40%) at the same post-infection time (13). These results suggested that SCV probably fuse with a type of lysosome lacking some of its 'expected' components.

During the time SCV fuse with lysosomes, 15 to 120 min post-entry, no late endosomal markers, such as mannose-6-phosphate receptors, are detected in the SCV (13). The late endosome, an organelle of the endocytic route, is an obligate intermediate organelle in the process of lysosome biogenesis (16, 21), and therefore the above results suggested that SCV fuse with lysosomes using an unusual trafficking route bypassing compartments of the endocytic route (16, 21). So, it is probable that SCV fuse with 'non matured lysosomes' that traffic to the plasma membrane via an exocytic route, preventing them from continuing their normal traffic route. These 'non matured lysosomes' may contain only part of their lysosomal contents, and only when they reenter the endocytic route from the plasma membrane, incorporate other lysosomal markers, such as the lysosomal enzymes recognized by mannose-6-phosphate receptors. Further data confirmed that indeed SCV are disconnected from the endocytic route, as uptake of fluid endocytic tracers by infected cells does not result in delivery of the tracer to the SCV, and SCV do not fuse with matured lysosomes pulsed and chased with endocytic probes (13). Therefore, *S. typhimurium* reside in an atypic compartment, with certain lysosomal elements, but highly distinct of a classical phagolysosomal compartment containing the full repertory of lysosomal enzymes. These unique characteristics of the SCV may provide this compartment with optimal conditions for bacteria to initiate intracellular proliferation.

Conclusions

The biology of the intracellular lifestyle of *Salmonella* within eukaryotic cells is starting to be defined by the use of vesicle markers, including endosomal and lysosomal markers. This experimental approach has changed the conclusion regarding interaction of *S. typhimurium* with lysosomes of the host cell. First, it was suggested that *S. typhimurium* was resistant to lysosomal activities since no inhibition of lysosome-fusion was evident (3, 5). Later studies showed that phago-

some–lysosome fusion was blocked by *S. typhimurium*, and this property was correlated to the capacity of the pathogen to survive within macrophages (4, 20). Nevertheless, the most recent studies analyzing distribution of lysosomal markers in *S. typhimurium*-infected cells, have shown that phagosomes containing this pathogen have some lysosomal markers, both in macrophages and epithelial cells (1, 13, 14, 15). For example, it has been shown that lysosomal membrane glycoproteins are massively present in *Salmonella* phagosomes (13, 14, 15). These molecules, which are the major components of the lysosomal membrane (10), are also present in phagosomes containing other intracellular pathogens, such as *Mycobacterium avium* (26, 30), *M. tuberculosis* (26), *Yersinia* (14), and the protists *Leishmania* (26) and *Toxoplasma gondii* (27). It has been postulated these glycoproteins could protect the lysosomal membrane from hydrolytic activities present in the lumen of the lysosome (10), and a similar function could have these glycoproteins in the phagosomal membrane. Intracellular pathogens may have some predilection to fuse with vacuolar compartments containing these molecules. However, the strategy of obtaining a safe 'coat' for the phagosome may have some detrimental consequences for the pathogen if other lysosomal components, such as lysosomal enzymes, are also delivered to the phagosome upon fusion with a 'classical' secondary or mature lysosome.

S. typhimurium has selected a unique strategy to avoid certain lysosomal enzymes, as has been recently demonstrated in epithelial cells (13). The *Salmonella*-containing vacuole (or SCV) fuses with compartments containing lysosomal membrane glycoproteins but devoid of certain lysosomal enzymes such as cathepsin D. Moreover, SCV does not intersect with the endocytic route and bypass an intermediate organelle in lysosome biogenesis, as it is the late endosome. This unusual intracellular targeting route has also been postulated for *Mycobacterium* species (26, 30). These aspects also revealed for the first time the capacity of intracellular pathogens to select a certain type of lysosome and form a highly specialized phagosome where to survive and proliferate. Without any doubt, this type of studies will provide a great amount of data related to the mechanisms used by intracellular pathogens and, at the same time, will increase our knowledge of the cell biology of the host eukaryotic cell.

Attempts are currently being carried out to identify the eukaryotic and bacterial factors involved in the specific interaction between the phagosome and lysosomes. These approaches include: (i) the purification of intact phagosomes and characterization of phagosomal membrane components, successfully performed for *Mycobacterium* and *Leishmania* phagosomes (30), and, (ii) in vitro studies on the effect of extracts obtained from infected cells in the functionality of eukaryotic proteins involved in vesicular budding and fusion (25). In a few years, we will certainly have a more detailed picture, at a molecular level, of the mechanisms that control fusion of pathogen-containing vacuoles with lysosomal compartments.

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Molecular determinants of *Yersinia* pathogenesis

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Summary

The genus *Yersinia* contains three pathogenic species: *Yersinia pestis, Y. pseudotuberculosis* and *Y. enterocolitica.* Even though the three species use different routes to infect their host and provoke diseases of different intensity, they share a common tropism for the lymphoid tissue and they are able to resist the primary immune response of the host. The main genetic determinants involved in this resistance are encoded by a highly conserved 70-kb virulence plasmid. The genes harbored by the pYV plasmid encode the lipoprotein YlpA, the outer membrane protein YadA, and a group of at least 11 secreted proteins called Yops. The pYV plasmid also encodes the apparatus necessary for the secretion of the Yop proteins, as well as those involved in the regulation of Yop synthesis. The Yop proteins are secreted by a specific secretion system which is considered as the archetype of a new secretion pathway called type III. After their secretion they are immediately internalized into the cytosol of a target eukaryotic cell, which represents a new phenomenon in microbial pathogenesis. The chromosome of *Y. enterocolitica* completes the virulence panoply of the bacteria by encoding an enterotoxin called Yst, fibrillae named Myf and an invasin called Inv.

Key words: *Yersinia*, Yop proteins, Myf fibrillae, Yst enterotoxin, invasins

Resumen

El género *Yersinia* contiene tres especies patógenas: *Yersinia pestis*, *Y. pseudotuberculosis* y *Y. enterocolitica.* Aunque las tres especies utilizan rutas diferente para infectar a su hospedador y provocar enfermedades de intensidad diversa, comparten un tropismo común por el tejido linfático y son capaces de resistir la respuesta inmunitaria primaria del hospedador. Los principales determinantes genéticos implicados en esta resistencia están codificados por un plásmido de virulencia altamente

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conservado (70 kb). Los genes que contiene el plásmido pYV codifican la lipoproteína YlpA, la proteína de membrana externa YadA y un grupo de al menos once proteínas secretadas denominadas Yops. El plásmido pYV codifica el sistema necesario para la regulación de la síntesis y la secreción de las proteínas Yop. Las proteínas Yop son secretadas por un sistema específico que se considera el arquetipo de una nueva vía de secreción denominada tipo III. Después de su secreción, las proteinas Yop son internalizadas inmediatamente en el citosol de la célula eucariótica diana, lo que representa un nuevo fenómeno en la patogenicidad microbiana. El cromosoma de *Y. enterocolitica* aporta los dispositivos complementarios de virulencia de la bacteria, codificando una enterotoxina denominada Yst, fibrillas denominadas Myf y una invasina denominada Inv.

Introduction

Yersinia pestis, the etiological agent of plague, enters the host via the bite of an infected flea and travels to the nearest lymphoid tissue where it proliferates. The inflammatory response of the host produces swelling of this lymphatic node and the formation of a bubo which gives bubonic plague its name. From infected lymph nodes, bacteria can spread via the blood stream, reach the lung and multiply in lung macrophages. This stage of the disease (pneumonic plague) is the most contagious one because the bacterium can easily be transmitted by aerosols. The disease resulting from the inhalation of aerosols progresses very rapidly, probably because bacteria already express all the virulence factors, and is often fatal in a few days.

Infections due to *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are acquired by the ingestion of contaminated food or water. Then, the two organisms cross the epithelial layer in the terminal ileum through the M cells (35), and multiply in the gut associated lymphoid tissue. Both species are widely distributed in the environment and they contaminate wild and domestic animals as well as humans. *Y. enterocolitica* causes gastrointestinal syndromes of varying intensity ranging from mild self limited diarrhea to mesenteric adenitis evoking an appendicitis. Reactive arthritis and erythema nodosum are common complications (24). The main cause of infections by *Y. enterocolitica* in Europe is pork meat (86, 94). Several cases of septic shock due to transfusion of blood contaminated with *Y. enterocolitica* have also been reported (87). *Y. pseudotuberculosis* is essentially a rodent pathogen but it occasionally provokes infections in humans. The disease caused by *Y. pseudotuberculosis* is similar to that caused by *Y. enterocolitica* but systemic complications are more common, and diarrhea is usually not a symptom.

Even though the three *Yersinia* species use different routes to infect their host, and provoke diseases of very different intensity, they share a common tropism for the lymphoid tissue and they are able to resist the nonspecific immune response of the host (Fig. 1). The main genetic determinants involved in this resistance are highly conserved and are encoded by a 70-kb virulence plasmid common to all pathogenic strains. In *Y. enterocolitica* several chromosome encoded genes are involved in the first step of the infection. This review is mainly devoted to *Y. enterocolitica* and the differences with the other species will be mentioned throughout. We will mainly focus on pathogenicity factors and interactions of the pathogen with its host. The regulation of expression of virulence factors has been recently reviewed by ourselves and by other authors (23, 84). (For a complete overview of yersiniae, see 12, 13, 18, 21, 22).

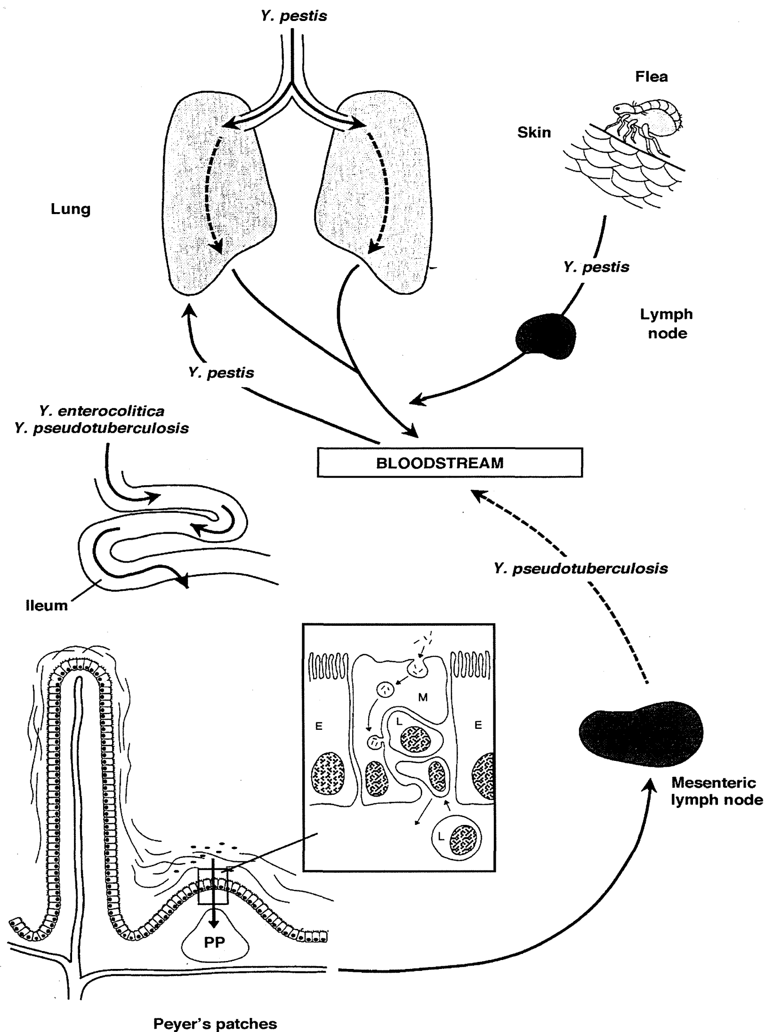

FIG. 1. Schematic representation of the routes of entry and localization of *Yersinia pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. *Y. pestis* infections can be acquired by direct transmission via aerosols or by flea bite. *Y. enterocolitica* and *Y. pseudotuberculosis* enter the gastrointestinal tract via the ingestion of contaminated food or water. They cross the epithelial cell layer in the terminal ileum through the M cells. The three species share a common tropism for the lymphoid tissue. Systemic complications due to *Y. pseudotuberculosis* and *Y. enterocolitica* are not common (dashed line).

The chromosome encoded virulence functions of *Yersinia enterocolitica*

The enterotoxin Yst. The most common syndrome observed in young children after infection by *Y. enterocolitica* is diarrhea. The same syndrome can be reproduced in the laboratory by inoculating *Y. enterocolitica* to young rabbits. One of the factors involved in the production of diarrhea seems to be the small thermostable enterotoxin Yst (27, 28, 62, 72, 85). Yst is encoded only by the chromosome of pathogenic serotypes of *Y. enterocolitica*, but not by *Y. pseudotuberculosis* or *Y. pestis* (27). It is similar to the heat-stable enterotoxin (ST1) of *Escherichia coli* and both stimulate the intestinal guanylate cyclase (72). Yst and ST1 resemble guanylin, an endogenous activator of intestinal guanylate cyclase (25), suggest-

ing that the genes encoding these toxins could have been acquired by horizontal transfer from a eukaryotic host.

The Myf fibrillae. To exercise its action, the enterotoxin should be delivered in close proximity to the enterocytes. Thus, the ability of enterotoxin-producing bacteria to adhere to the intestinal epithelium is another important factor in the onset of diarrhea. In enterotoxigenic *E. coli* and *Vibrio cholerae*, this adhesion is mediated by specific appendages called fimbriae or fibrillae. Some time ago, Diaz and co-workers described a temperature-induced surface antigen common to pathogenic serotypes of *Y. enterocolitica* (29, 88). This antigen is synthesized when bacteria are grown at 37°C on a solid medium supplemented with a metabolizable sugar (29, 88). Electron microscopy studies showed that this surface antigen is a fibrillar structure which closely resembles the colonization factor of enterotoxigenic *E. coli* (40), suggesting that Myf could be the enterocyte adhesion factor required for Yst action (Fig. 2A). We cloned and characterized the genes involved in the production of the Myf fibrillae. These genes are situated on the chromosome and they are organized as an operon that strongly resembles those involved in the production of fimbriae in enterotoxigenic *E. coli* (Fig. 2B). Gene *myfA* encodes the subunit of the Myf antigen while *myfB* and *myfC* encode an assembly machinery related to those involved in assembly of other surface appendages. MyfB is a putative periplasmic chaperone that belongs to the PapD family and MyfC is an outer membrane protein probably acting as an usher directing export and assembly of the pilin subunits. Interestingly, the sequence of the *myf* genes resembles the sequence of the genes encoding CS3 pili encountered in strains of *E. coli* colonizing the human intestine (40).

The role of Myf in the pathogenesis of *Y. enterocolitica* has not been clarified yet. Some data suggest that Myf could indeed play a role in the infection. First, antibodies against the Myf antigen are detected in rabbits and mice experimentally infected with *Y. enterocolitica* pre-grown at room temperature, indicating that this antigen is synthesized in vivo (Iriarte and Cornelis, unpublished observations). Second, *myfA*, the gene encoding the subunit of the Myf antigen, is only present in the chromosome of *Y. enterocolitica* serotypes associated to pathogenesis (40). A possible and appealing role for Myf would be to promote the colonization of the intestine and so to allow the delivery of the enterotoxin Yst at the surface of the enterocytes. In this hypothesis, the pathogenesis of *Y. enterocolitica* diarrhea would mimic that of enterotoxigenic *E. coli* or *V. cholera*. However, MyfA is 44% identical to PsaA the major subunit of the pH6 antigen from *Y. pestis*, a fibrillar antigen which is involved in systemic infection (53, 54, 70). Thus we can not exclude that Myf plays a role as an antiphagocytic or protective surface antigen.

The invasin Inv. *Y. enterocolitica* and *Y. pseudotuberculosis* synthesize a 100-kDa outer membrane protein called Inv (44, 60). This protein promotes the entry of *Yersinia* into non-phagocytic cultured mammalian cells after binding to β1 integrins. Entry process involves reorganization of the host cytoskeleton and is hypothesized to occur by zippering of the host cell surface around the invasin-coated bacterium (10, 46, 63, 89, 96). However the role of Inv in the pathogenesis of *Yersinia* infection is not so clear. In *Y. enterocolitica* Inv seems to be involved in the first steps of intestine penetration, but a Inv- strain is able to penetrate and colonize the Peyer's patches to the same extent as the wild type strain though more slowly (64). *Y. pestis* does not produce Inv and a *Y. pseudotuberculosis* double mutant unable to synthesize the invasin and YadA (see below) is surprisingly more virulent than the wild type strain (73). These observations suggest that Inv does not play a crucial role in the infection process.

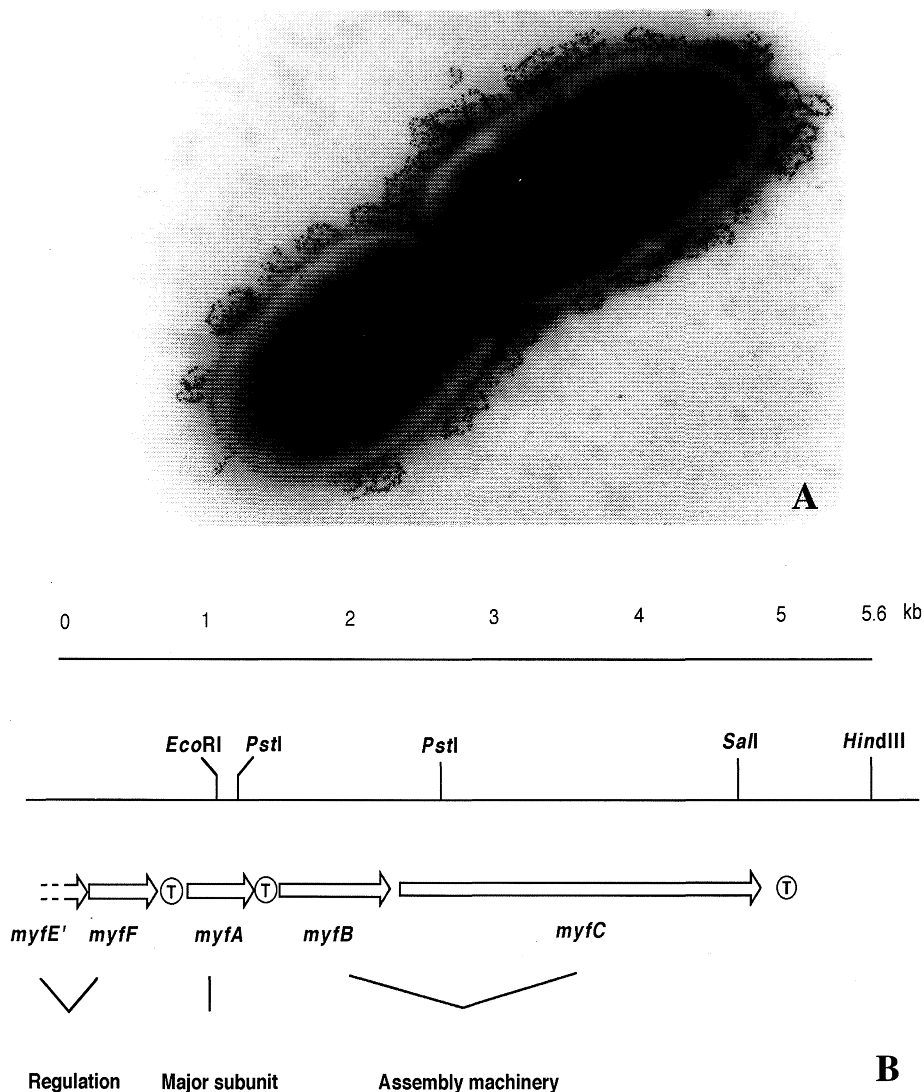

FIG. 2. The Myf fibrillae of *Yersinia enterocolítica*. (A) Electron micrograph showing the structure of the Myf fibrillae revealed by immunogold. (B) Schematic representation of the chromosomal locus containing some of the genes involved in the synthesis of the Myf antigen.

Regulation of the expression of chromosomal genes involved in pathogenesis. Chromosomal virulence genes are under the dual control of bacterial growth-phase and environmental variables.

Inv, *yst*, and *myf* are not expressed during the exponential growth. Expression of *inv* and *yst* begins when cells enter late-exponential phase to early stationary phase and continues throughout the stationary phase (59, 65). By contrast, *myfA* transcription occurs during transition between late exponential phase and early stationary phase and then decreases gradually throughout the stationary phase (42). Growth-phase dependent gene regulation is a highly complex phenomenon far from being completely understood. In *E. coli*, expression of many stationary-phase genes requires the alternative sigma factor RpoS (for a review see 39,

49). In order to determine the role of this factor in transcription of *yst*, *inv* and *my*f genes, we have isolated and mutagenized the homolog of *rpoS* in *Y. enterocolitica* (42). In such a mutant, *yst* expression is reduced though not completely abolished indicating that the *Y. enterocolitica* homolog of RpoS is involved in the expression of *yst* (42). However, as for many *E. coli rpoS*-regulated genes, additional control mechanisms responding to growth-phase, must also be involved. In agreement with this, *yst* expression is modulated by osmolarity and by the histone-like protein YmoA (see below) (59). The mechanism of action of RpoS in *yst* regulation appears thus to be quite complex. So far, it is impossible to conclude whether RpoS regulates *yst* directly by binding to the promoter region, or indirectly through other regulatory proteins. Expression of *myf* and *inv* is not significantly affected in a *rpoS* mutant (7, 42), indicating that they do not depend on RpoS for their transcription.

The three chromosomal virulence genes are also thermoregulated though not always in the same direction. The host temperature of 37°C reduces expression of *inv* and *yst* as compared to ambient temperature (27, 45, 59, 67). This may seem surprising for virulence functions but temperature upshift is not the only stimulus which triggers *Y. enterocolitica* once they enter the host. Several environmental cues also affect the expression of *inv* and *yst*. The most significant ones in the intestinal tract are osmolarity, pH, oxygen and ions concentrations. In vitro, one can observe *inv* expression in *Y. enterocolitica* at 37°C when pH goes below 7 and in high salt. Thus, in vivo, the acidity of the stomach, and the high Na^+ concentrations close to the enterocytes brush border could allow continous *inv* expression at the host's body temperature (65). In vitro, *yst* transcription can be induced, at 37°C by increasing Na^+ and K^+ ions concentration as well as pH to values normally present in the ileum lumen (59). Thus, although *inv* and *yst* are preferentially expressed in vitro at low temperature, it is very likely that they are expressed at 37°C in vivo.

The expression of Myf is regulated at the transcriptional level by pH and temperature. Gene *myfA* is transcribed only at 37°C and acidic pH (41). Myf production is thus downregulated when *Y. enterocolitica* are in the environment and it is stimulated when they enter their host. After entering the host, Myf synthesis is thus turned on presumably by the acidic pH of the stomach and nondividing bacteria could keep their fibrillae when they colonize the intestine at alkaline pH. This interpretation, which is sustained by our hypothesis that Myf plays a role in intestinal colonization, is supported by the fact that *Vibrio cholerae* enterotoxin-associated pili are also expressed at 37°C and low pH. Tight regulation by acidity suggests that the regulating network controlling *myf* expression is more specific and probably more sophisticated that those controlling the expression of *yst* and *inv*. Transcription of *myfA* requires at least two genes *myfF* and *myfE* situated immediately upstream from *myfA*. The *myfF* product does not show any similarity to any known regulatory protein but the topology of the protein strikingly evokes ToxS, a protein involved in regulation of Tcp pilus in *Vibrio cholerae* (41). Genes *myfE* and *myfF* are presumably part of a whole network because other chromosomal mutations, not yet analyzed, also affect expression of Myf (Iriarte and Cornelis, unpublished data).

The pYV plasmid

The three pathogenic *Yersinia* species carry a common virulence plasmid of 70 kb called pYV (Fig. 3). Strains that have been cured of their virulence plasmid have a decreased capacity to disseminate to deeper organs. The genes encoded by the pYV plasmid are highly conserved among

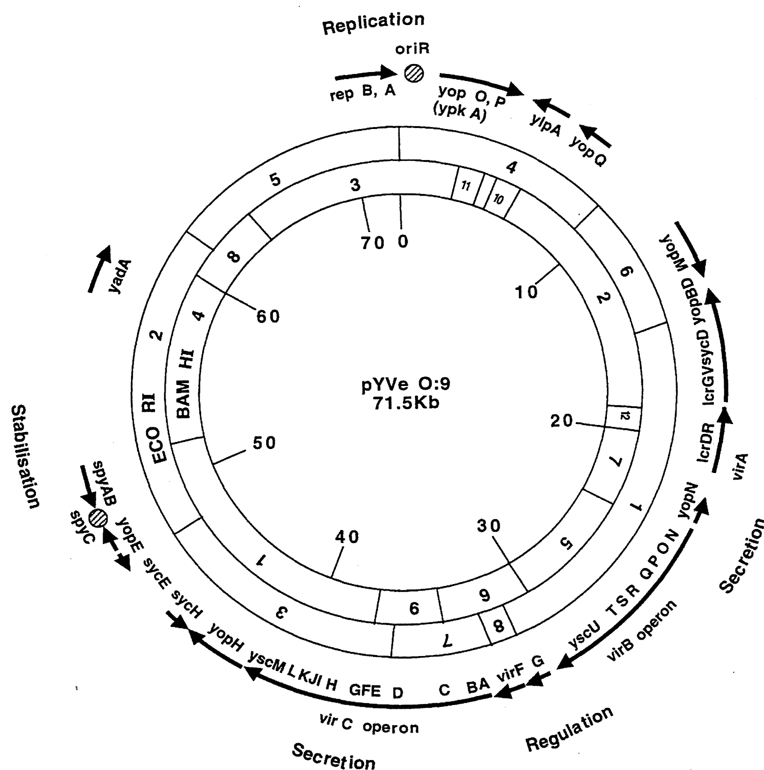

FIG. 3. The pYV plasmid of *Yersinia enterocolitica*. The virulences genes and the organization of the pYV plasmid are highly conserved in the three pathogenic species of *Yersinia*.

the three species. Their products include the lipoprotein YlpA, the outer membrane protein YadA, and a group of at least 11 secreted proteins called Yops. The pYV plasmid also encodes the apparatus necessary for the secretion of the Yops proteins, and proteins involved in the regulation of Yop synthesis (for review see 22, 83).

The pYV plasmid is a very well organized machinery. Apart from one quadrant, all the genes are disposed in a very compact way and the intergenic regions are very short. Some of the *yop* genes and the *ysc* genes encoding the secretion machinery are organized as operons and within these operons the stop codon of one gene often overlaps with the start codon of the downstream gene.

Regulation of pYV encoded functions. The expression of the pYV-encoded genes is thermoregulated. Genes are strongly transcribed at 37°C and poorly at low temperature. Temperature regulation is not completely understood but it involves YmoA, a histone-like protein encoded by the chromosome (20). YmoA is the homolog of Hha, a regulator of hemolysin production in *E. coli* (61) and both proteins are the first representatives of a new class of histone-like proteins regulating the expression of topologically sensitive promoters (58). In addition, transcription of many genes also requires the product of *virF*, a 30-kDa transcriptional activator of the AraC family (19, 50). Finally the expression of the *yop* genes in vitro is downregulated by a high Ca^{2+} concentration. In vitro, the massive production of Yops at 37°C and in the absence of Ca^{2+}, correlates with a severe growth restriction. This phenomenon is currently described as "Ca^{2+}-dependency" (for review see 23, 82, 84).

The outer membrane proteins YadA and YlpA. YadA is an outer membrane protein thought to form a fibrillar structure at the surface of *Y. enterocolitica* and *Y. pseudotuberculosis* (48, 97). *Y. pestis* contains the *yadA* gene but it is inactivated by a point mutation (73). YadA is only produced by bacteria grown at 37°C. It forms a polymer of 200–240 kDa by association of 4–5 subunits of 50 kDa, addressed via the classical Sec export pathway (79).

The presence of YadA in the outer membrane considerably increases the hydrophobicity of the bacterial surface and mediates a strong adherence to various substrates including mammalian cells. This suggests that YadA can act as a colonization factor, but some observations indicate that, at least in *Y. enterocolitica*, YadA plays a major role in the defence against nonspecific immune responses. It confers resistance to the bactericidal activity of human serum by promoting the fixation of factor H (16), which leads to the degradation of C3b deposited at the bacterial surface (68) and prevents C3-mediated phagocytosis and killing by polymorphonuclear leukocytes (17).

YlpA is a 29-kDa lipoprotein related to the TraT proteins encoded by the *E. coli* sex factor F, and by the virulence plasmid of *Salmonella typhimurium*. The expression of *ylpA* is regulated like that of the *yadA* and *yop* genes but the role of YlpA in the pathogenesis of *Y. enterocolitica* has not been determined yet (15).

The Yops proteins. The Yop proteins are important virulence factors playing a central role in the antiphagocytic response of *Yersinia* either by interfering with the signal transduction (YopH) or by attacking the host cytoskeleton (YopE).

There are at least eleven Yops, but functions have been assigned only to a few of them (for review see 83). YopH is a protein tyrosine phosphatase (36) acting on multiple substrates in the cytoplasm of macrophages. Since dephosphorylation of tyrosine residues is one of the early events of phagocytosis, it has been suggested that YopH counteracts these early signals and prevents phagocytosis of *Yersinia* by macrophages (9, 74). YopO (called YpkA in *Y. pseudotuberculosis*) is a protein serine-threonine kinase with extensive similarity to eukaryotic serine-threonine protein kinases (34). YopE has a cytotoxic effect on eukaryotic cells leading to depolymerization of the actin network by an unknown mechanism (75, 76). The function of YopE during infection may be to kill phagocytes. YopM is a 41-kDa protein sharing significant similarity with the thrombin-binding domain of the α-chain of human platelet membrane glycoprotein Ib (α-GP1b). Accordingly, YopM binds thrombin (51, 71), inhibits platelet aggregation (52) and may prevent platelet-mediated host defense events of the inflammatory response. No cytotoxic or enzymatic activity has been reported so far for YopB, YopD, YopN, YopP, YopQ, LcrV, and YopR. Yops alone do not have any cytotoxic effect. To exert their activity they need to be injected by the bacteria directly inside the eukaryotic cell (see below). Interestingly, two major Yops, YopB and YopD have transmembrane domains (37), and are involved in this translocation process (see below).

A new secretion pathway. The Yop proteins are secreted by a specific secretion system which is considered as the archetype of a new secretion pathway called Type III (Fig. 4). The signal required for secretion is situated in the N-terminal region but it does not have the characteristics of a classical signal peptide, and it is not cleaved off during secretion (55, 56). There is no similarity between the secretion domains of the secreted Yops with respect to amino acid sequence, suggesting that the secretion signal is essentially conformational.

The machinery required for Yop secretion is encoded by at least 20 genes of the pYV plasmid, organized in three operons *virA, B* and *C* (2, 3, 8, 32, 57, 69, 95; for review see 22). Most of the

proteins of the secretion machinery seem to be situated in the inner membrane, while YscC is inserted in the outer membrane. The latter protein shares significant homology with other multimeric outer membrane proteins, including PulD required for secretion of pullulanase by *Klebsiella pneumoniae* (26) and PIV involved in the assembly of filamentous bacteriophages (11). The energy required for Yop secretion could be provided by YscN, a 48-kDa protein containing a putative ATPase domain (95). Many of the proteins involved in Yop secretion have homologous in export systems from other animal and plant pathogens like *Salmonella* (47), *Shigella* (1, 5), *Xanthomonas campestris* (31), *Pseudomonas syringae* (38) and *Pseudomonas solanacearum* (6). Related genes are also encountered in loci involved in flagellum synthesis and assembly (4, 14; for review see 90). The *Yersinia* Yop secretion pathway could thus derive from the system involved in the export of the flagellum components; however, despite the similarity between both machineries the two systems are regulated by different sigma factors (43).

The Syc chaperones. One important peculiarity of Yop secretion is that it makes use of cytoplasmic proteins called Syc for Specific yop chaperone (for review see 93). Three chaperones have been identified till now: SycE specifically assists YopE secretion, SycH serves YopH and SycD is required for secretion of both YopB and YopD (33, 66, 91, 92).

Unlike most of the cytoplasmic chaperones, the Syc proteins do not have an ATP binding domain and they are presumably devoid of ATPase activity. The Syc chaperones are weakly related in terms of amino acid sequence; nevertheless, they share some characteristics: a small size (15–20 kDa), an acidic pI, and a putative amphipathic α-helix in the C-terminal portion (for review see 93). Each Syc protein binds specifically to its target

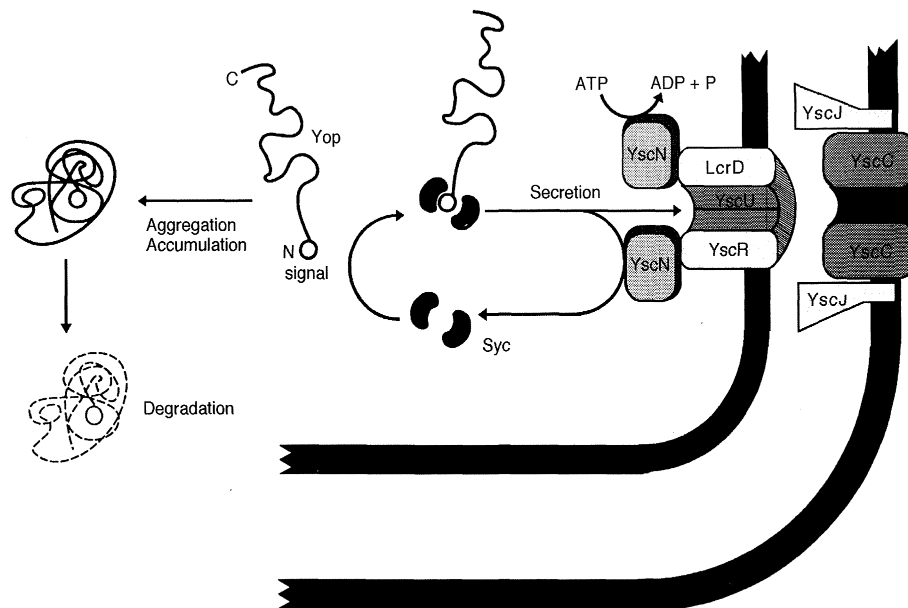

FIG. 4. Secretion of the Yops proteins. The secretion signal is amino terminal but is not cleaved during secretion. The Syc chaperones could act as pilots, antiassociation or antifolding factors. The secretion machinery is constituted by several proteins situated in the inner membrane among them LcrD, YscR and YscU. YscC probably forms a channel in the outer membrane and acts in association with the lipoprotein YscJ.

Yop but the exact role of the chaperone in the secretion process is not clear. They can act as targeting factors driving the Yop proteins to the secretion machinery, or they can prevent interaction between the Yop proteins themselves in the bacterial cytosol. Finally, they can also act as antifolding factors protecting intracellular Yops from degradation.

A new relationship between bacteria and its target cell

Histological studies of mice infected with *Yersinia* show that bacteria remain mainly extracellularly located (78). In agreement with this in vivo observation, Yersinia resist in vitro phagocytosis by polymorphonuclear leukocytes and macrophages (17, 30, 74). However at least two Yops (YopE and YopH) have intracellular targets. This observation suggests that somehow *Yersinia* are able to inject the Yops from outside into the cytosol of the target cell. This hypothesis has been confirmed by two different approches, confocal microscopy (66, 77) and the use of an enzymatic reporter gene (80, 81) (Fig. 5). Sory et al. (81) infected eukaryotic cells with recombinant *Yersinia* producing YopE and YopH fused to the active domain of the adenylate cyclase from *Bordetella pertussis*. This enzyme is dependent on calmodulin, a ubiquitous eukaryotic protein which is not synthesized by bacteria. Hence, accumulation of cAMP inside eukaryotic cell cultures infected with recombinant *Y. enterocolitica* marks the presence of the hybrid protein in the cytosol of the eukaryotic cell. This method conclusively showed that YopE and YopH are indeed translocated from extracellular bacteria across the membrane of the target cell (80, 81). This phenomenon requires an intact secretion apparatus as well as the secretion of two other Yops, YopB and YopD (77, 80) and only occurs when bacteria are firmly attached to the eukaryotic cell by Inv or YadA. The internalization of Yops into eukaryotic cells occurs in a polarized manner dependent on YopN (66,77). In a *yopN* mutant , Yops are detected mainly in the extracellular medium rather than in the cytosol of the target cell. The contact between bacteria and the cell is the signal triggering the secretion of the Yops out of bacteria and their internalization in

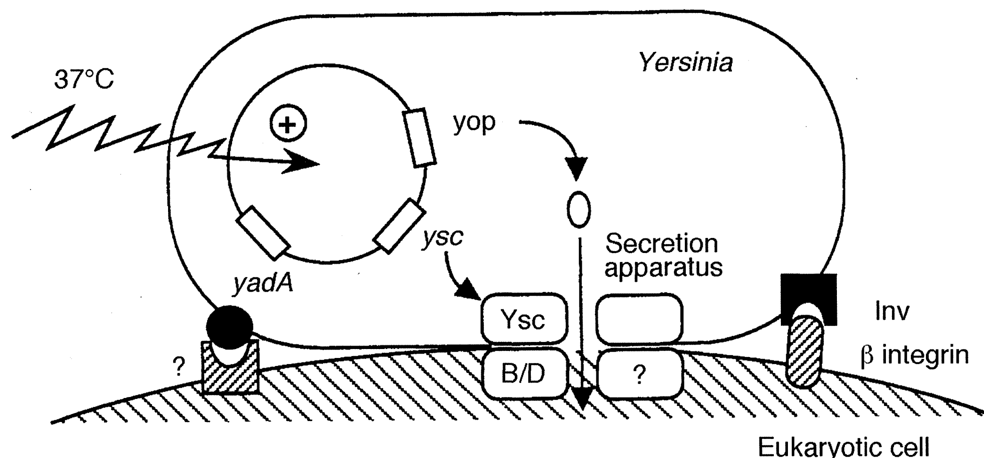

FIG. 5. Interaction between *Yersinia enterocolitica* and a eukaryotic cell. *Yersinia enterocolitica* adheres to the surface of the eukaryotic cell via YadA and Inv. Upon contact Yops are secreted and they are translocated across the membrane of the eukaryotic cell. The *ysc* genes encode the secretion apparatus. YopB and YopD are involved in translocation.

eukaryotic cells. All these observations suggest that *Yersinia* builds some kind of specific apparatus that allows the internalization of Yops inside the eukaryotic cell. This appealing hypothesis is supported by the fact that the proteins involved in Yop secretion have counterparts in the flagellum and filamentous phage assembly system.

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Cytolytic toxins from Gram-negative bacteria

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Summary

Many Gram-negative bacterial pathogens synthesize cytolytic toxins as virulence factors. Most of these toxins generate pores in eukaryotic cell membranes, but there are apparently several different mechanisms of pore formation. Cytolysins of Gram-negative bacteria are usually synthesized as precursor proteins which are converted to the active toxins by modification or proteolytic processing. Such a requirement for activation is not common for cytolysins produced by Gram-positive bacteria. The extracellular secretion of cytolytic toxins from Gram-negative bacteria depends on specific transport systems.

Key words: cytolysins, RTX-toxins, haemolysins, bacterial pathogenicity, virulence factors

Resumen

Muchas bacterias patógenas Gram negativas sintetizan toxinas citolíticas como factores de virulencia. La mayoría de estas toxinas originan poros en las membranas de las células eucarióticas, a través de varios mecanismos. Las citolisinas de las bacterias Gram negativas se sintetizan normalmente como proteínas precursoras, que posteriormente son convertidas a la forma activa de la toxina por modificación o por procesamiento proteolítico. Esta activación no es frecuente, en cambio, en las citolisinas producidas por bacterias Gram positivas. La secreción hacia el medio extracelular de toxinas citolíticas por las bacterias Gram negativas depende de sistemas específicos de transporte.

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Introduction

Cytolytic toxins are synthesized by a variety of Gram-negative bacteria associated with diseases in humans and animals. Many of these toxins have been shown to represent important virulence factors, but their exact functions in the pathogenesis of infections are poorly understood. Most known cytolysins from Gram-negative bacteria form pores in the cytoplasmic membrane of eukaryotic cells. In hypotonic media, these pores cause the osmotic lysis of the target cells. Sublytic concentrations of pore-forming cytolysins may also induce membrane-associated reactions in host cells leading to cellular disfunction. Some cytolytic toxins from Gram-negative bacteria do not form transmembrane pores, but damage target cell membranes by an enzymatic (hydrolytic) or detergent-like activity.

The most common cell used for assaying the activity of cytolysins is the red blood cell. Many cytolytic toxins, which are able to lyse erythrocytes, are therefore called "haemolysins", although they are often active against a wide range of cell types. However, a few cytolytic toxins from Gram-negative bacteria exhibit a high target cell and host species specificity.

The RTX-toxin family

The RTX (repeats in toxin)-toxins represent a family of structurally and functionally related pore-forming cytolysins which require Ca^{2+} for activity. They are widespread among Gram-negative bacterial pathogens. The most striking structural feature of these toxins is a series of glycine-rich nonapeptide repeats with the consensus sequence L-X-G-G-X-G-N/D-D-X, which is present in the C-terminal half of the toxin protein. The RTX-toxins are synthesized as inactive proteins and activated by modification via an accessory protein prior to secretion from the bacterial cell. The secretion of the RTX-toxins is accomplished by a specific, highly conserved transport mechanism involving a noncleavable, C-terminal secretion signal in the toxins. The genes required for synthesis, activation and secretion of an RTX-toxin are usually localized on the bacterial chromosome and clustered in an operon.

α-Haemolysin from *Escherichia coli*. α-Haemolysin, the most extensively studied RTX-toxin, is frequently produced by *E. coli* strains causing extraintestinal infections. The contribution of α-haemolysin to the virulence of these *E. coli* strains was established in several animal models (25, 92), but the pathophysiological function of this toxin is still unclear. In addition to its haemolytic activity, α-haemolysin displays strong cytotoxic activity against a variety of nucleated cell types including leukocytes, endothelial cells and renal tubular epithelial cells (9, 37, 79). This probably provides a mechanism for extraintestinal *E. coli* isolates to impair and counteract the host immune defence system and to cause tissue damage. Cell death caused by α-haemolysin is most likely due to the rapid and irreversible depletion of cellular ATP following formation of the transmembrane pores (9). Sublytic concentrations of α-haemolysin modulate normal functions of several types of host cells. They induce, for example, the release of inflammatory mediators such as leukotrienes, 12-hydroxyeicosatetraenoic acid, histamine and serotonin, from leukocytes and other cell types (39, 40), cause a contraction of endothelial cells (79) and stimulate the release of interleukin 1 from monocytes (9). Many of these host cell responses to sublytic doses of α-haemolysin are most likely triggered by a signal transduction cascade which is activated by the passive influx of Ca^{2+} into the target cells following transmembrane pore formation (39, 79). However, some of the cellular reactions induced by α-haemolysin, like the gen-

eration of superoxide in granulocytes, appear to precede the pore formation (10).

The genetic determinant of *E. coli* α-haemolysin is either localized on the chromosome (especially in uropathogenic isolates) or on large plasmids, and contains four structural genes arranged in an operon in the order *hlyC*, *hlyA*, *hlyB* and *hlyD* (Fig. 1a) (18, 28). Gene *hlyA* encodes the 110 kDa haemolysin protein (HlyA), which represents an inactive precursor of the mature toxin. HlyC, a cytoplasmic protein of 20 kDa, is required for the posttranslational activation of HlyA to the haemolytically active haemolysin. The proteins encoded by *hlyB* (HlyB, 80 kDa) and *hlyD* (HlyD, 55 kDa) are essential for the extracellular secretion of α-haemolysin (18, 28). The transcription of the *hlyCABD* operon starts upstream of *hlyC* and results in the generation of two mRNA species: the major transcript comprises *hlyCA* and the minor transcript *hlyCABD* (94). In addition, the transcription is positively regulated by sequences localized further upstream of the promoter region, which may act as binding sites for regulatory proteins (17, 88). Interestingly, the *hlyC*-proximal sequences of chromosomally encoded and plasmid-encoded haemolysin determinants share little sequence homology, indicating that there are differences in the regulation of these determinants.

The HlyC-mediated activation of α-haemolysin takes place in the cytoplasm of *E. coli* and is achieved by a covalent, acyl carrier protein-dependent fatty acid acylation of two internal lysine residues (Lys_{564} and Lys_{690}) of the haemolysin protein (Fig. 1b) (36, 75). HlyC possibly acts as a fatty acyl transferase in this process. The activated α-haemolysin is secreted directly across both the inner and the outer membrane of *E. coli* without accumulation in the periplasmic space. This se-

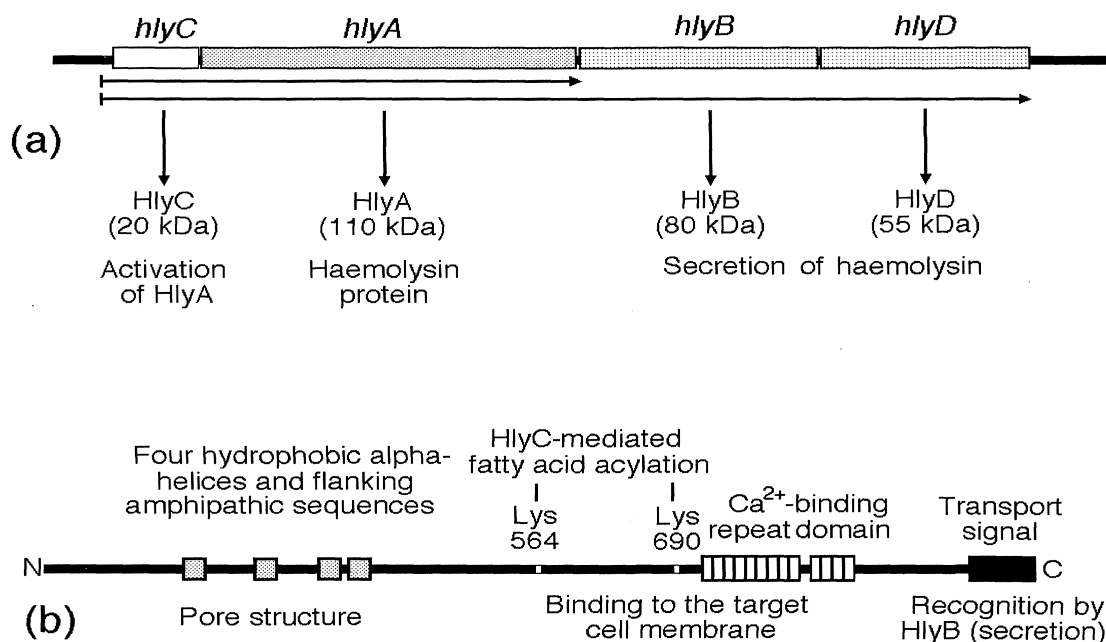

FIG. 1. (a) Structure of the α-haemolysin determinant of *Escherichia coli* and function of the *hly* gene products. The transcriptional organisation of the *hlyCABD* operon is indicated. (b) Model of HlyA from *Escherichia coli* showing the location of functional domains.

cretion is independent of the general export pathway (*sec* pathway). It is rather accomplished by a specific transport apparatus consisting of at least three proteins, HlyB, HlyD and TolC (21, 42, 90, 91). These proteins probably form a transenvelope complex which spans both membranes of *E. coli*. HlyB and HlyD are integral proteins of the cytoplasmic membrane (21, 91). TolC is a common 52 kDa outer membrane protein of *E. coli*, which is encoded by a chromosomal gene not belonging to the *hly* gene cluster (90). Interestingly, HlyB is a member of the superfamily of ATP binding cassette (ABC) transporters. It provides energy for the translocation of HlyA by binding and hydrolyzing ATP (42). The secretion signal of HlyA is localized within its C-terminal 50 to 60 amino acid residues, but the exact structural features of this signal are poorly understood (38, 102). The transport signal of HlyA is not proteolytically removed during secretion.

The binding of α-haemolysin to target cell membranes depends on the activation of HlyA by HlyC (11) and in addition on the binding of Ca^{2+} to the repeats which are clustered distal from both activation sites (Fig. 1b) (11, 48). The repeat domain of HlyA contains 13 tandemly arranged repeat units (18, 28). The transmembrane pore seems to be formed by a domain in the N-terminal half of HlyA, which contains four putative hydrophobic transmembrane α-helices flanked by amphipathic sequences (49). Studies employing planar lipid bilayer membranes demonstrated that the α-haemolysin pore is cation-specific, water-filled and unstable, i.e. it has a short lifetime of a few seconds (7). From the single-channel conductance, a lower limit of about 1 nm was estimated for the effective pore diameter (7).

Other RTX-toxins from *Enterobacteriaceae*. Recently, a novel haemolysin which is homologous, but not identical to α-haemolysin, was identified in enterohemorrhagic *E. coli* (EHEC) strains (68). The EHEC haemolysin (Ehly, Ehx) is endowed with haemolytic and leukotoxic activity, but it exhibits a higher target cell specificity than α-haemolysin (5). Haemolysins highly related to *E. coli* α-haemolysin are also produced by a number of other *Enterobacteriaceae*, including *Proteus vulgaris*, *Morganella morganii* and *Enterobacter cloacae* (41, 93).

Leukotoxin from *Pasteurella haemolytica*. *Pasteurella haemolytica* biotype A serotype 1 is the etiological agent of bovine pneumonic pasteurellosis. Among the various potential virulence factors of this pathogen is an extracellular leukotoxin belonging to the RTX-toxin family. This leukotoxin, LktA, specifically damages ruminant leukocytes (71) but exhibits only a weak haemolytic activity against ruminant erythrocytes (30). LktA appears to play a key role in the pathogenesis of *P. haemolytica* infection (58). Its presumed function is the destruction of leukocytes at the site of infection, which reduces the capacity of the host to perform an efficient immune response. In addition, destructive enzymes released from lysed leukocytes into the lung tissue may contribute to the severe necrosis observed in *P. haemolytica* infections.

The chromosomal *lktCABD* operon required for the synthesis and secretion of LktA resembles the α-haemolysin determinant of *E. coli*. The four genes *lktC*, *lktA*, *lktB* and *lktD* encode proteins of 20 kDa, 102 kDa, 80 kDa and 55 kDa, respectively, which are homologous to HlyC, HlyA, HlyB and HlyD (47, 77). The lower molecular mass of LktA as compared to that of HlyA is largely due to a shorter repeat domain in LktA, consisting of only eight repeat units (47). The expression of the *lkt* genes of *P. haemolytica* A1 seems to be similarly regulated as that of the *hly* genes of *E. coli* (30, 78). It was also shown that HlyB and HlyD can accomplish the secretion of LktA, when the leukotoxin is expressed in *E. coli* (30). Furthermore, LktA can

be activated by HlyC from *E. coli* without altering the activity or target cell specificity of LktA, although LktC cannot completely replace HlyC in the activation of HlyA (30). This suggests that the activation of HlyA and LktA by their corresponding C proteins is mechanistically similar but that there are differences in detail. Indeed, one of the two lysine residues identified in *E. coli* HlyA as the acylation sites, Lys_{690}, is not conserved in LktA (47).

RTX-toxins from *Actinobacillus* species. A variety of RTX-toxins are synthesized by species of the genus *Actinobacillus*. Among the twelve serotypes of *A. pleuropneumoniae*, three different RTX-toxins, ApxI, ApxII and ApxIII, were detected (19). These toxins are discussed in detail in another article of this issue of the journal (Tascón et al., Virulence factors of the swine pathogen *Actinobacillus pleuropneumoniae*). *A. suis*, an opportunistic pathogen which causes septicaemia in young swines, produces a haemolysin which is genetically and structurally almost identical to ApxII (13).

The 115 kDa leukotoxin from *A. actinomycetemcomitans* represents another member of the RTX-toxin family (43, 44, 74). This leukotoxin is mainly produced by *A. actinomycetemcomitans* strains causing juvenile periodontitis and other periodontal diseases, suggesting that it is a major virulence factor in the pathogenesis of these infections. The leukotoxin of *A. actinomycetemcomitans* is unique among the known RTX-toxins because it lyses specifically human and primate polymorphonuclear leukocytes and monocytes. Other human cells or leukocytes of other species are not killed by this toxin (83). It is presumed that the leukotoxin inhibits neutrophil defence mechanisms at the site of infection.

The chromosomal determinant of the leukotoxin of *A. actinomycetemcomitans* represents a typical RTX-toxin operon and contains four genes in the order *lktC*, *lktA*, *lktB* and *lktD* (also designated *ltxC, A, B, D* or *AaLtC, A, B, D*) (12, 43, 44, 74). Interestingly, the proteins encoded by these genes are more related to HlyC, HlyA, HlyB and HlyD, respectively, from *E. coli* than to LktC, LktA, LktB and LktD from *P. haemolytica*. A complete *lkt* (*ltx*) gene cluster is also present in the nonleukotoxic strains of *A. actinomycetemcomitans*. However, the leukotoxin genes are only inefficiently transcribed in these strains, due to a different promoter region upstream of *lktC* (12, 74).

In contrast to the other known RTX-toxins, the leukotoxin of *A. actinomycetemcomitans* is not secreted extracellularly but remains associated with the bacterial cell surface (44, 83). This is apparently caused by ionically binding of the leukotoxin to DNA localized on the cell surface of *A. actinomycetemcomitans* (53).

Adenylate cyclase toxin from *Bordetella pertussis*. *Bordetella pertussis*, the causative agent of whooping cough, produces several virulence factors that are involved in the pathogenesis of the disease. Among these virulence factors is an RTX-toxin known as adenylate cyclase toxin (AC toxin, CyaA). This toxin differs from the other members of the RTX family in that it is a bifunctional toxin endowed with calmodulin-dependent adenylate cyclase activity and haemolytic activity. CyaA is synthesized as a large protein of 1706 amino acid residues with an apparent molecular mass of 200–220 kDa, which is secreted without proteolytic processing into the medium (6, 23, 29, 64). The adenylate cyclase activity and the haemolytic activity of CyaA are structurally and functionally independent of each other. Particularly, the N-terminal ~400 amino acids of CyaA constitute the catalytic domain, whereas the remaining C-terminal ~1300 residues represent the haemolytic domain (6, 66). Both domains are essential for the virulence of *B. pertussis* (24). The haemolytic domain of CyaA shares 25% homology with *E. coli*

α-haemolysin, but in contrast to HlyA it contains almost 40 repeat units (23).

AC toxin binds to human leukocytes and other cell types, followed by the penetration of the N-terminal catalytic domain through the target cell membrane. Within the cell, the adenylate cyclase domain is activated by calmodulin and apparently split off from the C-terminal domain. Finally, the 45 kDa adenylate cyclase fragment catalyzes the uncontrolled formation of supraphysiological levels of cAMP from endogenous ATP. This is toxic and causes the debilitation of the bactericidal functions of immune effector cells (16, 29, 65). The binding of AC toxin to the target cell membrane and the translocation of its catalytic moiety depend on the haemolytic domain (6). In addition, the penetration of the catalytic domain into the cell depends on conformational changes in the AC toxin molecule, which are induced by mM concentrations of extracellular Ca^{2+} (29, 65). The small transmembrane pore formed by the haemolysin portion of CyaA is most likely not directly involved in the translocation of the AC domain through the target cell membrane (8).

The AC toxin determinant consists of five closely linked chromosomal genes arranged in the order *cyaC*, *cyaA*, *cyaB*, *cyaD* and *cyaE* (4, 23). *CyaA*, the structural gene encoding the toxin protein, and the three genes downstream of *cyaA* form an operon which is transcribed from a strong promoter localized in the intergenic *cyaC-cyaA* region (45). The transcription from this promoter is indirectly activated in response to environmental stimuli by the BvgA/BvgS two component system of *B. pertussis* (45). Most transcripts terminate 3' to the *cyaA* structural gene, although there is also a low level of readthrough resulting in full-length *cyaABDE* transcripts. A second, weaker promoter localized upstream from *cyaB* is not dependent on BvgA/BvgS and allows a low level of constitutive expression of *cyaB*, *D* and *E* (45). CyaB, CyaD and CyaE are necessary for the extracellular secretion of CyaA. CyaB and CyaD share striking homology with HlyB and HlyD from *E. coli* (23). CyaE probably has a function analogous to that of TolC in the secretion of *E. coli* α-haemolysin, although there is only very little similarity between these two proteins (23, 90). In contrast to the known B and D proteins involved in RTX-toxin secretion, TolC and CyaE are synthesized with cleavable N-terminal signal sequences. The gene *cyaC* is homologous to *hlyC* of *E. coli* and required for the posttranslational activation of CyaA. However, *cyaC* is oriented oppositely from *cyaA* and transcribed from its own promoter (4). Nonactivated AC toxin possesses adenylate cyclase activity but it is devoid of invasive and haemolytic activities (4, 64). Interestingly, CyaA is activated in *B. pertussis* by fatty acylation (palmitoylation) of only a single lysine residue, Lys_{983}, which corresponds to Lys_{690} of HlyA from *E. coli* (26).

Evolutionary aspects of RTX-toxins. It is obvious that the RTX-toxin determinants found in Gram-negative bacteria have a common origin. The dissemination of these determinants was probably caused by horizontal gene transfer between different species, possibly via plasmids and insertion sequence (IS)-mediated transfer mechanisms. The observation that the α-haemolysin determinants of various *E. coli* strains are localized on plasmids and that these plasmid-encoded determinants are often flanked by IS-like elements is consistent with this supposition (101).

Several Gram-negative bacteria produce extracellular proteins which are secreted by transport systems homologous to those of the RTX-toxins, but do not act as pore-forming toxins. These proteins include various proteases from *Erwinia chrysanthemi*, *Serratia marcescens* and *Pseudomonas aeruginosa*, the nodulation protein NodO from *Rhizobium leguminosarum* and iron-regulated proteins from *Neisseria meningitidis*

(22, 82). All these proteins are only partially related to the RTX-toxins, but they contain a C-terminal secretion signal and a tandem array of nonapeptide repeats homologous to those of the repeat toxins. It is tempting to speculate that the RTX-toxins and these more distantly related proteins arose from a single ancient protein which developed differently to acquire haemolytic, leukotoxic or proteolytic activity. In the case of the adenylate cyclase toxin of *B. pertussis*, an adenylate cyclase gene, possibly of eukaryotic origin, most likely fused to the original toxin gene.

Related haemolysins from *Serratia marcescens* and *Proteus* species

Serratia marcescens is an opportunistic pathogen which may cause various extraintestinal infections in humans. Almost all strains of *S. marcescens* produce a haemolysin/cytolysin which contributes to the virulence of this species. This haemolysin is an extracellularly secreted, pore-forming protein not related to the RTX-toxins. Its activity is independent of Ca^{2+} and other divalent cations (60, 67, 69).

Synthesis and secretion of the *S. marcescens* haemolysin is determined by the chromosomally encoded *shlBA* operon (60). The transcription of this operon from a promoter localized upstream of *shlB* is negatively regulated by iron, probably via a protein that is functionally analogous to the *E. coli* Fur repressor (59). The genes *shlA* and *shlB* encode proteins of 165 kDa and 62 kDa, respectively. These proteins represent precursors containing N-terminal signal peptides which are removed during transport across the cytoplasmic membrane. ShlA (162 kDa in processed form) is the haemolysin protein itself (60). The outer membrane protein ShlB (60 kDa) is required for the activation of ShlA and for its translocation from the periplasm into the culture supernatant (67). Both processes seem to be tightly coupled.

The mechanism of the activation of ShlA is not known upto now, but it is presumed to involve a modification of the protein (54). This activation seems to induce a conformational change in ShlA that is required for binding to the erythrocyte membrane. Functional analyses of ShlA demonstrated that both the activation site and the signal allowing the ShlB-dependent secretion across the outer membrane are localized in the N-terminal region of the haemolysin protein. The C-terminal half of ShlA, on the other hand, is involved in membrane insertion and formation of the transmembrane pore (54, 60, 67). In planar lipid bilayer membranes, ShlA generates water-filled pores which vary in size with inner diameters between 1 and 3 nm (69). The small pores seem to be formed by ShlA monomers, but the larger pores are probably generated by oligomers which most likely form within the membrane by association of variable numbers of ShlA molecules (69).

A cytolysin highly related to the haemolysin of *S. marcescens* is produced by almost all strains of *Proteus mirabilis* and *Proteus vulgaris* (80, 93). These bacteria frequently cause urinary tract infections in humans. The *hpmBA* operon required for the synthesis of this cytolysin shares striking similarity with the *shlBA* operon of *S. marcescens* and encodes two proteins, HpmA and HpmB, which are structurally and functionally homologous to ShlA and ShlB (85). Like ShlA, HpmA is endowed with Ca^{2+}-independent haemolytic activity (85). It was also shown that HpmA is cytotoxic for a variety of nucleated cells (80).

El Tor haemolysin from *Vibrio cholerae*

Vibrio cholerae serotype O1, the etiological agent of cholera in humans, is subdivided into the

classical biotype and the El Tor biotype. Strains of the El Tor biotype are generally capable of synthesizing an extracellular haemolysin (cytolysin), whereas isolates of the classical biotype are usually considered to be nonhaemolytic. The gene *hlyA* required for the production of El Tor haemolysin encodes a protein of 82 kDa which represents an inactive prepro form of the mature toxin (1, 62, 98). During secretion across the cytoplasmic membrane, the preprotoxin is converted to the 79 kDa protoxin by removal of an N-terminal signal peptide. The protoxin is then further translocated through the outer membrane into the medium and processed into the 65 kDa active haemolysin by release of the N-terminal 15 kDa fragment (1, 98). This proteolytic activation is probably accomplished by a secreted protease of *V. cholerae* (98). The translocation of the protoxin from the periplasm into the culture medium seems to require a 60 kDa protein which is putatively localized in the outer membrane. The gene *hlyB* encoding this protein was identified downstream of *hlyA* in the chromosome of a *V. cholerae* El Tor strain (2). Both genes, *hlyA* and *hlyB*, appear to be separate transcriptional units (1, 2, 62, 98).

The regulation of the expression of El Tor haemolysin seems to be complex. It was shown that the transcription of *hlyA* is activated by a small regulatory protein of 12 kDa which is encoded by the *hlyU* gene of *V. cholerae* (95). In addition, the production of El Tor haemolysin is induced under conditions of iron limitation (76). It was further reported that the expression of *hlyA* is controlled by a locus designated *hlyR*, which maps distant from *hlyA* on the chromosome of *V. cholerae* (89). *V. cholerae* El Tor strains which produce little or no haemolysin were repeatedly isolated. In addition, a haemolytic phenotype variation, i.e. the conversion from a nonhaemolytic to a haemolytic phenotype and vice versa, was observed in individual El Tor strains (62). This variability of the haemolytic phenotype seems to be caused by sequences that are located outside of the *hlyA* gene and its promoter region (62).

Interestingly, the *hlyA* gene is not only present in the chromosome of El Tor strains. DNA sequences homologous to *hlyA* were also detected in all tested classical strains of *V. cholerae* serotype O1. However, the *hlyA* genes of the classical strains analysed so far contain a common internal deletion of 11 bp which causes a frame shift resulting in the expression of a C-terminally truncated HlyA polypeptide of 27 kDa (1, 62). This truncated HlyA protein retains only a weak activity on chicken and rabbit erythrocytes (63).

Non-O1 *V. cholerae* strains, which usually cause gastroenteritis, are also often haemolytic and secrete a haemolysin that is structurally and immunologically indistinguishable from El Tor haemolysin (98). A cytolysin partially related to El Tor haemolysin is produced by *Vibrio vulnificus* (99).

Thermostable direct haemolysin (TDH) from *Vibrio parahaemolyticus* and related cytolysins

Vibrio parahaemolyticus is a marine bacterium that has been recognized as an important agent of seafood-borne gastroenteritis. Most clinical isolates of this pathogen, but only 1–2% of the environmental strains, exhibit a defined characteristic called Kanagawa phenomenon, which is manifested by the formation of clear haemolytic zones around the colonies growing on a special blood agar (Wagatsuma agar). This haemolytic activity is due to the "thermostable direct haemolysin" (TDH), also known as Kanagawa phenomenon (KP)-associated haemolysin, which seems to be a major virulence factor of *V. parahaemolyticus* (32, 50). In addition to the haemolytic activity,

TDH possesses several other biological activities including cytotoxicity, cardiotoxicity, lethal toxicity for small experimental animals and enterotoxigenicity (32). TDH is an extracellular, heat-stable, pore-forming toxin. It is composed of two identical or very similar, noncovalently associated protein subunits of about 21 kDa (165 amino acid residues) (32, 50, 84).

KP-positive strains of *V. parahaemolyticus* contain two nonidentical, but very similar chromosomal copies of the TDH structural gene, designated *tdh1* (or *tdhS*) and *tdh2* (or *tdhA*). The homology between these gene copies is 97% (35, 52). Both genes encode highly related proteins of 189 amino acid residues which represent precursors of the mature TDH protein subunits containing N-terminal signal peptides of 24 amino acids (35, 52). Interestingly, *tdh2* contributes to the production of more than 90% and *tdh1* to less than 10% of the total extracellular TDH of *V. parahaemolyticus* (52). This seems to be caused by differences in the transcriptional control of these two genes. Indeed, the DNA sequences of the promoter regions of *tdh1* and *tdh2* differ significantly (52). Recently, it was shown that the transcription of *tdh2* is directly activated in response to environmental signals by the Vp-ToxR/ToxS system which is highly homologous to the ToxR/ToxS system of *V. cholerae*. In contrast, *tdh1* is not significantly affected by Vp-ToxR/ToxS (46).

Most of the KP-negative *V. parahaemolyticus* strains apparently do not possess *tdh* genes. However, in some of these strains a single chromosomal *tdh* gene copy was detected. One of the rare clinical KP-negative isolates even contained a plasmid-encoded *tdh* gene (designated *tdh4*) in addition to a single chromosomal *tdh* gene (*tdh3*) (52). Interestingly, these *tdh* genes of KP-negative strains share high sequence homology with *tdh1* and *tdh2* of KP-positive strains and encode haemolytically active TDH in *E. coli* when fused to exogenous promoters. However, the *V. parahaemolyticus* strains carrying these genes do not efficiently synthesize the corresponding TDH proteins. This seems to be due to an inefficient transcription of the *tdh* genes in these strains (52).

Almost half of the clinically isolated KP-negative strains of *V. parahaemolyticus* produce a TDH-related haemolysin, designated TRH (31, 72). TRH exhibits similar biological activities as TDH, but the spectrum of haemolytic activity against various erythrocytes is different for the two toxins. In addition, TRH is heat-labile (31). Like TDH, TRH has a dimeric structure. The two identical protein subunits of TRH consist of 165 amino acid residues, and share striking homology with the subunits of TDH (31, 51). The *trh* gene is strongly associated with clinical isolates of *V. parahaemolyticus,* and shows significant sequence variations in these strains (72). Some clinical isolates of *V. parahaemolyticus* carry a *tdh* (or *tdh*-like) gene and in addition a *trh* gene and may produce TDH and TRH simultaneously (72, 97).

Among other *Vibrio* species causing gastroenteritis, a few strains of *V. cholerae* non-O1, *V. mimicus* and *V. hollisae* produce haemolysins related to TDH of *V. parahaemolyticus*. Two of these haemolysins, NAG-rTDH from *V. cholerae* non-O1 and Vm-rTDH from *V. mimicus*, are thermostable, but Vh-rTDH from *V. hollisae* is thermolabile (3, 100). The gene NAG-*tdh* encoding NAG-rTDH was cloned from a plasmid of a *V. cholerae* non-O1 strain, while the genes Vm-*tdh* and Vh-*tdh* were cloned from the chromosomes of *V. mimicus* and *V. hollisae*, respectively. All three genes are highly homologous to the *tdh* genes of *V. parahaemolyticus* (3, 100). The sequence of NAG-*tdh* is even identical to that of the plasmid-encoded *tdh4* gene of *V. parahaemolyticus* (3). In addition, the plasmids carrying *tdh4* and NAG-*tdh* are very similar, suggesting that the *tdh* gene was disseminated between *Vibrio* species by

a plasmid-mediated transfer (3). The presence of IS-like elements in the DNA sequences flanking the *tdh* genes of *Vibrio* species further indicates that the duplication of the *tdh* gene and the transfer of this gene between chromosomes and plasmids was accomplished by an IS-mediated mechanism (81).

Aerolysin from *Aeromonas hydrophila* and *Aeromonas sobria*

Aeromonas hydrophila and *A. sobria* have received increasing attention because of their frequent association with human diseases including diarrhea, wound infections, septicaemia and meningitis. Both species secrete a cytotoxic, pore-forming haemolysin called aerolysin (33, 34), which appears to be largely responsible for the virulence of these bacteria (14).

The chromosomal gene *aerA* required for the synthesis of aerolysin is highly homologous in *A. hydrophila* and *A. sobria* and encodes a protein of 54 kDa (33, 34, 86). This protein represents an inactive precursor of aerolysin designated preproaerolysin, which is proteolytically processed in two steps to yield the active toxin. First, the N-terminal signal peptide is removed cotranslationally during transport of the preprotoxin across the inner bacterial membrane, yielding proaerolysin (52 kDa). In the periplasm, the proaerolysin molecules fold and form dimers which are then further translocated into the culture supernatant (27). This transport across the outer membrane requires several additional gene products. The proaerolysin dimers, still haemolytically inactive, bind with high affinity to the transmembrane protein glycophorin on the surface of mammalian erythrocytes (57, 87). Proaerolysin is activated by proteolytic nicking at a position approximately 45 amino acids from the C-terminus, yielding the mature aerolysin protein with a molecular mass of 47.5 kDa (86). This removal of the C-terminal fragment, which is probably carried out by host proteases, induces the formation of very stable aerolysin oligomers on the target cell surface (87). The oligomerization of aerolysin is apparently accompanied by the exposure of hydrophobic patches (86) which may provide a driving force for the penetration of the oligomer into the target membrane, resulting in pore formation and osmotic cell lysis. Thus, the oligomerization of the aerolysin molecules is essential for pore formation and it seems to precede membrane insertion (87).

A low-resolution model of the aerolysin channel was obtained by image analysis from electron micrographs of two-dimensional crystals of aerolysin in lipid membranes. This model suggests that an oligomer consisting of seven aerolysin molecules encloses a central pore with an inner diameter of 1.7 nm (96). Structural analyses of proaerolysin employing X-ray crystallography further indicated that the domain of the mature aerolysin protein, which inserts into the target membrane, consists almost entirely of β-strands (57). The pore-forming structure of the aerolysin oligomer most likely adopts a barrel topology similar to that found in outer membrane porins of Gram-negative bacteria (57). The uniform, stable transmembrane pores generated by aerolysin in planar lipid bilayer membranes are indeed reminiscent of porin pores (15).

Haemolysins from *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an important opportunistic pathogen that has been recognized as a common cause of nosocomial infections. Among the numerous extracellular products of *P. aeruginosa* are two haemolysins, i.e. a heat-stable and a heat-labile haemolysin (20, 61). Interestingly, both haemolysins do not belong to the group of pore-

forming toxins. The heat-stable haemolysin consists of a mixture of two haemolytically active acidic glycolipids with the chemical structure rhamnose-β-hydroxydecanoic acid-β-hydroxydecanoic acid and rhamnose-rhamnose-β-hydroxydecanoic acid-β-hydroxydecanoic acid, respectively (20). The haemolytic activity of these rhamnolipids is due to the dimer of β-hydroxydecanoic acid, which seems to disrupt the erythrocyte membrane by a detergent-like mechanism (20). The heat-labile haemolysin of *P. aeruginosa* is a haemolytically active phospholipase C (PLC-H), which lyses human and sheep erythrocytes. PLC-H (78 kDa) catalyses the hydrolysis of phosphatidylcholine and other phospholipids containing quaternary ammonium groups, which are found primarily in eukaryotic cell membranes (61).

The production of the heat-stable haemolysin and the expression of PLC-H is induced in *P. aeruginosa* growing in phosphate (P_i)-deficient media and repressed in P_i-sufficient media (61, 73). The same type of regulation is also involved in the expression of several other proteins of *P. aeruginosa*, including an extracellular alkaline phosphatase and an extracellular nonhaemolytic phospholipase C (PLC-N), which is 40% identical to PLC-H (56, 73). It has been suggested that these P_i-regulated substances function cooperatively in liberating inorganic phosphate from phospholipids of the host (73). Such a P_i-scavenging system may be critical to the pathogenesis of *P. aeruginosa* infections. Indeed, PLC-H significantly contributes to the virulence of *P. aeruginosa* in experimental animal models, but the precise role of this protein in the pathogenesis remains to be determined (55).

The haemolytic phospholipase C of *P. aeruginosa* is encoded by the chromosomal *plcSR* operon (61, 70). The transcription of this operon is activated under conditions of phosphate limitation by a *P. aeruginosa* homologue of the *E. coli* PhoB protein (61, 73). The first gene of the *plcSR* operon, *plcS*, encodes a 82.6 kDa precursor protein of PLC-H containing a putative N-terminal signal peptide of 38 amino acids (61). This signal sequence is most likely removed upon translocation of the protein through the cytoplasmic membrane. Downstream of *plcS* are two in-phase overlapping genes, *plcR1* and *plcR2*, which encode proteins of 23 kDa and 17 kDa, respectively (70). The gene product of *plcR1* contains a potential N-terminal signal sequence but PlcR2 lacks a transport signal. The function of PlcR1 and PlcR2 is not clear, but preliminary studies suggested that these proteins may be involved in the regulation or activation of PLC-H (55, 56, 70).

Concluding remarks

It is striking that the production of cytolysins is not obvious in facultatively intracellular (invasive) Gram-negative pathogens like *Yersinia*, *Salmonella* or *Shigella*, when these bacteria are grown under in vitro cultivation conditions. However, this does not rule out the existence of cytolysin-encoding genes in these organisms. Indeed, the intracellular lifecycle of invasive bacteria involves the lysis of membranes, for example when the bacteria evade the phagosome or when they leave the host cell. It is tempting to speculate that these steps may be performed by cytolytic proteins. The expression of the genes encoding such hypothetical cytolysins is possibly only induced under specific intracellular conditions.

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Genetic tools in pathogenic nocardioform actinomycetes

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Summary

Nocardioform actinomycetes are Gram-positive bacteria with high G+C content. Several species of *Mycobacterium*, *Corynebacterium*, *Nocardia* and *Rhodococcus* are important human or animal pathogens. Transposon mutagenesis and homologous recombination are powerful genetic tools used for the identification of pathogenicity determinants. Transposable elements with potential application to mutagenize pathogenic nocardioform actinomycetes are described. Homologous recombination experiments which have been recently achieved in mycobacteria and related actinomycetes are commented on.

Key words: pathogenic nocardioforms, actynomycetes, transposon mutagenesis, homologous recombination, phospholipase D

Resumen

Los actinomicetes nocardioformes son bacterias Gram positivas con un alto contenido en G+C. Varias especies de los géneros *Mycobacterium*, *Corynebacterium*, *Nocardia* y *Rhodococcus* son importantes patógenos humanos o animales. La mutagénesis por transposón y la recombinación homóloga son dos herramientas genéticas poderosas que posibilitan la identificación de los determinantes de patogenicidad. Se describen brevemente varios elementos transponibles que se han utilizado o podrían utilizarse para mutagenizar especies patógenas de actinomicetes nocardioformes. Se comentan experimentos de recombinación homóloga realizados recientemente en micobacterias y actinomicetos relacionados.

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Introduction

Gram-positive bacteria can be divided into two large groups on the basis of their DNA composition. The Gram-positive bacteria whose G+C content is lower than 50% are part of the *Bacillus-Clostridium-Streptococcus* group. The group called actinomycetes includes Gram-positive bacteria with a high G+C content (over 55%). As many as nine suprageneric groups of actinomycetes have been defined according to their morphology, chemical properties and 16S rRNA sequence: Actinobacteria, Actinoplanetes, Maduromycetes, Micropolysporas, Multilocular sporangia, Nocardioforms, Nocardioides, Streptomyces and Thermomonosporas (11). The nocardioform actinomycetes group includes five genera: *Caseobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia* and *Rhodococcus* (19). In this group, there are several important animal and human pathogens, most of them opportunist (Table 1).

The reappearance worldwide of tuberculosis, which was once thought to be eradicated, has intensified the research efforts on the genetics of mycobacteria (3). At the same time, the improvement in methods of microbiological diagnosis has increasingly permitted the identification of "coryneform" microorganisms as agents responsible for infections, whereas previously they had been mostly regarded as contaminating agents in laboratory samples. The development of genetics tools in these microorganisms opens up the possibility of identifying pathogenicity determinants, of improving diagnostic methods, and of developing vaccines against these microorganisms. The aim of this article is to review some of the systems that have been developed in the last few years for the genetic analysis of nocardioform bacteria.

Transposon mutagenesis

Transposable elements are mobile DNA segments. They can be inserted at a new site within the genome of the organism of which they form part in a process that is independent of the homologous recombination system of this organism. Transposition is a different process from site-specific recombination because insertion of a transposable element into a new genomic site does not require homology between the ends of the element and the site of insertion. Almost all bacteria contain transposable elements in their genome. Transposable elements are useful tools for identifying bacterial genes involved in virulence. Since insertion of a mobile element inactivates the targeted gene, transposable elements can be used advantageously to create isogenic mutants defective in virulence-associated phenotypes. If the virulence gene is part of an operon, insertion produces a polar effect. Several virulence operons have been mapped by means of transposon mutagenesis. Many transposable elements contain genetic markers which facilitate the cloning of the genes into which they are inserted. They can also be used to introduce foreign genes into the bacterial genome. An illustrative example of the use of a transposable element to identify a pathogenicity gene is the isolation of non-hemolytic mutants in *Listeria monocytogenes* by inserting Tn*1545* (9). These mutants are nonvirulent since they cannot escape from the phagolysosome to the cytoplasm. For a detailed description of the use of transposable elements as genetic tools, the reader is referred to articles by Berg et al. (2) and Kleckner et al. (18).

While highly efficient transposon mutagenesis systems have been developed in many Gram-negative and in low G+C content Gram-positive pathogenic bacteria, these systems are not yet available for high G+C content Gram-positive

TABLE 1. Some significant pathogenic nocardioforms in human and veterinary medicine

Pathogen	Host	Disease
Corynebacterium diphteriae	human	diphteria
Corynebacterium pseudotuberculosis	sheep, goat	caseous limphadenitis
Mycobacterium tuberculosis	human	tuberculosis
Mycobacterium avium–intracellulare complex	birds, human	cavitary pulmonary disease
Mycobacterium bovis	cattle	tuberculosis
Mycobacterium leprae	human	leprosy
Nocardia asteroides	human	pulmonary nocardiosis
Rhodococcus equi	horse, human	purulent cavitary pneumonia

bacteria. For this reason, many laboratories have screened the genome of these bacteria in a search for transposable elements functional in nocardioform and related organisms.

In most cases, transposition occurs at very low frequency so that traps must be set to capture the transposable elements. These traps consist of marker genes introduced into the host bacterium and whose disruption produces an easily detectable phenotypic change.

Examples of these traps are *lac*Z (5), *sacB* (28) and an apramycin resistance gene under control of *c*I857 phage λ promoter (27).

For a transposable element to be used to identify pathogenicity genes, it must meet the following criteria: (i) active transposition in the bacterium which is the target of mutagenesis, (ii) high frequency of insertion, (iii) random distribution of insertions throughout the genome, and (iv) the transposable element is not present in the genome of the bacterium which is the target of mutagenesis.

Moreover, an efficient system of DNA introduction must be available in the pathogenic species. Transposable elements can be cloned into a suicide vector, usually a plasmid which is unable to replicate in the pathogen. This plasmid is used to transform the pathogen, and selection is made for a transposon marker (usually an antibiotic resistance gene). In this type of experiment, two factors limit the number of mutants obtained: transformation efficiency and transposition frequency of the element. If transformation efficiency is low, a conditionally replicative plasmid (e.g., a thermosensitive plasmid) can be used instead as a vehicle. The plasmid carrying the transposon is propagated in the pathogen under permissive conditions. Then, the plasmid is eliminated by shifting the culture to nonpermissive conditions, under which persistance of the transposon selection marker indicates insertion into the host genome. This strategy is independent of transformation efficiency, and the only limiting factor is transposition frequency.

The ultimate aim is to obtain a collection of insertion mutants that will allow the identification of genes involved in virulence. The frequency of auxotrophs is a good indicator of the quality of the collection obtained; an optimum proportion of auxotrophs is considered to range between 0.2 and 0.5%. Table 2 lists some transposable elements (insertion sequences or transposons constructed from them) which have been used or could be used to mutagenize pathogenic nocardioforms. We shall now comment on some of these systems.

IS*1096*. This insertion sequence was identified when it was inserted into the *lac* Z gene, which had been previously introduced as a marker into the chromosome of *Mycobacterium smegmatis* (5). Its

TABLE 2. Transposable elements with potential use for mutagenesis of pathogenic nocardioforms

Transposable element	Host	Application	Reference
IS*31831*, Tn*31831*	*C. glutamicum*	*B. flavum*	28
IS*1096*, Tns*5366-8*	*M. smegmatis*	*M. bovis* BCG	5, 22
Tn*610*, Tn*611*	*M. fortuitum*	*M. smegmatis*	21
IS*1110*	*M. avium*	unknown	15
IS*6120*	*M. smegmatis*	unknown	12
IS*1137*	*M. smegmatis, M. chitae*	unknown	10
IS*204*	*N. asteroides*	unknown	30
IS*1166*	*Rhodococcus* spp.	unknown	8

length is 2,260 bp, it is randomly inserted into the *lac* Z gene, and its transposition frequency is of the order of 10^{-4}/cell. IS*1096* was found in the genome of *Mycobacterium smegmatis* but is also functional in *Mycobacterium bovis* BCG. By introducing a kanamycin-resistant gene into regions of IS*1096* not required for transposition, artificial transposons have been constructed and used to obtain a library of mutants in *M. bovis* BCG (22). A suicide vector containing one of these transposons (Tn*1096)* was introduced by electroporation into *M. bovis* BCG. Because of the low transformation efficiency by electroporation in *M. bovis* BCG, the frequency of mutants was very low (colonies were obtained in only 5 of the 20 experiments performed). If a conditionally replicative plasmid is used instead of the suicide vector, the number of mutants might be larger. Thermosensitive plasmids have been constructed and assayed in *M. smegmatis* (13), and have been used to obtain mutants by transposition of Tn*611* (14). Tests are currently being carried out with these thermosensitive plasmids in *M. bovis* BCG and *M. tuberculosis*.

IS*1110*. When the presence of plasmids was being studied in strains of *M. avium* isolated from patients with AIDS, an increase in size of one of the plasmids present in the LR541 strain was observed. This phenomenon was the result of the insertion of a hitherto undescribed 1,457-bp transposable element designated IS*1110* (15). This insertion sequence was not detected by using a system of transposon capture, but by examining random colonies, which indicated its high transposition frequency. This property and the fact that the sequence only forms part of the genome of *M. avium* and possibly *M. chitae* make it an outstanding candidate for both the construction of artificial transposons and their use in the mutagenesis of pathogenic mycobacteria and other nocardiaceae.

IS*6120*. It was identified in *M. smegmatis* by its ability to transpose into different sites in the λ phage repressor gene *c*I857, which controls the expression of an apramycin resistance gene (12). IS*6120* is present in *M. smegmatis* genome but was not found in other species, including *M. tuberculosis* and *M. bovis*. Therefore, IS*6120*-derived elements with selective markers could be used for transposon mutagenesis.

IS*1137*. This insertion sequence was found during a search for the *M. smegmatis* superoxide-dismutase gene (10). Since IS*1137* is not present either in *M. tuberculosis* or in *M. bovis* BCG, it is also a good candidate for transposon mutagenesis in nocardioform bacteria.

IS*204*. During the characterization of *Nocardia asteroides* transformants with the plasmid pCY104, it was observed that the plasmids isolated from some colonies spontaneously became larger, and this occurred at a high frequency (10^{-1}–10^{-2}). The

increase in size was due to the insertion of a transposable element which was called IS*204* (30). IS*204* contains an 1134 bp open reading frame, which encodes a putative transposase similar to that found in IS*1096*. Its high transposition frequency makes it a good candidate for mutagenesis in nocardioform as well.

IS*1166*. Two insertion sequences were detected by sequencing the region contiguous to a cluster of three sulfur oxidation genes that reside on a plasmid in *Rhodococcus* sp. strain IGTS8 (8). One of these sequences, denominated IS*1166*, is closely related to IS*6120* from *M. smegmatis*. IS*1166* was present in four copies in the strain IGTS8. Two mutants of this strain were isolated in which an additional copy of IS*1166* was present, suggesting that transposition of at least one copy of this element had occurred. IS*1166* could be a good candidate for transposon mutagenesis of pathogenic species of *Rhodococcus*, since this element does not naturally occur in *R. equi*.

Homologous recombination

Homologous genetic recombination involves the exchange of homologous regions of DNA between two DNA molecules. The RecA protein is essential for homologous recombination. In nature, recombination is a source of genetic variation. In the laboratory, homologous recombination is a powerful technique to investigate gene function.

Homologous recombination can be directly used to mutagenize a gene. A portion of the gene is introduced into a suitable vector and pathogenic bacteria are subsequently transformed with the plasmid obtained in that way. A crossing-over at a single point generates a knock-out mutant of the gene in which the complete plasmid is integrated into the chromosome by means of homologous recombination.

A gene which is thought to play a major role in virulence is mutated, either by transposon mutagenesis or by other procedures (disruption with an antibiotic resistance cassette, deletion or site-specific mutagenesis if the gene is well characterized). Once mutation has been achieved and the inactivation of the gene has been demonstrated, the wild-type gene is replaced by the mutant gene in the pathogenic bacterium. The mutant obtained in this way is called an isogenic mutant. The isogenic mutant is then subjected to virulence tests in order to determine the role the gene plays.

The mutant gene is introduced into the pathogenic bacterium by means of a suitable vector. Because of the existing homology between the sequences of the wild-type and mutant alleles, recombination will take place between the vector and the chromosome of the pathogenic bacterium. Most of the recombinant events will occur by a crossing-over at a single point. This results in the integration of the vector into the chromosome, and the bacterium will have a copy of each allele. Subsequent recombination between the two alleles can occur producing either a wild-type or a mutant bacterium. However, in a few cases, the crossing-over will occur at two points, with the wild-type and the mutant alleles interchanging between the vector and the chromosome. The vectors for these allele replacement experiments carry a marker whose absence can readily be detected. Moreover, if mutation produces an easily identifiable phenotype, the mutant bacterium can be selected by analyzing a not excessively large number of clones (a good ratio is 1/1000). The only limiting factor in this type of experiment is the length of the homologous sequences required for the crossing-over to occur. RecA-mediated homologous recombination is most efficient at regions where DNA is single stranded (29). So, linearization of the vector with an enzyme which generate single and/or double-stranded breaks can favor recombination between chromosome and vector.

Allelic exchange by homologous recombina-

tion has been achieved in *M. smegmatis* (16). The exchanged gene was *pyr F*, encoding for orotidine monophosphate decarboxylase, which provides a nutritional marker that allows both positive and negative genetic selections (4). The *pyrF* gene was disrupted by a DNA cassette containing the *aph* gene from the transposon Tn*903* and this construct was incorporated into a vector capable of replication in *Escherichia coli* but not in *M. smegmatis*. The vector was integrated into the *M. smegmatis* genome at the *pyrF* locus by homologous recombination.

Homologous recombination is less efficient in slow-growing pathogenic mycobacteria. *M. leprae* and members of the *M. tuberculosis* complex code for an 85 kDa RecA precursor protein which is converted by protein splicing into the mature RecA protein (40 kDa) (6, 7). The uncommon structure of these *recA* genes has been invoked as the cause of poorly efficient recombination in slow-growing mycobacteria. When an allelic exchange standard experiment has been attempted, in most cases illegitimate recombination events between the vector and chromosome have been detected (17). This high level of non-homologous events may mask homologous recombination. The fact that efficient homologous recombination has been achieved in *M. intracellulare* (20), a slow-growing mycobacteria which has no intein in its RecA protein (7), supports these arguments.

Both homologous recombination and gene replacement have been recently demonstrated in *M. bovis* BCG by using replicating vectors (25). Intramolecular homologous recombination was tested in *M. bovis* BCG by using a shuttle plasmid, pUS950. This plasmid contains two overlapping fragments of the Tn*903* kanamycin phosphotransferase gene (*aph*), separated by a functional hygromycin resistance gene. This vector allows for positive selection of homologous recombination between the two *aph* gene fragments, resulting in kanamycin resistance. If recombination is intramolecular, hygromycin resistance will be lost. The pUS950 plasmid was introduced into *M. bovis* BCG by electroporation, with initial selection for hygromycin resistance. Transformant colonies were tested for recombination by plating on media with and without kanamycin. Analysis of the structure of the km^r hyg^s putative recombinant plasmids confirmed that resistance was due to the plasmid (as opposed to spontaneous mutation), and established that homologous recombination does indeed occur in *M. bovis* BCG. The gene *accBC* encoding for a subunit of acyl-CoA carboxylase was chosen for a gene replacement experiment in *M. bovis* BCG. In this case, a replicating vector was used, which extends the time over which recombination can occur. Despite poor efficiency, the experiment was successful and opens up the possibility of applying this powerful tool to *M. tuberculosis*. This possibility has been recently confirmed by the work of Reyrat et al. (26). They have used a *M. tuberculosis* disrupted *ureC* gene incorporated into a suicide vector to construct a urease-negative *M. bovis* BCG mutant by allelic exchange. Both the use of an appropriate marker gene, such as *ureC*, and linearization of the vector before transformation have probably contributed to the success of this experiment.

A good example of the application of homologous recombination to study bacterial pathogenicity is the work of McNamara and associates, in which they demonstrated the role of the phospholipase D gene (*pld*) in the virulence of *Corynebacterium pseudotuberculosis* (23). This nocardioform bacterium is the agent responsible for caseous lymphadenitis, a disease affecting sheep and goats and thus producing substancial financial losses in many countries. *C. pseudotuberculosis* produces an exotoxin, phospholipase D (PLD), which had for a long time been considered a virulence determinant because of its dermonecrotic action (1). PLD also originates synergistic hemolysis with extracellular products from *Rhodococcus equi* (24).

The gene that codes for PLD was cloned in *E. coli* and mutagenized by deleting 2 bp of its coding sequence. The absence of PLD activity in *E. coli* cells containing the mutant *pld* gene confirmed that deletion caused loss of *pld* gene function. The vector for allele exchange was then constructed. This vector, designated pJM11ar, contains the origin of replication from the ColE1 plasmid, which is non-functional in *C. pseudotuberculosis*, an erythromycin- resistant gene (Em^r), which is functional in both *E. coli* and *C. pseudotuberculosis*, and the mutant *pld* gene. The vector was introduced into *C. pseudotuberculosis* by electroporation, and the Em^r phenotype was selected. Em^r colonies were grown in the absence of antibiotic to enhance excision of the plasmid. Selection of the *pld* mutants was made by screening for the loss of synergistic hemolysis with *R. equi*. Out of 3000 colonies screened, three were non-hemolytic mutants and one of these was Em^s. Subsequent genetic and biochemical characterization of this mutant verified the deletion of 2 bp, which prevented PLD synthesis. Allele replacement had occurred in this mutant. Finally, virulence tests were performed in mice, and reduced virulence was found for the *pld* mutant compared with the wild-type strain.

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Epílogo y agradecimientos

Del 7 al 11 del pasado mes de agosto de 1995, y en el marco de los XI Cursos de Verano de Laredo, se celebró el I Curso sobre "Bases moleculares de la patogenicidad bacteriana". Fueron cinco días intensos, en los que todos los participantes tuvimos la oportunidad de disfrutar de unas excelentes sesiones científicas en un entorno geográfico privilegiado. La experiencia fue enormemente gratificadora, tanto para los organizadores como para los conferenciantes, por la entusiástica respuesta que tuvo la convocatoria: 42 alumnos inscritos, de todas las titulaciones biomédicas. Ni el sofocante calor que reinaba en la abarrotada aula del centro cultural Dr. Velasco del Ayuntamiento de Laredo, ni la especialmente tentadora opción de escaparse a la cercana playa, disuadieron al alumnado de asistir a las sesiones, lo cual es prueba del interés que éstas despertaron.

El programa, ciertamente, merecía la pena. Fernando Baquero (Hospital Ramón y Cajal, Madrid) abrió las sesiones con una conferencia en la que, con el entusiasmo y la amenidad que le caracterizan, disertó sobre la interacción hospedador–patógeno desde un punto de vista ecológico y dialogístico. A continuación, los organizadores del curso —y editores del presente número de *Microbiología SEM*— presentamos sendas charlas introductorias sobre los mecanismos generales de la patogenicidad bacteriana y las aproximaciones experimentales para su estudio. Posteriormente, Francisco García del Portillo (Universidad Autónoma de Madrid) nos habló sobre el tráfico intracelular de *Salmonella* y las interacciones de estos microorganismos con el aparato lisosómico; Jesús Agüero (Universidad de Cantabria, Santander) presentó una interesante charla sobre diagnóstico molecular y mecanismos de patogenicidad de *Brucella*; y Maite Iriarte (Universidad Católica de Lovaina, Bélgica) nos explicó los mecanismos de interacción de *Yersinia* con las células eucarióticas y el papel que juegan en este sentido las proteínas Yop. Albrecht Ludwig (Universidad de Würzburg, Alemania) presentó una exhaustiva revisión sobre las toxinas citolíticas producidas por bacterias Gram negativas; y Francisco Leyva-Cobián (Hospital Marqués de Valdecilla, Santander) se encargó de recordarnos que las células del sistema inmunitario juegan un papel fundamental en la interacción hospedador–agente infeccioso. Patrick Berche (Hospital/Facultad de Medicina Necker-Enfants Malades, París) y Trinad Chakraborty (Universidad de Giessen, Alemania) nos deleitaron con dos seminarios sobre la fisiopatología molecular y biología celular de la infección por *Listeria*, durante los que tuvimos la oportunidad de admirar bellas imágenes de microscopía confocal de la motilidad bacteriana intracelular por polimerización de actina. Como broche final, Jorge Galán (Universidad del Estado de Nueva York, Stony Brook, EE. UU.) nos ofreció un magnífico resumen de sus trabajos sobre la biología de la infección por *Salmonella*, poniendo énfasis en la transducción de señales durante la interacción con la célula eucariótica. Realmente ha sido para nosotros un privilegio contar con investigadores de tanto prestigio científico, y una entrañable experiencia compartir con ellos y con el alumnado unos días de ciencia y amistad. El éxito de esta iniciativa nos ha motivado de tal forma que tenemos la voluntad de organizar, de forma regular y en los próximos años, nuevas ediciones del curso.

Movidos por este entusiasmo, así como por el interés y la calidad científica de los temas abordados, creímos que sería una buena idea tratar de difundir el curso a través de una publicación que recogiera la esencia de sus contenidos. Esta publicación se ha materializado en el presente número monográfico sobre

Participantes en el I Curso "Bases moleculares de la patogenicidad bacteriana"

"Bases moleculares de la patogenicidad bacteriana". Los once artículos que lo componen revisan aspectos actuales del máximo interés en el campo de la patogenia molecular de las infecciones bacterianas. Aunque abordando cuestiones puntuales, las contribuciones aquí recogidas constituyen paradigmas que aportan una idea general sobre el objeto y los métodos de estudio de la patogénesis microbiana como área científica, e ilustran sobre los enormes progresos que ha experimentado esta nueva disciplina que emerge, con gran pujanza, desde el campo de la microbiología. La mayoría de los artículos fueron redactados por el profesorado del curso, y otros pocos lo fueron por especialistas invitados. Confiamos en que los lectores de *Microbiología SEM* encuentren interesante este número, y que disfruten de sus artículos tanto como nosotros lo hicimos con los seminarios del curso de verano.

Para terminar, queremos expresar nuestro agradecimiento a todos los autores que, generosa y desinteresadamente, aceptaron colaborar en este número, invirtiendo su esfuerzo y su escaso tiempo. Igualmente, también queremos agradecer muy sinceramente a la revista *Microbiología SEM*, y en particular a Ricard Guerrero, director-coordinador de la revista, por permitirnos usar estas páginas como vehículo de expresión, y por la excelente labor editorial realizada. Y, finalmente, no queremos olvidarnos de expresar nuestro reconocimiento a la organización de los XI Cursos de Verano de Laredo (Universidad de Cantabria–Ayuntamiento de Laredo), representada por sus directores Juan Antonio Michell Martín y Lourdes Royano Gutiérrez, por su magnífica gestión; a la DGICYT, por contribuir a la financiación de nuestro curso a través de una "Acción Especial"; y a Sigma-Aldrich Química S.A. y bioMérieux España S.A. por brindarnos su apoyo económico para este proyecto editorial. A todos ellos reiteramos nuestro profundo agradecimiento.

José Antonio Vázquez-Boland y **Jesús Navas Méndez**
Editores del número monográfico "Bases moleculares de la patogenicidad bacteriana"

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Breve historia de la Sociedad Española de Microbiología, IV. De 1983 a 1987

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Summary

In this fourth chapter of the Spanish Society for Microbiology (SEM) short history, the major activities carried out from 1983 to 1987 are described. During that period, the 9th and 10th SEM National Congresses took place in Valladolid (September 1983) and Valencia (September 1985), respectively. A monographic meeting on postgraduate training in microbiology was held in Sigüenza (June 1987), partially sponsored by the University of Alcalá de Henares. Other meetings were organized by the Specialized Groups of the Society. It was during that period that the journal *Microbiología SEM* started being published (September 1985), an award for young researchers was set up, and the by-laws of the Society were amended twice.

Resumen

En este cuarto capítulo sobre la breve historia de la Sociedad Española de Microbiología (SEM) se describen las actividades desarrolladas entre los años 1983 y 1987. Se celebraron el noveno (Valladolid, septiembre 1983) y décimo (Valencia, septiembre1985) Congresos Nacionales, la reunión monográfica sobre formación postgraduada en microbiología (Sigüenza, junio 1987), en la que colaboró la Universidad de Alcalá de Henares, así como diversas reuniones correspondientes a los Grupos Especializados. En este período se comenzó la publicación de la revista *Microbiología SEM* (septiembre 1985), se instauró el premio bienal para científicos jóvenes y se efectuaron dos enmiendas consecutivas de los Estatutos de la Sociedad.

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IX Congreso Nacional de Microbiología

Celebrado en Valladolid durante los días 6 al 10 de septiembre de 1983 y organizado por Antonio Rodríguez Torres, el IX Congreso Nacional tuvo lugar en las Facultades de Medicina y Ciencias, de la Universidad de Valladolid (Fig. 1). Pronunció la conferencia inaugural David Vázquez y la de clausura Amadeo Foz. El congreso contó con tres Simposios internacionales, en los que intervinieron diferentes especialistas nacionales y extranjeros. Contó también con diecisiete Mesas Redondas y más de quinientas comunicaciones libres, repartidas en forma oral y en paneles. Participaron cerca de mil microbiólogos. Este congreso introdujo la novedad de publicar todas las aportaciones científicas en forma de artículos completos (que constituyeron tres voluminosos libros, el primero, con las comunicaciones, de 1140 páginas, en dos tomos, y el segundo, con las contribuciones por invitación, de 740 páginas).

En la Junta General ordinaria celebrada durante el IX Congreso Nacional, se expusieron las actividades más destacadas desarrolladas por la Junta Directiva durante el bienio anterior, y las de cada Grupo Especializado. Estas últimas fueron expuestas por sus respectivos Presidentes. En el caso del Grupo de Microbiología Industrial, con motivo de la renovación de su Junta Directiva, Federico Uruburu se había incorporado como nuevo Presidente. En el Grupo de Taxonomía Bacteriana fue nombrado Presidente Alberto Ramos Cormenzana.

El Presidente de la SEM, César Nombela, propuso la celebración, durante los Congresos Nacionales, de una conferencia que llevaría el nombre de un científico destacado, y que sería impartida por un microbiólogo joven. Después de un debate entre los presentes, y dado que el siguiente congreso iba a celebrarse en Valencia en 1985, se aceptó la propuesta de Julio R. Villanueva de llamarla conferencia "Jaime Ferrán", por la doble circunstancia de impartirse en la ciudad donde Ferrán realizó la mayor parte de su labor científica, y de cumplirse en 1985 el centenario de su gran campaña de vacunación contra el cólera. Por último, el Presidente informó sobre la elección efectuada durante el presente congreso del anagrama de la SEM, realizado por Paloma Párraga, que sería utilizado a partir de ese momento.

En cuanto a la CECT, debido a su crecimiento y a las cada día mayores relaciones internacionales, pues acababa de incorporarse a la ECCO (European Culture Collection Organization), Federico Uruburu solicitó apoyo financiero a la SEM para poder dotar a la colección de una infraestructura más sólida. La CECT también recibiría apoyo económico de la Generalitat Valenciana, el FIS, la Unesco, etc.

Bienio 1984–1985

Reanudando sus actividades habituales, la Junta Directiva de la SEM consideró que la nueva etapa de publicación de la revista *Microbiología Española* en colaboración con el CSIC no estaba siendo todo lo fructífera que se esperaba, por lo que habría que tratar de impulsarla para conseguir su total consolidación. Por el contrario, el *Boletín Informativo*, a cargo de Pilar Pérez Breña, estaba publicándose a plena satisfacción. Con respecto a la conferencia bienal de la SEM, se acordó que la comisión de selección de los futuros conferenciantes, que serían a la vez galardonados con el Premio de la SEM para investigadores jóvenes, estaría constituida por los Presidentes de los Grupos Especializados, junto con el Presidente y el Secretario de la SEM. Los candidatos al Premio de la SEM deberían ser científicos destacados en el campo de la Microbiología, que tuvieran menos de 40 años y que fueran socios de la SEM. Como otra nueva actividad de este período, el Presidente, César Nombela, propuso la creación de un programa de

FIG. 1. Sesión inaugural del IX Congreso Nacional de Microbiología (Valladolid, 1983). A la derecha, Antonio Rodríguez Torres, organizador del mismo; a continuación, César Nombela, Presidente de la SEM, y diversas autoridades.

enseñanza de la microbiología adaptado a las nuevas tecnologías de vídeo y ordenador, de cuya gestión se encargaría Andrés Chordi.

Los Grupos Especializados y Regionales continuaron organizando las reuniones correspondientes al bienio en curso. El Grupo de Microbiología Industrial preparaba su II Reunión en Arganda del Rey (Madrid), a cargo de Francisco Garrido, y el Grupo de Microbiología de los Alimentos hacía lo propio con su IV Reunión, a celebrar en Pamplona bajo la dirección de José M. Arcos Santo Domingo; este Grupo había renovado su Junta Directiva y Benito Moreno era su nuevo Presidente. Por su parte, el Grupo de Taxonomía Bacteriana, de reciente creación, había iniciado sus actividades con la celebración de la primera reunión.

Con motivo de la renovación de la Junta Directiva del Grupo de Aragón, Rioja, Navarra y Soria, Rafael Gómez Lus, elegido Presidente del mismo, se incorporó de nuevo a la Junta Directiva de la SEM. El Grupo de Virología se encontraba organizando su VI Reunión, a celebrar en Valencia en 1984, a cargo de Rafael de Andrés Medina. El Grupo de Microbiología Clínica, al frente del cual se encontraba Luis Arcalís como Presidente en funciones, trataba de conseguir su consolidación después del paréntesis producido por la creación de la SEIMC. Paralelamente, el Grupo del Noroeste, presidido por José Ángel García Rodríguez, intentaba su reactivación.

Por su parte, la CECT estaba ultimando la edición de su Catálogo de Cepas. Continuando con su política de expansión, había participado en la reunión de colecciones organizada en Brno (Checoslovaquia) e intervendría en la siguiente reunión, a celebrar en Norwich (Gran Bretaña).

Para atender la petición del Ministerio de Sanidad de nombrar dos representantes de la SEM para las Comisiones Nacionales de cada especialidad médica, la Junta Directiva acordó refrendar

a sus dos representantes, Rafael Sentandreu y José Ángel García Rodríguez, y nombrar como suplentes a Miguel Gobernado y a Rafael Nájera. El Ministerio dio curso a la propuesta y, en base al Real Decreto del año 1984, nombró a José Ángel García Rodríguez y Miguel Gobernado, representantes de la SEM en la Sección de Microbiología de la Comisión Nacional de Especialidades Médicas. Respondiendo a otra solicitud del Ministerio para que la SEM propusiera vocales para los tribunales de plazas de microbiólogos en la Seguridad Social, el Presidente, dada la premura de tiempo, fue consultando a diferentes miembros de la Junta para solucionar cada caso particular en su momento.

Las relaciones con la FEMS estaban aumentando gradualmente, debido a la organización en Madrid, en 1984, del simposio FEMS sobre "Microbial cell wall synthesis and autolysis", a cargo de César Nombela, y al reciente nombramiento de Carlos Hardisson como miembro del consejo de redacción de la revista *FEMS Microbiology Letters*. Con motivo del XIV Congreso Internacional de Microbiología, a celebrar en Manchester en 1986, la FEMS concedió a cada una de sus sociedades integrantes dos becas de asistencia a investigadores jóvenes, para lo cual la SEM debía realizar la selección correspondiente.

Como consecuencia del aumento paulatino de actividades de la SEM, el Presidente, César Nombela, consideró necesaria la informatización de la secretaría administrativa de la Sociedad, y así poder abarcar tanto la gestión correspondiente a la SEM y a los Grupos Especializados, como la del boletín y la revista. También se puso en marcha la actualización de los Estatutos, para lo cual se propuso la formación de una comisión integrada por Antonio Portolés, Ricardo Guerrero y Sara I. Pérez Prieto.

Ante la correspondiente renovación de la Junta Directiva, y habiéndose recibido varias candidaturas, como resultado de la votación se incorporaron a la misma las siguientes personas: Secretaria, Concepción García Mendoza; Tesorero, Juan Antonio Leal Ojeda; y Vocales, Francisco Ruiz Berraquero, M. Luisa Gómez-Lus Centelles, Bernabé Sanz y Andrés Chordi (Fig. 2). Recién constituida la nueva Junta, se procedió a la instalación de un equipo informático IBM en la nueva sede de la SEM, un local cedido por el CSIC situado en el número 8 de la calle Vitruvio.

Con motivo de la recepción de sendas propuestas para nombrar Socios de Honor a Julian Davies (Wisconsin, EE. UU.) y a David Hopwood (Norwich, Gran Bretaña) por su reconocida labor científica y colaboración con distintos grupos de microbiólogos españoles, se acordó informar a los socios de dichas propuestas, para que, como indican los Estatutos, manifestaran su opinión. El nombramiento se haría durante el Congreso Nacional de Valencia. También en este congreso se efectuaría la entrega del I Premio de la SEM para microbiólogos jóvenes a Juan Ortín, elegido para pronunciar la conferencia plenaria "Jaime Ferrán" por el jurado constituido al efecto.

A la vista del reiterado incumplimiento de los plazos de publicación por parte de la revista *Microbiología Española*, la Junta Directiva de la SEM acordó su desvinculación definitiva de la misma y decidió la publicación de su propia revista, que se denominaría *Microbiología SEM*. Se podría preparar el primer número para el Congreso Nacional de Valencia, y se encargó de tal gestión a Rubens López, que fue nombrado Editor-Jefe. [Véase el artículo sobre la historia de la revista de la SEM en el número anterior, 12(1), 117–125.] Dada la premura de tiempo para cumplir el plazo de publicación, el Editor-Jefe presentó la lista de nuevos Editores especializados en áreas determinadas. Por su parte, el Presidente de la SEM envió un escrito a todos los miembros de la Junta Directiva, en el que se establecían las bases para la redacción de las normas de publicación de los artículos. Con la introducción del nuevo cargo de Editor-Jefe se hacía necesaria la correspondiente

FIG. 2. Junta Directiva de la SEM (enero de 1985). Arriba (de izquierda a derecha): Andrés Chordi, Juan Antonio Leal, José M. Arias, Bernabé Sanz, Rosa Peñalva (secretaria administrativa), Concepción García Mendoza (Secretaria entrante), Eulalia Cabezas de Herrera (Secretaria saliente), César Nombela (Presidente), Miguel Gobernado, Ricardo Guerrero y Benito Moreno. Debajo (de izquierda a derecha): José Ramón Díaz, Rafael Sentandreu, Pilar Pérez Breña, Rafael Gómez Lus, M. Luisa Gómez-Lus, Carlos Hardisson y Francisco Ruiz Berraquero, y (delante) Alberto Ramos Cormenzana.

enmienda en los Estatutos, que ya estaba en fase de estudio por la comisión designada al efecto. Esta enmienda debería presentarse en la Junta General Extraordinaria que, a la vez que la Junta General Ordinaria, o Asamblea, se celebraría en Valencia durante el Congreso Nacional. Para facilitar la realización de ambas Juntas se decidió enviar a los socios la convocatoria, junto con un resumen del Acta de la Asamblea anterior, un extracto económico del bienio y, en este caso, la propuesta de subida de cuota con motivo de la publicación de la nueva revista. También se envió la propuesta de modificación de Estatutos, con la actualización necesaria en ese momento, que incluía la sustitución del cargo de Bibliotecario por los de Editor-Jefe de la revista y Coordinador del *Boletín Informativo*. Para este último cargo se propuso a Rosalina Pomés, por cese de su antecesora. También se decidió que, en lo sucesivo, la secretaria administrativa de la SEM asistiese a los Congresos Nacionales con objeto de atender consultas de los socios, ayudar en cuestiones administrativas del congreso y, en este caso, colaborar en el reparto del primer ejemplar de la revista.

El Comité de la IUMS reunido en Budapest había decidido que el XIII Simposio Internacional de Microbiología de los Alimentos se celebrase en España en 1986. Aunque al principio trató de organizarse en Salamanca, después del Congreso Internacional de Microbiología de Manchester, finalmente se decidió que fuese en Zaragoza, junto con la Reunión del Grupo Especializado corres-

pondiente. La reunión estaría a cargo de Rafael Gómez Lus y de David A. A. Mossel (Univerteit Utrecht), promotor del mismo. Por su parte, el Grupo de Aragón, Rioja, Navarra y Soria estaba preparando su próxima reunión en Pamplona, bajo la dirección de Ramón Díaz. El Grupo de Virología, a raíz de sus últimas reuniones celebradas durante el Congreso Internacional de Virología de Sendai (Japón) y en la reunión del Grupo, realizada en Valencia, intentaba la creación de la nueva Sociedad Española de Virología. El Grupo de Micología acababa de celebrar su II Reunión, en Barcelona, a cargo de M. Ángeles Calvo, conjuntamente con la AEEM. El Grupo de Taxonomía Bacteriana estaba organizando su II Reunión en Barcelona, bajo la dirección de Francisco Congregado y M. Dolores Simón. Paralelamente, el Grupo de Microbiología Industrial estaba organizando su correspondiente Reunión, conjuntamente con la Sociedad Española de Biotecnología, en León, de cuya organización se había encargado el Presidente de la nueva Sociedad, Juan Francisco Martín. Todos los Grupos, además, se habían encargado de la organización de diferentes Mesas Redondas de su especialidad para el próximo Congreso Nacional de Valencia. Finalmente, y ante la petición de Rafael Nájera de informar durante la próxima Asamblea sobre la creación de la Sociedad Española de Virología, punto no incluido en el orden del día, se decidió que fuese el Presidente de la SEM quien leyera una nota al respecto al final de su informe.

X Congreso Nacional de Microbiología

El X Congreso Nacional de la SEM se celebró en la Facultad de Medicina de la Universidad de Valencia, entre los días 30 de septiembre y 3 de octubre de 1985, organizado por Federico Uruburu. La conferencia inaugural, o Conferencia "Jaime Ferrán", fue pronunciada por Juan Ortín, quien había sido distinguido con el I Premio de la SEM por su labor científica. La conferencia de clausura corrió a cargo de Lynn Margulis (Boston University). El congreso constó de seis Simposios y veintitrés Mesas Redondas. Hubo 674 comunicaciones libres, que se presentaron en forma de panel, y participaron unos mil microbiólogos españoles y algunos destacados microbiólogos extranjeros.

En la Junta General Ordinaria celebrada durante el X Congreso Nacional, el Presidente, César Nombela, informó sobre el cumplimiento de los principales objetivos marcados durante el bienio anterior, como la dotación de una sólida infraestructura administrativa a la Sociedad, el nombramiento de Socios de Honor a Julian Davies y a David Hopwood (este último asistió a la entrega de su galardón durante el congreso) y la instauración de la Conferencia y Premio de la SEM, esta vez en honor de Jaime Ferrán, dándole el realce de conferencia inaugural. Seguidamente, procedió a la lectura de una nota informativa acerca de la creación de la nueva Sociedad Española de Virología.

La presentación de la nueva revista *Microbiología SEM* corrió a cargo del Presidente y del Editor-Jefe, Rubens López. Tanto el aspecto como el contenido de la revista constituían un auténtico logro. Finalmente, la SEM presentó su adhesión al homenaje que la Sociedad Española de Bioquímica dedicaba a Severo Ochoa en esos días en Valencia, con ocasión de su ochenta cumpleaños.

Las demás actividades realizadas durante los años 1983–85, descritas anteriormente, fueron expuestas por sus respectivos representantes en la Junta Directiva. Finalmente, se debatió la sede del siguiente Congreso Nacional, que, con reservas acerca de su capacidad hotelera, podría ser Oviedo y Gijón conjuntamente, bajo la dirección de Carlos Hardisson.

A continuación se celebró la Junta General Extraordinaria, en la que se debatió la enmienda de los Estatutos en sus artículos 2, 3, 10, 11, 15, 19 y

20, aprobándose la nueva redacción de los mismos. Finalmente, se ratificó la presente Junta Directiva.

Bienio 1986–1987

De vuelta a sus actividades, aquella Junta Directiva consideró importante la organización de una reunión monográfica sobre "Enseñanza postgraduada de la Microbiología", en la que intervendrían, además de miembros de dicha Junta, varios invitados; trataría áreas muy variadas, desde la organización de cursos especializados hasta posibilidades de empleo, etc. Para cubrir las tendencias más representativas de la microbiología se nombró una comisión gestora que incluyó, además del Presidente de la SEM como coordinador, a Andrés Chordi para la estructuración de encuestas, Benito Moreno por el área de Microbiología de Alimentos, Luis Arcalís por Microbiología Clínica, Ernesto García por el CSIC, José Luis Fernández Puentes por la industria farmacéutica, Rafael Nájera por Sanidad y Enrique García Maiquez o Jaime Conde por fermentaciones industriales. Seguidamente, el Presidente informó de su participación en la conferencia sobre "Organismos manipulados genéticamente", celebrada en Londres con asistencia de los presidentes de la mayoría de Sociedades de Microbiología europeas. Dado el interés de promover la continuidad de este tipo de reuniones, se constituyó un comité preparatorio, con Juan Ortín al frente del mismo. Con relación al Congreso Internacional de Microbiología, a celebrar en Manchester en 1986, se propusieron, a requerimiento del entonces Secretario General de la IUMS, Stuart W. Glover, como delegados de la SEM para asistir a la Asamblea, a César Nombela, Federico Uruburu y Bernabé Sanz, entre varios de los futuros asistentes a dicho congreso.

Por su parte, los Grupos Especializados continuaban organizando sus respectivas reuniones. Así, la III del Grupo de Micología se celebraría en Jarandilla de la Vera (Cáceres), en 1986, bajo la dirección de Germán Larriba y en colaboración con la AEEM. El simposio internacional sobre Microbiología de los Alimentos, que debía realizarse en Zaragoza también en 1986, junto con la reunión del Grupo, se canceló inesperadamente, celebrándose únicamente dicha reunión, dirigida por Rafael Gómez Lus. El Grupo de Taxonomía Bacteriana estaba intentando organizar un simposio FEMS sobre "Bacterias halófilas", a propuesta de Francisco Rodríguez Valera y Antonio Ventosa, para lo cual la Secretaria de la SEM debería hacer las gestiones pertinentes.

Con relación a la gestión administrativa, también se estaban introduciendo en el fichero de socios las palabras clave de sus especialidades, para poder suministrar esta información a los organismos oficiales que lo estaban solicitando, como era el caso de la CAICYT. Además, debido a la instauración del IVA en todas las facturaciones, la SEM estaba intentando conseguir su exención, lo que le facilitaría tanto la gestión como la disminución de los gastos.

Celebrado el Congreso Internacional de Manchester, en el que tuvieron lugar tanto la Asamblea de la IUMS como el Consejo de Delegados de FEMS, el Presidente de la SEM, que había actuado como representante de la Sociedad en ambas reuniones, informó sobre la favorable acogida, por parte de FEMS, que habían tenido tanto la propuesta de organización del "workshop" sobre "Bacterias halófilas" (a cargo de Francisco Rodríguez Valera y Antonio Ventosa) como la aceptación de Ricardo Guerrero como miembro del Consejo Editorial de la nueva revista *FEMS Microbiology Ecology*. Con respecto a la IUMS, en la correspondiente Asamblea —en la que también representó a nuestra Sociedad Fernando Baquero—, se pudo constatar que la SEM sólo era superada en número de representantes por las sociedades de EE. UU., Inglaterra y Rusia, ya que

contaba con cinco votos sobre un máximo de seis: tres en razón de cada una de las Divisiones de Bacteriología, Micología y Virología, y los otros dos debido al número de socios. Después de la votación pertinente, se acordó que la siguiente asamblea tuviese lugar en Berlín en 1990, coincidiendo con el Congreso Internacional de Virología. Igualmente, se acordó que las Divisiones de Bacteriología y Micología celebrasen sus congresos cada cuatro años, mientras que la de Virología lo hiciera cada tres. Fue también en estos momentos cuando la IUMS solicitó el nombramiento de un nuevo representante de la SEM en el Comité Internacional de Bacteriología Sistemática (ICSB), para lo cual se refrendó el nombramiento previo de Alberto Ramos Cormenzana.

Paralelamente, la CECT, que había finalizado la distribución de su segundo Catálogo de Cepas, planteó la urgente necesidad de informatización para poderse conectar con las colecciones extranjeras. Con motivo de la reunión de colecciones de Londres, celebrada en fechas contiguas al congreso de la IUMS de Manchester, se negoció la colaboración de la colección inglesa para la informatización de la CECT.

Próximo a celebrarse el XI Congreso Nacional, la Junta Directiva acordó que la conferencia bienal llevase en esta ocasión el nombre de "David Vázquez", anterior Presidente de la SEM, fallecido recientemente. Reunido el jurado, resultó elegido para tal premio Enrique Herrero. También durante el congreso se entregaría el galardón de Socio de Honor concedido anteriormente a Julian Davies, que en aquellos momentos se encontraba trabajando en el Institut Pasteur de París.

La revista *Microbiología SEM* continuaba su andadura ascendente y la Sociedad había suscrito un contrato con una empresa privada para que gestionase una publicidad discreta que ayudase a su financiación. Por otra parte, la revista *Microbiología Española*, conjuntamente con el Instituto Jaime Ferrán, había presentado un recurso para que se suspendiera el registro de la cabecera de *Microbiología SEM*, basándose en una supuesta duplicidad de denominación. Poco después, dicho Instituto desapareció, y su personal se integró en el Centro de Investigaciones Biológicas. El Director del CIB, a instancias de la SEM, retiró notarialmente la demanda, adjuntando un certificado del Secretario General del CSIC que acreditaba la capacidad del Director para tal actuación. Se hacía constar que el nombre de nuestra revista no era un término genérico, como se había argumentado, sino un nombre bien delimitado.

Con respecto a la petición de exención del IVA, también la SEM consiguió el apoyo del CSIC, de forma que el Vicepresidente para Asuntos Internacionales certificó que la SEM se encontraba bajo la protección del CSIC y que, de acuerdo con la CEE, debía estar exenta de impuestos fiscales. Continuando con las gestiones administrativas, con motivo de la solicitud por parte del Ministerio de Hacienda de documentación adicional acreditativa de las potestades del cargo de Secretario en la tramitación de la citada exención del IVA, la entonces Secretaria, Concepción García Mendoza, expuso la necesidad de una nueva enmienda de los Estatutos, debido a la laguna jurídica existente en cuanto a la falta de definición de competencias de los cargos directivos de la SEM. Para tal fin, se solicitó el asesoramiento jurídico pertinente. El Presidente informó acerca de la negociación llevada a cabo con la Sociedad Española de Bioquímica para la utilización conjunta del equipo informático de la SEM, pero con la financiación de la SEB en cuanto a la ampliación del programa correspondiente; para ello, ambas secretarías, situadas en locales contiguos del CSIC, se comunicarían y compartirían una línea telefónica independiente con contestador automático, con el consiguiente ahorro económico para ambas.

Correspondiendo estatutariamente la renovación parcial de la Junta Directiva, y solicitadas las

FIG. 3. Reunión monográfica en Sigüenza (junio de 1987). Junta Directiva de la SEM e invitados (de izquierda a derecha y de arriba hacia abajo): Bernabé Sanz, Carlos Hardisson, Francisco Ruiz Berraquero, Luis Domínguez, José M. Echevarría, Fernando Laborda, Luis Arcalís, J. F. Pellón, José Carlos Montilla, Benito Moreno, Alberto Ramos, Juan Antonio Leal, F. Perdigones, Juan Carlos Nieto, Andrés Chordi, Rubens López, Ernesto García, Ernesto Gallego, César Nombela, Concepción García Mendoza, Enrique Montoya, Julio R. Villanueva, Sara I. Pérez Prieto, Rafael Sentandreu y Rafael Gómez Lus.

candidaturas necesarias completas o incompletas, tras las votaciones quedaron elegidos: Presidente, César Nombela; Vicepresidente, Miguel Gobernado; y Vocales, Enrique Montoya y Sara I. Pérez Prieto.

Respondiendo a la petición del Ministerio de Sanidad acerca del nombramiento de un representante de la SEM en la Comisión de Especialidades Farmacéuticas en Microbiología, la Junta Directiva acordó proponer a Carmen Domínguez como representante titular y a Aurora Sánchez Sousa como representante suplente.

La celebración de la reunión monográfica sobre "Formación postgraduada en Microbiología", tuvo lugar finalmente en Sigüenza en junio de 1987, y fue financiada parcialmente por la Universidad de Alcalá de Henares (Fig. 3). Contó como invitados, además del Vicerrector de la citada Universidad, P. García Corrales, con diferentes representantes de la administración central, la industria, la universidad, el CSIC, etc. En la reunión, César Nombela, propuso la realización de un curso intensivo sobre "Introducción a la investigación microbiológica", para veinte o treinta estudiantes, seleccionados por sus méritos académicos, que realizasen el último curso de las licenciaturas que incluyen estudios de microbiología. El curso sería impartido por unos diez microbiólogos activos y responsables de grupos de investigación, que expondrían, durante al menos media jornada de trabajo, sus diferentes experiencias. Los pormenores de esta iniciativa se perfilarían durante las sucesivas reuniones de la Junta Directiva y se presentarían a los socios de la SEM durante la Asamblea del siguiente Congreso Nacional.

Las actividades de los Grupos Especializados

habían cristalizado en la preparación de las siguientes reuniones para 1988: la IV Reunión de Micología, en colaboración con la AEEM (en La Manga del Mar Menor, Murcia), bajo la dirección de Mariano Gacto y de Mario Honrubia; la VI Reunión de Microbiología de los Alimentos (en Madrid), coordinada por Bernabé Sanz; y la reunión del Grupo de Microbiología Industrial, que se celebraría dentro del II Congreso Nacional de Biotecnología (que tendría lugar en Barcelona en el mes de junio y que sería presidido por Ricardo Guerrero). Por último, el Grupo de Taxonomía Bacteriana iba a participar en el simposio FEMS sobre "Arqueobacterias" que se celebraría en las Islas Azores (Portugal), con Antonio Ventosa como representante español.

La CECT había participado en la reunión de Ioannina (Grecia), en la que se decidió el funcionamiento del MINE (Microbial Information Network Europe), para coordinar las distintas colecciones europeas. La siguiente reunión sobre el tema tendría lugar en Budapest, con objeto de ir incorporando las colecciones de países no integrados en la CEE.

Finalmente, antes de la celebración del XI Congreso Nacional, se pudo apreciar la buena acogida por parte de los socios de la propuesta de nombrar Socio de Honor a Hans G. Trüper (Universität Bonn), por su vinculación con diferentes grupos españoles y por su colaboración con la revista *Microbiología SEM*; este nombramiento tendría lugar durante el congreso. En el XI Congreso Nacional se celebraría una Junta General Extraordinaria para discutir la enmienda de Estatutos, con la propuesta de crear los cargos de Presidente y Secretario electos, tal como existen en diversas sociedades extranjeras. Éstos serían elegidos dos años antes de su toma de posesión, con objeto de hacer más fácil el relevo de los respectivos cargos.

Y hasta aquí, la cuarta entrega de la breve historia de la SEM. En el próximo número de la revista narraremos los principales acontecimientos de nuestra Sociedad entre los años 1987 y 1991.

MICROBIOLOGÍA SEM **12** (1996), 317–319

Elena N. Kondratieva (1925–1995)*

Ruslan N. Ivanovsky

Department of Microbiology, Moscow State University, Moscow, Russia

Elena Nikolaievna Kondratieva (16.12.1925–26.04.1995) was an outstanding Russian microbiologist, one of the greatest specialists in the field of phototrophic bacteria, microbial physiology and biochemistry. She was an active member of the Russian Academy of Sciences, president (1975–1985) and vice-president (1985–1995) of the All-Union (USSR) Microbiological Society, vice-chairperson (1985–1988) of the Federation of European Microbiological Societies, and Honorary Member of a number of European microbiological societies. Since 1989 she had been the head of the Department of Microbiology of Moscow State University.

Elena N. Kondratieva graduated from Moscow State University in 1949, having specialized in microbiology. At that time the department was headed by professor (academician) Vladimir N. Shaposhnikov, a famous researcher of the physiology and biochemistry of microorganisms and one of the creators of the so-called technical microbiology (now called biotechnology) in Russia.

After Kondratieva's graduation from Moscow State University, V. N. Shaposhnikov offered her the opportunity to begin the study of physiology and metabolism of phototrophic purple and green bacteria. At that time these groups of microorganisms were studied rather poorly, and some of the available data were controversial; their serious and profound study in Russia was just at its beginning. Kondratieva was the first scientist in Russia who started a systematic study of the physiology and biochemistry of phototrophic bacteria. This subject became the major branch of her scientific work. Her PhD (1952) and Doctorate (1963) theses were devoted to the study of phototrophic anoxygenic bacteria. The creation of a culture collection of phototrophic bacteria, many of which were isolated by Kondratieva herself and by her disciples, had a great scientific and practical value. Her laboratory was the alma mater of many well-known Russian microbiologists of the present time.

The work led by Kondratieva is a great contribution to the study of diversity and evolution of

* Note of the Editor-in-Chief: It was with deep sorrow that we learnt of the passing of our colleague and friend, distinguished Russian microbiologist Elena N. Kondratieva. *Microbiología SEM* wishes to pay homage to this leading microbial physiologist and ecologist by publishing the obituary written by Ruslan N. Ivanovsky, and received through Prof. Hans G. Trüper, member of our Editorial Board.

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phototrophic microorganisms and to the understanding of the mechanisms of photosynthesis. The pathways of carbon, nitrogen and sulfur metabolism in a number of green and purple bacteria (*Chlorobium limicola*, *Thiocapsa roseopersicina*, *Ectothiorhodospira shaposhnikovii*, *Rhodobacter sphaeroides*, and others) were described.

The study of a wide number of phototrophic bacteria from different taxonomic groups led to a conclusion of the close connection existing between the systems of autotrophic CO_2 assimilation and the metabolism of organic compounds. The reasons for obligate autotrophy of some phototrophic bacteria were discovered.

It was shown that the role of organic compounds in the metabolism of phototrophic bacteria depended on the functioning of the tricarboxylic acid cycle (TAC) and the glyoxylate shunt. Those species that have TAC and glyoxylate shunt may utilize organic compound not only as carbon sources, but also as electron donors. The species lacking some enzymes of TAC and glyoxylate shunt are usually autotrophs. Thus, they show a limited capacity for utilization of organic compounds, and use them only as an additional carbon source. It was shown that besides CO_2 assimilation via the ribulose-bisphosphate carboxylase of Calvin cycle, purple bacteria can assimilate CO_2 using other carboxylases that are more variated than in other microorganisms.

It was shown that CO_2 assimilation in the green sulfur bacterium *Chlorobium limicola* is carried out not via the Calvin cycle, but via a perfectly new pathway, a reductive TAC with a key enzyme: ATP-dependent citrate synthase. This enzyme had not been found in bacteria before. Later it was discovered that the reductive tricarboxylic acid cycle is functioning in non-phototrophic bacteria, too.

The results of the study on CO_2 assimilation pathways in filamentous green bacteria *Chloroflexus aurantiacus* and *Oscillochloris trichoides* are of special interest. It was shown that autotrophic CO_2 assimilation in *Chloroflexus aurantiacus* is carried out via a special cyclic mechanism called reductive dicarboxylic acid cycle. The product of assimilation in this cycle is glyoxylate, and the key enzyme is malate lyase. The functioning of this cycle was demonstrated for the first time. Despite *O. trichoides* referring to the group of green bacteria, it carries out CO_2 fixation via the Calvin cycle. This is also the first evidence of the functioning of the Calvin cycle in green phototrophic bacteria.

One major aspect of investigation was the study of the metabolism of phototrophic bacteria in the dark. The work of Kondratieva's group showed the capacity of some phototrophic bacteria for aerobic and anaerobic respiration, as well as for fermentation. Thus, the energy processes in these bacteria are more diverse than in other microorganisms. These data are important to determine the place of photosynthetic bacteria in the system of prokaryotes and their evolution.

Kondratieva believed that the discovery of the capacity of purple sulfur bacterium *Thiocapsa roseopersicina* and some others to switch from photosynthetic to autotrophic growth in the dark at the expense of oxidation of inorganic compounds (i.e., chemolithoautotrophy) had an outstanding evolutionary significance.

Kondratieva was one of the first in Russia who began the study of methylotrophic bacteria utilizing methanol and other monocarbon compounds. The efficacy of ATP synthesis during utilization of C_1 compounds was shown in a number of methylotrophic bacteria. It was shown that during the growth of methylotrophs on C_1 compounds their electron transfer chain acquires a branched character; the formation of two terminal oxydases one of which is cyanide- and formate-resistant is carried out. As a result, formate, an intermediary product of oxidation of another C_1 compound, does not inhibit the growth of these bacteria.

The study of nitrogen fixation and H_2 formation and of the intensification of these processes has a great scientific and practical importance. The mechanisms of regulation of nitrogen fixation and ammonium assimilation in a number of purple bacteria, green bacteria and cyanobacteria were carried out under the supervision of Kondratieva. As a result, significant procedures for production of H_2 and ammonia using phototrophic bacteria on the basis of bioconversion of solar energy were elaborated.

During her last few years, Kondratieva paid much attention to the study of physiology and metabolism of new species of extremely thermophilic anaerobic bacteria, active H_2 producers.

Kondratieva led a number of biotechnological programs aimed at the production of amino acids, enzymes, etc., by microorganisms. Her publications consisted of more than 250 scientific papers, 5 books (2 monographs and 3 textbooks), and 9 author's patents. Her book "Phototrophic bacteria" (1963) was translated into English and published in Israel (Jerusalem). She was awarded the M. V. Lomonosov prize (1970), the S. N. Vinogradsky prize (1980) and the USSR State Award (1988).

Kondratieva was a brilliant lecturer, a popularizer of science and teacher. More that 30 dissertations were successfully defended under her supervision. Many of her students became well-known scientists and professors. The term "School of E. Kondratieva" appeared in the Russian microbiological world. Kondratieva gained incontestable authority among her colleagues, and she was frequently addressed for consultations to edit manuscripts or to review dissertations. She carried out such a work almost continuously. She had an outstanding capacity for work and high professional exactingness that she combined with benevolence, optimism, a sense of humour and exceptional hearty charm.

Elena N. Kondratieva was a person of many talents. Not all of her colleagues knew that all her life she had been highly involved with equestrian sport. She became the master of sports of the USSR, the champion of the USSR in equestrian triathlon. The photographs of young Elena on horseback were reproduced on postal stamps and postcards. Later Kondratieva became a member of the Russian National Olympic Committee. She was regulary invited to judge international equestrian competitions, including Olympic Games (1984, 1992), World Championships (1986, 1990), Dressage World Cup Finals (1990, 1991, 1994), et cetera.

The life of Elena N. Kondratieva and her family was closely connected with the history of Russian scientific intelligentsia, and with its most tragic periods. Her father, Nikolai D. Kondratiev, was one of the greatest Russian mathematicians and economists. He was the author of the well-known theory of "economic waves". Nikolai D. Kondratiev became a victim of Stalin's repressions and was shot as a "public enemy" in 1938. Only after the end of the Soviet Union he was fully rehabilitated. Despite the danger involved, Elena N. Kondratieva preserved handwritings of her father, including his last works written in prison. During the last years, the interest in the economic theories of Nikolai D. Kondratiev increased, first in Europe and then in Russia. Thus, Elena N. Kondratieva devoted much of her efforts, energy and enthusiasm to the reconstitution of her father's good name. The publication of Nikolai D. Kondratiev's writings and the international scientific conference on his scientific heritage devoted to his 100th anniversary were organized with her active participation and assistance. Elena N. Kondratieva was a member of the International Kondratiev Foundation, the activity of which includes the support of talented scientists-economists, the publication of books, dissemination and propagation of scientific knowledge.

V ENAMA / I Encontro Nordestino de Microbiologia Ambiental

Fortaleza - Ceará (Brasil)
2–5 de diciembre de 1996

Del 2 al 5 de diciembre de 1996 tendrá lugar en Fortaleza-Ceará (Brasil), el V ENAMA / I Encontro Nordestino de Microbiologia Ambiental. El congreso está patrocinado por las Universidades Federales de Ceará y de Paraíba, y contará con la participación como conferenciantes invitados de:

—Erko Stackebrandt, Deutsche Sammlung von Microorganismen, Braunschweig, Alemania
—Takor Patel, Memorial University Newfoundland, Canadá
—Ricard Guerrero, Universidad de Barcelona, España
—Johana Dobereiner, URRJ-EMBRAPA, Brasil
—Jan Dirk van Elsas, Institute for Soil Fertility, Holanda

Se organizarán sesiones de pósters sobre microbiología acuática y microbiología terrestre, así como mesas redondas y simposios sobre diferentes aspectos de la microbiología ambiental. Entre ellos:

—Biotecnología Ambiental. Coord: Rosana F. Vazoller, UFSCarlos, Brasil
—Ecosistemas Acuáticos. Coord: Victoriano Campos, Univ. de Valparaíso, Chile
—I Encontro Regional de Microbiol. Ambiental. Coord.: José Siqueira Jr., UFPb, Brasil
—Ecosistemas Terrestres. Coord: Allen Norton Hagler, UFRJ, Brasil

Los días 2, 3 y 4 de diciembre se celebrarán reuniones de trabajo que estarán coordinadas por diferentes especialistas, tanto invitados como de la organización del congreso: Biotecnología Ambiental (P. Sánchez, Brasil), Ecosistemas Acuáticos (R. B. de Oliveira, Brasil), Ecosistemas Terrestres (M. A. Rezende, Brasil).

Puede solicitarse información a:

Profa. Dra. Regine H. S. dos Fernandes Vieira
Presidente do V ENAMA / I Encontro Nordestino de Microbiologia Ambiental
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OPINION

So you think there should be more microbiology in the media...?

Bernard Dixon*

Positively, the media have a voracious appetite, renewed on every revolution of the planet, for material to entice and entertain their customers. With exceedingly rare exceptions, writers and broadcasters do not invent their "stories". Science correspondents are wholly dependent upon you, the practitioners of science, to find things out and to explain why they should interest a wider audience. Come up with the right stuff, and you are guaranteed an eager and receptive response—not only from science journalists but also from the gatekeeping editors in newspapers, radio and television to whom they must sell their ideas before they ever appear in print or on the air.

So what is "the right stuff"? The sensational, the threatening and the weird may well qualify. A hot virus, rampant in Barnstaple, could fit all three criteria. But journalists are not preoccupied by these qualities alone. There is at least as great an appetite for the useful (a novel method of bioremediation), the counter-intuitive (food poisoning attributable to organic farming), the mind-stretching (bacteria from way back when) and the inherently fascinating (an organism that lives on nothing).

A "peg" helps enormously too. The onset of winter for a broadsheet feature article on influenza vaccines, a North Sea oil spill as a reason for the BBC's *Science Now* to set up a discussion of natural cleansing, an historic date (such as last year's SGM Golden Jubilee and anniversary of Pasteur's death)—all of these help to tweak the noses of journalists and editors, as well as arousing our interest as readers and listeners. Publication in a journal is itself a peg ("...in this week's *Nature*").

The wrong stuff might include ionic bonds, cell wall polymers, an improved method of Gram staining, 6-phosphogluconolactone and the phylogenetic relations of endophytic microfungi in Borneo. None of these things are automatically excluded from media attention. They do lack charisma, however, and would qualify for attention

* This short article appeared in the *Society for General Microbiology Quarterly*, February 1996, p. 3. It is published here with permission. Bernard Dixon is a microbiologist and science books writer. Editor of *New Scientist* from 1969 to1979; he also is European Editor of *Biotechnology* since 1989. His numerous books include the well known *Magnificient Microbes* (1976), *Beyond the Magic Bullet* (1978), *Health and the Human Body* (1986), *Genetics and the Understanding Life* (1993), and *Power Unseen. How Microbes Rule the World* (1994). The latest has been reviewed by Lynn Margulis, in Spanish, in this journal, vol. **10** (4), 449–450 (1994).

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only if their applications or implications lifted them out of the vast mass of daily science and gave them significance for the ordinary reader or listener.

So there is discouragement to set alongside the everlasting appetite of the media for material. You may find the feedback inhibition of glutamate dehydrogenase activity to be utterly absorbing. It may even be your life's work. But even some other scientists will find the same topic extremely boring. Certainly it will never make tomorrow's headlines unless a journalist can persuade a news editor of its practical relevance or intrinsic fascination.

Consider, for an illuminating comparison, what sort of observations from the Hubble space telescope might attract your own attention in the press. New heavenly bodies and hitherto unknown phenomena, probably yes. New insights into the origin of the cosmos, almost certainly. Yet many other Hubble discoveries, all immensely engaging to specialists and all duly published in the appropriate specialized journals, would bore you to tears. It is for this very reason that they are not publicized at all through newspapers or the air waves. Journalists are not obsessed with novelty, immediacy and practicality for their own peculiar reasons. They seek these characteristics on behalf of all of us, as readers, listeners and viewers.

A second discouragement is the intensity of the struggle to get anything into the media. Newspapers have such limited space, and radio and television such limited time, that they can accommodate only a tiny fraction of the enormous amount of material that flows into editorial offices day by day, minute by minute. Even in the best of all possible worlds, microbiologists would be bound to feel that there was insufficient microbiology in the media. Particle physicists too would continue to grumble about the neglect of particle physics, while hockey and lacrosse enthusiasts would go on complaining that their games received short shrift in the sports pages.

And even if there were to be a huge increase in media microbiology, this could spawn a further generation of complaints from specialists. Bioremediators and bioreactor buffs would imagine that there were too many stories about untreatable infections, giving a distorted picture of the true significance of micro-organisms in the world, while medical microbiologists would resent the fashionable emphasis on biotechnology. Taxonomists, as always, would grow even more angry about the lack of public recognition of taxonomy.

To end on an up-beat. Despite those restrictions on column inches and air minutes, and the intense competition by countless sectorial interests to secure some of them, despite also the inappropriateness of much science as media fodder, microbiology is an unusually prolific source of material for science journalists. This is why there is indeed a lot of microbial activity in the media already—whether it's BSE and CJD, the burgeoning problem of antibiotic resistance, the release of GMOs to control agricultural pests or the discovery of extremophiles in the Earth's most bizarre environments. Although none of this is labelled *Microbiology* (for the same reason that we do not come across newspaper sections headed *Economics* or *Industrial Relations*), it is undoubtedly microbial.

There could, of course, be even more column inches and air time devoted to micro-organisms (with some marginal loss in the coverage of particle physics, hockey and lacrosse). If so, what topics and angles would be suitably gripping? I'm sure that the SGM *Quarterly* editor, Dave Roberts, would be interested to hear any suggestions. I certainly would.

Revisión de libros

Vocabulario científico y técnico, 3ª ed.

Real Academia de Ciencias Exactas, Físicas y Naturales

Editorial Espasa Calpe, S. A., Madrid, 1996. 1628 pp. ISBN 84–239–9407–4

Hace pocas semanas se celebró el acto de presentación de esta tercera edición del *Vocabulario científico y técnico*. La sesión, que contó con la asistencia de SS. MM. los Reyes y diversos representantes del mundo académico, tuvo lugar en la sede de la Real Academia de Ciencias Exactas, Físicas y Naturales, en Madrid. No es vano el significado de actos como éste, y en realidad no tendría que serlo para ningún libro de este tipo, sino sencillo reconocimiento que se merece el resultado de años de trabajo, estudio, discusión y, sobre todo, dedicación y amor por la lengua y por la ciencia.

Han pasado trece años desde la primera edición del *Vocabulario*, seis desde la segunda. Los 35.000 términos incluidos en la segunda edición se han convertido en más de 50.000 en la presente y, dadas las posibilidades de la informática para acumular información y acceder fácilmente a los distintos campos terminológicos, todo hace pensar que su expansión, tanto cuantitativa como cualitativa, va a continuar.

Todos los idiomas con los que nos relacionamos en nuestro quehacer diario han sufrido el influjo del inglés en la expresión científica. No vamos a discutir aquí si es procedente o no; es un hecho evidente, que hay que aceptar. Pero, junto al conocimiento imprescindible para todo investigador de la lengua inglesa, existe la necesidad de disponer, conocer y manejar los términos equivalentes en el idioma de expresión habitual, con objeto de mantener una correcta comunicación oral y escrita. Y esto se hace tanto más necesario ahora que son muchos los libros que se traducen en todas las materias científicas, y en las cuales los avances que se están produciendo necesitan neologismos que los expresen.

Esa necesidad se da también en la propia lengua inglesa, a partir de la cual se traducen la mayor parte de textos, pero el inglés tiene una extraordinaria facilidad para producir términos nuevos —substantivando y adjetivizando verbos, convirtiendo nombres en verbos, añadiendo preposiciones y sufijos a palabras ya existentes, etcétera. Puede decirse que una de las características de la lengua inglesa es la economía, pero que ésta afecta al término, no al significado. Los avances en biología molecular, por ejemplo, comportan una serie de nuevos conceptos y términos en inglés, a los cuales hay que buscarles la traducción correcta en otros idiomas. Disponer de una obra como el *Vocabulario* facilita enormemente la tarea en español.

Con relación a sus antecesoras, esta tercera edición del *Vocabulario* incorpora un vocabulario español-inglés, inglés-español, sin duda una novedad práctica y adaptada al indiscutible carácter prioritario de la lengua en la que primero se nombran los avances científicos. Se han amplia-

do, entre otras, las áreas de biomedicina y de nuevos materiales y se ha conseguido una mayor sistematización de otras.

No estaría de más considerar, para futuras ediciones del *Vocabulario*, la incorporación de una lista de principales «falsos amigos», esas palabras inglesas —en el caso que tratamos, las hay en todos los idiomas— parecidas a las nuestras —por su origen latino—, pero con significado diverso. Es el caso de «injury, silicon, billion, evidence, egregious, actually, remove» y muchas más. Como tampoco sería difícil, a mi juicio, promover la creación de un servicio de consulta y asesoramiento rápidos en el que los usuarios pudieran exponer dudas que no han logrado resolver con los instrumentos (vocabularios y diccionarios) disponibles, a la vez que aportar sus propias sugerencias —que no «sugestiones», como dicen algunos textos traducidos.

Como se indica en el prólogo a esta edición, estamos ante una obra de carácter abierto, obligada a revisar, ampliar y actualizar de manera constante su contenido, a tenor de los avances científicos y tecnológicos. Y para ello, todos deberíamos aportar nuestros conocimientos y experiencias. El *Vocabulario* es una obra de todos y, por supuesto, todos debemos consultarlo y seguir sus indicaciones. Su contenido no es fruto de decisiones improvisadas, sino consecuencia de un detenido proceso de estudio. A pesar de la indisciplina proverbial de nuestra gente en lo que respecta al idioma, debemos seguir unas normas generales y cuidar el maltratado lenguaje científico, no sólo por afán de cultura, sino también por el respeto y cariño debidos al tesoro común que es la lengua.

El interés por el lenguaje científico y por la correcta expresión escrita en los ambientes científicos está adquiriendo cada día más importancia a escala internacional. Existe una gran cantidad de publicaciones dedicadas a este fin, editadas, las mejores, por sociedades científicas de prestigio. Los libros de estilo, que en nuestro entorno eran prácticamente inexistentes, o limitados a profesionales de las letras, hasta hace pocos años, van ocupando el lugar que les corresponde. Cualquier investigador sabe que entre las normas de publicación de las revistas internacionales figura la de presentar el texto correctamente escrito, y que no hacerlo puede ser causa de rechazo o devolución del trabajo para su mejora. Ésta debería ser una norma presente en el trabajo científico, independientemente del idioma en que se escriba, pero, en cualquier caso, sin menoscabar ni despreciar el propio.

Microbiología SEM publicó en su primer número de 1994 (vol. 10, pp. 203-204), la reseña de la segunda edición del *Vocabulario*. Se ponía ya entonces de manifiesto el interés de todo el equipo de la revista por las cuestiones lingüísticas y su efecto sobre la correcta expresión científica. Queremos ahora felicitar a todos los que han hecho posible esta nueva edición y animarles a que sigan trabajando. Y no hay mejor manera de hacerlo que dándole, para beneficio propio y de la comunidad científica, el uso para el que ha sido creado.

Finalmente, no puede acabar esta recensión sin mencionar a la persona a quien principalmente debemos el *Vocabulario*. Ángel Martín-Municio, actual presidente de la Real Academia de Ciencias Exactas, Físicas y Naturales, ha impulsado desde el primer día de su gestación esta extraordinaria tarea. Si bien es verdad que «los hombres pasan, pero las obras quedan», no es menos cierto que sin las personas responsables esas obras no habrían visto la luz. La aportación de Martín-Municio, de su equipo de trabajo en el *Vocabulario*, y de los demás académicos, es la contribución más importante, y más bella, que ningún colectivo ha hecho nunca a la terminología científica en lengua castellana.

Ricard Guerrero
Universidad de Barcelona

Guía de Terapéutica Antimicrobiana, 6ª ed.

Josep Mensa, Josep M. Gatell,
M. Teresa Jiménez de Anta, Guillem Prats,
Manuel Corachan, M. Carmen Escofet,
José A. Martínez, Laura Zamora
MASSON, S.A., Barcelona, 1996. 475 pp.
ISBN 84-458-0437-5.

«Love'em or hate'em, you have to take your hat off to the Americans when it comes to the production of encyclopedic textbooks and manuals...». Con estas palabras se refería recientemente D. Greenwood al *Manual of Clinical Microbiology* (ASM Press, 6th ed.). Algo similar podría decirse de los autores de este manual de bolsillo o "Guía Antimicrobiana'96", elaborada por un grupo de colaboradores —y asesores— de la Universidad de Barcelona, del Hospital Clínic i Provincial de Barcelona y de la Universidad Autónoma de Barcelona. En esta 6ª edición, queda patente el esfuerzo que desde 1991 viene realizando este grupo para incorporar las novedades que se producen en el campo de las enfermedades infecciosas. En un tema como éste la actualización se hace especialmente necesaria. Basta ojear las estadísticas que la Organización Mundial de la Salud publica periódicamente, para darse cuenta del problema epidemiológico que representa el resurgir de algunas enfermedades o la aparición de otras nuevas y, en consecuencia, la necesidad de nuevos agentes y medidas antiinfecciosas. Los autores han realizado una exhaustiva revisión bibliográfica, lo que garantiza una información científica rigurosa. Un mérito añadido de la *Guía* lo constituye el que se trata de un texto original en español, que aporta la experiencia obtenida en algunos de nuestros hospitales. Tal como se comenta en el prefacio, en nuestro país las infecciones presentan algunas características —etiológicas o de resistencias a antimicrobianos, por ejemplo— que no coinciden plenamente con la experiencia anglosajona. De ahí la relevancia de una publicación de este tipo.

Desde el punto de vista docente, la *Guía* es particularmente útil. De redacción clara y concisa, cada capítulo resume la información esencial en una panorámica global de cada uno de los antimicrobianos, microorganismos o estados patológicos que trata.

Todas las secciones han sido ampliadas en relación con las ediciones anteriores y se aprecia unidad y estructuración en el contenido de sus siete capítulos. Éstos cubren: las características de los antimicrobianos; el tratamiento de las infecciones producidas por microorganismos específicos; la etiología y tratamiento empírico de algunos síndromes de origen infeccioso; la prevención de las infecciones; algunos consejos generales para viajeros; la infección por VIH y SIDA; y, finalmente, un pequeño *Vademecum*. El acceso a cada tema es rápido gracias a un breve epígrafe en el margen del libro, que hace referencia a su contenido y orientación. Así, los capítulos pueden identificarse, respectivamente, como antibacterianos, microorganismos, síndromes, quimioprofilaxis, viajeros, VIH y SIDA, y *Vademecum*.

En el capítulo 1 se describen los antibacterianos, antivíricos, antifúngicos, antiparasitarios y antisépticos. Al principio de cada bloque o grupo se especifican la clasificación, los mecanismos de acción y de resistencia, los efectos secundarios o interacción con otros fármacos y su espectro antibacteriano específico. No faltan los datos referentes al peso molecular y fórmula empírica de cada uno. Además de los fármacos comercializados en España, se incluyen algunos que todavía no lo están o no han sido aprobados por la «Food and Drug Administration». En esta edición se han suprimido los que se han retirado del mercado en fechas recientes, y se incluyen los comercializados en 1995, entre los que se encuentran las

cefalosporinas de cuarta generación Cefpiroma y Cefepima, o el inhibidor de la proteinasa del VIH Saquinavir.

El capítulo 2 presenta todos los microorganismos conocidos que producen enfermedades en los humanos y se describe el tratamiento de elección y las posibles alternativas terapéuticas. Estos agentes se ordenan alfabéticamente, con indicación de las características taxonómicas y de la sintomatología que producen. Se han incluido hasta 20 nuevos microoganismos patógenos, entre virus, bacterias y hongos, y se ha adaptado la taxonomía de todos ellos a la nomenclatura más reciente. En el capítulo 3 se clasifican los síndromes alfabéticamente, dando especial importancia a la etiología y al tratamiento empírico de las infecciones. Se han incorporado nuevos síndromes (artritis infecciosa, faringitis, neumonía en el niño, neumonía en el anciano, neumonía necrotizante y absceso de pulmón, sinusistis, y úlcera cutánea). Mención especial merecen las tablas y comentarios que los acompañan y que se distribuyen por todo el libro.

Los capítulos 4, 5, y 6 se dedican, respectivamente, a la quimioprofilaxis y prevención de las infecciones, con un excelente apartado de vacunas; a las recomendaciones que deben seguir los viajeros, especialmente los que van a países tropicales; y a una monografía sobre el SIDA.

Finaliza la *Guía* con un capítulo que contiene los nombres comerciales y las formas de presentación de todos los principios activos que figuran en la misma y cuya información ha sido obtenida a partir del Vademecum Internacional *MEDICOM* (versión informatizada de 1995). En suma, este libro reúne una serie de características que lo configuran como un manual de consulta obligada para profesionales de las ciencias de la salud, docentes y estudiantes de microbiología.

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Manual de terapéutica médica

El manual Washington, 9ª ed.

Gregory A. Ewald, Clark R. Mckenzie

MASSON - Little, Brown, S. A., Barcelona, 1996. 735 pp. ISBN 84-8227-013-3.

De obligada referencia, el *Manual Washington* es uno de los más utilizados en terapéutica médica. Y qué mejor tarjeta de presentación que sus 28 ediciones (en versión original y 9 en español), alcanzadas, desde su introducción en 1944, por el Department of Internal Medicine, Washington University School of Medicine (St. Louis, Missouri, EE. UU.). Desde entonces, cada edición se ha revisado cada dos años, a fin de incorporar los avances que se han ido produciendo, especialmente en todo lo que se refiere al diagnóstico y al tratamiento de las enfermedades.

En la práctica médica, particularmente, la eficacia consiste en saber qué hay que hacer y cuándo debe hacerse. De ahí la importancia que tiene disponer de un manual actualizado y de fácil consulta que integre, de manera racional, las continuas modificaciones que se introducen en la práctica clínica. En este sentido, en el *Manual Washington* se exponen de forma clara y concisa la fisiopatología y el diagnóstico diferencial de las enfermedades más importantes; se ofrecen los criterios a seguir para su valoración y tratamiento, según las últimas normas aprobadas en materia sanitaria; y se añaden diversas recomendaciones preventivas efectuadas por diversas organizaciones acreditadas en la materia. Como ya es tradicional en esta obra, las enfermedades y sus tratamientos siguen una organización lógica por órganos y sistemas. Las tablas y algoritmos que acompañan cada capítulo, y las que constituyen los apéndices, facilitan la comprensión y la consulta rápida de este *Manual* de carácter eminentemente práctico.

La publicación de esta vigésimooctava edición surge como resultado de la experiencia diaria y del esfuerzo combinado de los especialistas del Barnes Hospital, del Children's Hospital y del Jewish Hospital, los cuales conforman el macrocomplejo hospitalario del campus de Washington University. Poco más puede añadirse acerca de este grupo de profesionales, sino que el cargo que ocupan supone un merecido reconocimiento a su labor.

Esta nueva edición consta de 25 capítulos —en lugar de los 26 de la edición precedente, dada la redistribución de la información contenida en ellos—, pero todos mantienen la dinámica de presentación que caracteriza la obra. Esto es, en cada capítulo se cubren desde los conceptos básicos y consideraciones generales de la valoración diagnóstica y tratamiento de las enfermedades, hasta aquellas otras específicas de la terapéutica farmacológica o medidas de prevención a aplicar en cada caso.

Se ha incluido, además, un capítulo nuevo sobre el paciente inmunodeprimido. En él se presta especial atención al tratamiento de la infección por el virus de la inmunodeficiencia adquirida y de las infecciones oportunistas asociadas a VIH, sean de etiología vírica o bacteriana. Si bien estos aspectos ya habían sido abordados en la edición anterior, ocupaban entonces menor extensión, cuando formaban parte del capítulo correspondiente al tratamiento de las enfermedades infecciosas en general.

Otra novedad la aporta el capítulo de medicina intensiva y urgencias médicas donde, bajo un mismo epígrafe, se resumen los procedimientos y conceptos anteriormente dispersos por diversos capítulos de otras ediciones y que, básicamente, habían sido agrupados en dos capítulos en la edición anterior (de 1993).

Desde el punto de vista microbiológico, merecen destacarse especialmente los capítulos referentes a los antimicrobianos, al tratamiento de las enfermedades infecciosas y, cómo no, del SIDA.

En ellos se exponen los principios de la terapia antimicrobiana, combinaciones e interacciones medicamentosas, mecanismos de acción y de resistencia de cada grupo de antimicrobianos, sus variables farmacocinéticas, dosificación, posología e indicaciones terapéuticas.

En cuanto a las infecciones, también se clasifican por sistemas. En cada caso se aborda el estudio de los síntomas característicos, describiendo las posibles causas independientemente de su carácter infeccioso, su diagnóstico y tratamiento. Esto permite una visión global e integrada del proceso de la enfermedad y de los agentes etiológicos implicados en el mismo, algo que hace tiempo se venía reclamando en el diagnóstico de cualquier tipo de enfermedad. Precisamente por este motivo, la consulta del *Manual* por parte de docentes y estudiantes de carreras sanitarias resulta muy útil y recomendable, lo que añade un interés particular a este libro, ya bien conocido en otros ámbitos.

Completan el *Manual* siete apéndices que resumen las fórmulas y valores de laboratorio más frecuentemente utilizados por el médico y en toda práctica clínica que persiga una actuación eficaz; las interacciones medicamentosas más frecuentes; los métodos de preparación de mezclas intravenosas y sus normas de administración parenteral; la adecuación de las dosis, efectos secundarios y características específicas; así como una serie de normas para el control y aislamiento de los enfermos infecciosos o en cuidados intensivos.

No cabe duda de que esta nueva edición del *Manual* será muy bien recibida por quienes ya conocen y acuden a las anteriores, y por los nuevos usuarios que van a descubrir en ella un instrumento de valor excepcional.

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Introductory Microbiology

J. Heritage, E. G. V. Evans,
R. A. Killington

Cambridge University Press, Cambridge, Great Britain, 1996. 234 pp. ISBN 0-521-44977-4.

El título de este libro, *Introductory Microbiology,* puede confundir al lector, ya que no se trata de una introducción a la microbiología en general, sino a puntos concretos de esa ciencia. El libro está configurado en tres partes, que corresponden a los tres grandes capítulos que lo componen. Cada capítulo se encuentra subdividido en diferentes secciones, coincidiendo con distintos aspectos propios de cada tema que se trata.

En el primer capítulo, *Microbial structure and mode of life*, los autores presentan una detallada comparación de las diferencias existentes entre las células eucariotas y las procariotas. Además, describen tres grandes grupos de microorganismos diferentes: hongos, bacterias y virus. Se centran principalmente en las particularidades de su crecimiento y metabolismo, al igual que en su morfología. Es de destacar que el libro trata con parecido detalle los hongos y los virus que las bacterias, cosa que no es frecuente en otros libros de microbiología.

El segundo capítulo, *Handling microbes*, explica la manipulación que se realiza a un cultivo para su control y estudio en el laboratorio. Describe la utilización de los diferentes métodos de desinfección, centrándose en la esterilización por agentes físicos y químicos. Explica también los distintos tipos de tinciones. En otra parte del capítulo detalla cómo controlar los factores ambientales que pueden afectar el cultivo —pH, temperatura, factores osmóticos, etc. También se describen los diversos usos de las técnicas aplicadas a la ciencia microbiológica, como los tipos de microscopía o las diferentes formas de enumeración o recuento de microorganismos, para conseguir con todo ello un control del crecimiento poblacional.

La última parte, *Isolation, classification and identification of microbes*, es una continuación del capítulo anterior. Explica los diferentes métodos utilizados para el aislamiento y clasificación de los microorganismos, empleando las clasificaciones propias de cada grupo, al igual que los ensayos y cultivos de laboratorio.

Al final de los tres capítulos, los autores aportan información de libros apropiados para completar y ampliar la lectura de *Introductory Microbiology*.

Además, el libro contiene un glosario bastante extenso, que incorpora, como ayuda adicional, las palabras técnicas utilizadas. El nivel del glosario (definiciones y terminología utilizadas) es superior al nivel general (bastante básico) del libro.

En resumen, a diferencia de otros libros de introducción a la microbiología, éste abarca de una forma más detallada aquellos puntos que los autores han considerado de mayor importancia o no suficientemente tratados en otros textos generales.

Es éste un libro de nivel básico, que incluye un gran número de figuras. Estas figuras son, a veces, excesivamente simples, lo cual puede inducir incluso a errores conceptuales y restar eficacia a lo que cabe esperar de una figura: que sea explicativa por sí misma. Parece dirigido a profesores de enseñanza media, o de formación profesional, interesados en poseer un libro de rápido asesoramiento, o para aquéllos que se quieren iniciar en el conocimiento del inmenso mundo de la microbiología. Este libro se encuentra dentro de la colección *Studies in Biology*; es el tercer libro de esta serie, que esperemos continúe creciendo en el número de materias tratadas, y mejorando cada vez más su calidad.

Antoni Navarrete
Universidad de Barcelona

Internet y medicina

Luis G. Pareras

MASSON, S.A., Barcelona, 1996. 702 pp.
ISBN 84-458-0450-2.

Esta obra se presenta como "la guía de referencia para el aprovechamiento máximo de los recursos médicos de Internet". Creemos que esta presentación es modesta, al tiempo que, aunque parezca contradictorio, excesiva. Internet es una red mundial de comunicación en expansión continua y resulta imposible que una obra impresa pueda estar completamente al día de su desarrollo. Por tanto, hablar de "aprovechamiento máximo" podría ser excesivo, cuando es probable que existan recursos actuales dentro de la red que quizá no eran ni un proyecto cuando se escribió el libro. Lo cual no significa que el libro carezca de utilidad. Por el contrario, la obra es bastante más de lo que se infiere a partir de la frase de presentación.

Nos hallamos ante un tratado muy completo sobre Internet, y una gran parte del libro puede ser de gran utilidad para profesionales de cualquier campo, no sólo médicos, o para aquellas personas cuya curiosidad les impela a escudriñar los entresijos de la red sin ningún plan determinado, como quien penetra en un inmenso centro de información que comprendiese bibliotecas, hemerotecas, videotecas, ludotecas, etc. y que dispusiese también de zonas de ocio, tertulia y discusión.

El autor del libro es un neurocirujano cuyo conocimiento de los mecanismos lógicos del pensamiento deben de haberle llevado a escrutar, como continuación lógica, los mecanismos de funcionamiento de los ordenadores y de la transmisión de información. Ganador de un premio de innovación tecnológica de la CIRIT (Comissió Interdepartamental de Recerca i Innovació Tecnològica, de la Generalitat de Catalunya) con un proyecto de simulación informática, ha publicado numerosos trabajos relacionados con la utilización de Internet en medicina.

Las 25 páginas iniciales de índices proporcionan una idea clara de lo que el lector podrá encontrar en las cinco partes en que se divide el libro, cuyo contenido es el siguiente:

(i) *La revolución está aquí.* Comprende un capítulo de introducción a Internet: cómo funciona, cómo nació, en qué se basa, cómo se puede acceder, etc.; y un capítulo que describe los usos potenciales que un profesional de la medicina puede hallar en la red, así como lo que cabe esperar de ella en el futuro.

(ii) *La comunicación en Internet.* Dedica cuatro capítulos a describir las diferentes maneras de comunicación que pueden realizarse a través de Internet: el correo electrónico —con sus ventajas e inconvenientes— en relación a otros sistemas de comunicación personal; las listas de distribución (listserv), que permiten el debate en foros virtuales, cuyos participantes reciben cualquier mensaje que se envíe a la lista; los foros propiamente dichos (newsgroups), que, a diferencia de los anteriores, se encuentran en algún lugar de Internet y pueden ser consultados libremente por cualquier persona que acceda a ellos desde cualquier lugar del mundo.

(iii) *Las herramientas del futuro*: El uso de la información a través de Internet se lleva a cabo mediante paquetes de software que se conocen como "herramientas", y que se describen aquí. FTP (File Transfer Protocol) permite al usuario de Internet importar a su ordenador archivos de otro ordenador que puede estar en cualquier rincón del planeta; TELNET, una de las primeras herramientas de Internet para acceder a ordenadores remotos, que aún conserva un gran potencial, a pesar de que no permita importar ficheros; ARCHIE, localizador de archivos en la red, que pueden ser luego importados mediante FTP; Gopher, que facilita el acceso a información de los ordenadores integrados en la red sin necesidad

de saber dónde se encuentra almacenada dicha información; Veronica, que permite la búsqueda en los menús gopher mediante la introducción de palabras clave; WWW (World Wide Web), la herramienta más popular de Internet, que hace posible la transmisión de documentos multimedia, es decir, con texto, gráficos, imágenes, secuencias de vídeo y sonido, así como el paso de un documento a otro que puede encontrarse en un ordenador situado a miles de kilómetros de aquél en que estaba el anterior, con un simple clic sobre una palabra o un dibujo en pantalla, e incluso el acceso a los diferentes recursos de la red; HTML (Hypertext Markup Language), el nuevo lenguaje de programación utilizado por WWW, que condensa toda la información en archivos de texto que pueden ser leídos por diferentes "intérpretes" (Netscape, Mosai, IBM Web Explorer, etc.). Termina esta parte con un capítulo que analiza las aplicaciones WWW de más utilidad en el campo de la medicina.

(iv) *Buscando información médica en Internet*. Los capítulos que integran esta parte describen cómo aprovechar los recursos de Internet mediante (a) la utilización de los servidores de información organizados o WAIS (Wide Area Information Servers), que buscan información introduciendo palabras clave; (b) el acceso a publicaciones electrónicas, que proporcionan información actualizada a un coste mínimo, y que, según algunos especialistas, llegarán a sustituir a las publicaciones en soporte papel; (c) la búsqueda de información en WWW utilizando directorios de recursos o herramientas de búsqueda, que en algunos casos, como MedWeb, Hospital Web, PharmWeb, etc., están dedicados exclusivamente a las ciencias de la salud; (d) localización de otros profesionales en la red con diferentes herramientas, como directorios, "páginas blancas" en WWW, etc.; (e) investigación bibliográfica con herramientas como MEDLARS-MEDLINE o Grateful MED, que utilizan palabras clave.

(v) *Recursos médicos en Internet*. La obra termina con un directorio de los recursos médicos más importantes clasificados por áreas temáticas, y con una recopilación final de las mejores direcciones y recursos de la red, y la descripción de algunos de dichos recursos. Las áreas contempladas son: neurociencias, medicina interna, cirugía, oncología, sida, imagen, genética, cardiología, pediatría, anatomía patológica, traumatología, ginecología, anestesiología y UVI, oftalmología, ORL. Si bien algunas áreas temáticas de ciencia básica, como la genética, están muy bien representadas, se echa en falta algunas de tanta trascendencia en medicina como pueda ser la microbiología. No obstante, el libro proporciona los conocimientos necesarios para encontrar en Internet información sobre muy diversos temas, sea médico o no. Es una lástima que no haya un índice final alfabético que facilite la localización.

Las numerosas capturas de pantalla que ilustran el libro facilitan la comprensión de las explicaciones, y los centenares de direcciones permiten el acceso directo a puntos de la red de gran utilidad. Faltaría, sin embargo, como acabamos de señalar, un índice general alfabético, que permitiese acceder a la información que buscamos casi tan rápidamente como las herramientas de búsqueda de Internet nos llevan a puntos remotos de la red global. El "peso informativo" que pudiese tener dicho índice compensaría el "peso físico" adicional que ocasionaría, probablemente imperceptible en un volumen en rústica que alcanza ya los 1,7 kilogramos.

Mercè Piqueras
Redacción Microbiología SEM

Instructions to authors

Microbiología SEM (the official journal of the Spanish Society for Microbiology, SEM) publishes original research articles, research notes and reviews covering all aspects of microbiology. All submissions should be written in English (preferably) or Spanish. The decision to accept manuscripts is made by the Editorial Board. Submission of an article to this journal is understood to imply that it has not previously been published and that it is not being considered for publication elsewhere. Consent will be given for reproduction of papers published in this journal if the source is credited.

ORGANIZATION AND FORMAT OF THE MANUSCRIPTS. Type every portion of the manuscript double-space with wide margin at the left on UNE A-4 format sheets. Only one side of the sheet should be used and the pages should be numbered sequentially. Articles must be restricted to a maximum of 16 printed pages, including figures and tables (this corresponds to approximately 25 typewritten pages).

The front page should include title, name(s) of the author(s), institution affiliation(s) and complete address(es). Three to five "key words" should also be included. Articles should be divided into: Abstracts in English and in Spanish (not exceeding 250 words each), Introduction, Materials and methods, Results, Discussion, Acknowledgments, and References. Results and Discussion can be combined.

Abbreviations and symbols should follow the recommendations of the IUPAC-IUB Commission. The *Système International d'Unités* (SI) is to be used throughout.

Cite each listed reference by number in the text. References should be numbered and arranged in alphabetical order as indicated in the following examples:

Miller, J. H. (1972). Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Seeberg, E., Nissez-Meyer, J., Strike, P. (1976). *den*V gene of bacteriophage T4 determines a DNA glycosilate specific for pyrimidine dimers in DNA. J. Virol. **35**, 790–797.

Tomasz, A. (1984). Building and breaking in the cell wall of bacteria. The role for autolysins. *In* Nombela, C. (ed.), Microbial Cell Wall Synthesis and Autolysis, pp. 3–12. Elsevier Science Pub., Amsterdam.

References to thesis, manuscripts not yet accepted for publication or meetings should be indicated in the text as follows: (García, P. et al. 1985, in preparation), (Smith, T. 1985. Ph. D. thesis, University of Massachusetts, Amherst) or (Suárez, A., González, F. 1975. V Congr. Nac. Microbiol., p. 1845).

Only those photographs which are strictly necessary for the understanding of the article should be submitted. Photoprints must be of sufficient quality to ensure good reproduction. They should be numbered on the back and identified with the first author's name written in pencil. Legends for line-drawings and photoprints must be typed double-space on a separate sheet. The size of the photographs should not exceed the printing area (13 × 20 cm). All elements in the drawing should be prepared to withstand reductions. Drawings and line figures should be drawn in black ink on tracing paper and should be prepared as indicated for the photographs. Colored illustrations are not accepted.

Tables should be compiled on separate sheets with a descriptive title and numbered independently of the figures using Arabic numerals. Please indicate with a soft pencil the approximate location of tables and figures in the left margin of the pages of the manuscript.

NOTES. Notes should be restricted to 6 typewritten pages and are intended to present experimental observations and descriptions of techniques or methodological changes of interest. They should be written according to the instructions given for articles, but without the heading divisions, and their abstracts should not exceed 50 words. Figures and tables should be restricted to a maximum of 2 figures and 1 table or vice versa.

MINIREVIEWS. Minireview articles should deal with microbiological subjects of broad interest. They will be written in English. Specialists will be called upon to write them. However, if some authors are interested in publishing minireviews, these can be submitted for publication. They should be between 12 and 20 double-spaced typewritten pages, including the space needed for figures and tables.

PROOFS CORRECTION. On acceptance of the article, galley proofs will be sent to the corresponding author to check for typesetting accuracy. The corrected proofs should be duly returned when indicated. If delayed beyond this time the proofs will be published as they have been sent. Broader changes implying recomposition of the text will be at the author's expense. Twenty five offprints of each article are supplied free of charge. Additional reprints will be billed at cost price if requested upon returning the corrected galley proofs.

Articles must be submitted, original and two copies on paper, to the following address: *Microbiología SEM*. Apartado 16009, 08080 Barcelona, Spain, or to one of the members of the Editorial Board according to the discipline represented. If the article is accepted for publication, a version in diskette will be requested.

Normas para los autores

Microbiología SEM (la revista científica de la Sociedad Española de Microbiología, SEM) acepta artículos y notas de investigación originales dentro del campo de la microbiología y, ocasionalmente, artículos de revisión. Textos en inglés (preferentemente) o español. La aceptación corresponde al Consejo Editorial. Sólo se admitirán trabajos inéditos que no estén pendientes de publicación en cualquier otra revista. Los originales publicados en *Microbiología SEM* podrán ser reproducidos siempre que se indique su origen.

PRESENTACIÓN DE LOS ORIGINALES. Los artículos estarán escritos a máquina, a doble espacio, en hojas UNE A-4 por una sola cara, numeradas correlativamente y con un amplio margen en la parte izquierda. No deberán exceder de 16 páginas impresas, incluyendo tablas y figuras (lo que corresponde aproximadamente a 25 hojas mecanografiadas). Los artículos incluirán una primera página en la que se indicará por este orden: Título del artículo, nombre y apellido del autor o autores, centro en el que se ha realizado el trabajo y dirección completa del mismo, así como de tres a cinco "palabras clave". En los artículos en español se deberá incluir una versión inglesa del título. Los artículos constarán de: Resúmenes en inglés y en español (de no más de 250 palabras cada uno), Introducción, Materiales y métodos, Resultados, Discusión, Agradecimientos y Bibliografía. Las secciones de Resultados y Discusión se podrán juntar en una sola.

Las abreviaturas, símbolos y siglas deberán seguir las recomendaciones de la Comisión IUPAC-IUB sobre nomenclatura bioquímica. Deberá emplearse siempre el Sistema Internacional de Unidades (SI).

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Miller, J. H. (1972). Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Seeberg, E., Nissez-Meyer, J., Strike, P. (1976). *den*V gene of bacteriophage T4 determines a DNA glycosilate specific for pyrimidine dimers in DNA. J. Virol. **35**, 790–797.

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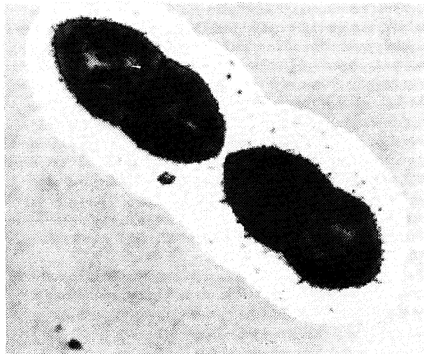

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