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Microbiología

SPECIAL ISSUE

Frontiers in antimicrobial resistance

Nuestro mundo es el suyo.

\ TERAPEUTICA DE
AYOR DIFUSION MUNDIAL

NOVEDAD 1996

Manual de
Terapéutica médica
ª edición

artment of Medicine **Washington University.** School of Medicine, St. Louis, Missouri

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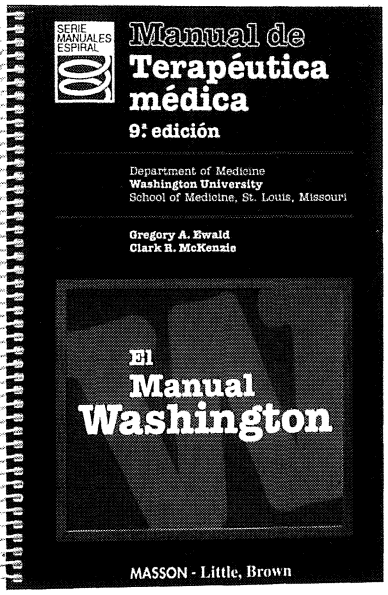

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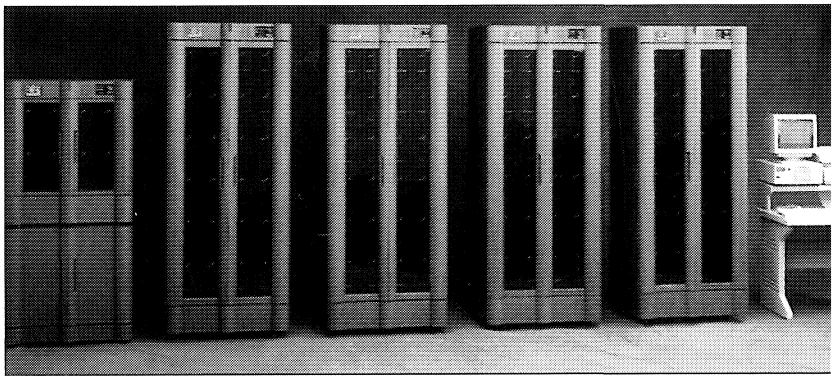

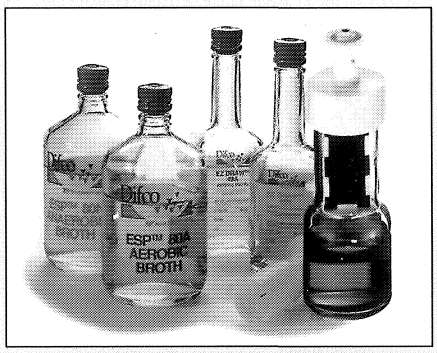

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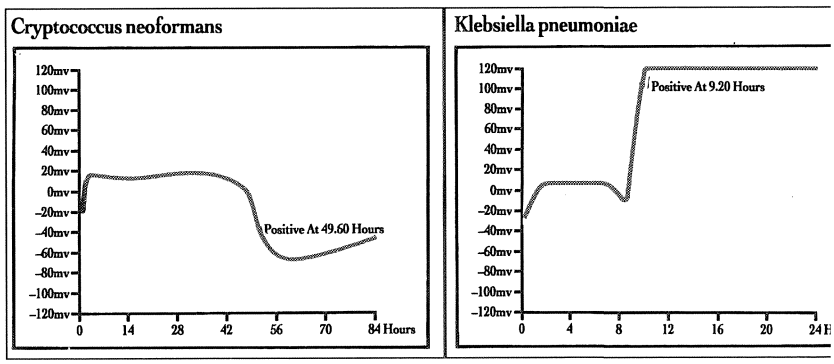

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(continued on next page)

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MICROBIOLOGÍA SEM **13** (1997), 267–270

Editorial*

Trayectoria y situación actual de la investigación biomédica en Argentina

La investigación biomédica en Argentina tiene una larga tradición, que se vio fortalecida con la creación en 1958 del Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). En el nacimiento de este organismo tuvo un papel esencial un hombre de extraordinarios conocimientos, que supo aplicarlos tanto en la investigación que él mismo desarrolló como en la planificación y organización de la ciencia. Fue Bernardo A. Houssay, fisiólogo que, sin ninguna duda, impulsó el auge de la biología no sólo en Argentina sino en los otros países de América Latina.

Al abrigo de la nueva organización se crea la carrera de investigador, que va a permitir a los científicos la dedicación exclusiva a la investigación, compartiendo esa actividad solamente con la enseñanza universitaria. La nueva situación supone una mejora con respecto a la anterior, y es mucho más adecuada para el desarrollo del trabajo científico y para el establecimiento de proyectos de colaboración. CONICET no incluye sólo la investigación biomédica, sino también las otras ramas de la ciencia y de la técnica. Se establece un sistema de becas para los jóvenes licenciados universitarios, que les permita iniciarse en las tareas de investigación y, una vez demostrados méritos y capacidad, incorporarse como investigadores. En líneas generales, se sigue el mismo sistema de la mayoría de países avanzados. Por esa misma época también se establece la figura del docente–investigador, con dedicación exclusiva al ámbito universitario.

En el país existen otras instituciones, como la Comisión Nacional de Energía Atómica (CNEA) y el Instituto Nacional de Tecnología Agropecuaria (INTA), dedicadas, fundamentalmente, al desarrollo tecnológico. Pero, sin ninguna duda, la creación del CONICET y la posibilidad de ejercer una dedicación exclusiva en las universidades, conforman las condiciones que permiten al licenciado universitario pensar en la investigación científica como una opción profesional. En ese período se inicia una actividad académica floreciente y es precisamente en el área biomédica donde se alcanza el mayor grado de desarrollo.

**Microbiología SEM* prosigue en este número la serie de editoriales dedicados al análisis de la situación de la ciencia en América Latina. (Véanse los números 12[1–4] y 13[1 y 2].) En esta ocasión, el estudio realizado por la Prof. Stella M. González Cappa se centra en la investigación biomédica en Argentina.

Es en esta rama de la ciencia en la que surgen dos premios Nobel. Bernardo Houssay obtiene en 1947 el de Medicina o Fisiología por sus trabajos sobre la acción de la hormona hipofisiaria sobre el metabolismo del azúcar. Lo comparte con los bioquímicos norteamericanos, de origen checo, Carl Ferdinand Cori y Gerty T. Cori, que habían centrado sus trabajos en el metabolismo catalítico del glicógeno. Luis F. Leloir es premiado con el Nobel de Química en 1970 por sus estudios sobre la biosíntesis de carbohidratos y el descubrimiento de los nucleótidos de azúcar. La labor experimental, los descubrimientos científicos, en definitiva, por los que ambos investigadores, Houssay y Leloir, alcanzan el galardón, fueron realizados en Argentina. Ambos, también, dieronorigen a escuelas de gran peso en nuestra vida académica e investigadora, con especial énfasis en la formación científica de los jóvenes, en la colaboración y establecimiento de intercambios, y en la proyección exterior. Los trabajos y la propia figura de Bernardo Houssay ejercieron una notable influencia sobre el desarrollo de la fisiología en todo el mundo y mantuvo una especial relación de trabajo y de amistad con científicos españoles que, a su vez, alcanzarían gran prestigio. El mismo Severo Ochoa, en sus memorias, evoca la impresión que le produjo cuando, siendo estudiante en la Facultad de Medicina, asistió a una de las conferencias que Houssay pronunció durante su visita a Madrid, en el año 1924.

Otros científicos argentinos, sin alcanzar el premio Nobel, logran notoriedad internacional; baste mencionar, entre otros, a Eduardo De Robertis, quien a su muerte, en 1989, se encontraba entre los mil autores contemporáneos más citados en el contexto total de las disciplinas científicas. César Milstein, otro premio Nobel de nacionalidad argentina, alcanza ese galardón en 1984 en Fisiología o Medicina por sus trabajos sobre la obtención de los anticuerpos monoclonales. Nacido y formado en Argentina, obtuvo su primer doctorado en la Universidad de Buenos Aires en 1957. Los trabajos que le aportaron notoriedad y prestigio los realizó en Inglaterra. Esto no es casual, ya que en la década de 1970 comienza un período de decadencia, marcado por vaivenes, interrupciones de la vida democrática, crisis económicas y ajustes indiscriminados e irracionales, ausencia de políticas científicas y diferente grado de interés genuino de los gobiernos de turno hacia la ciencia y la tecnología.

En esa década, se produce una gran emigración de ciudadanos argentinos de muy diversas profesiones que, por razones de distinta índole, buscan otros horizontes. Muchos de ellos ocupan hoy posiciones destacadas fuera de Argentina. El país pierde de este modo una parte importante de su capital humano y esto, en determinados campos del conocimiento, genera brechas casi imposibles de reparar. Algunos otros, como Leloir y De Robertis, continuaron trabajando en el país o regresaron a él a pesar de la inestabilidad política e institucional y de la falta de apoyo económico sostenido que, obviamente, dificultaba la realización de proyectos. Sin embargo, esta persistencia obstinada de quienes permanecieron y continuaron trabajando evitó la desaparición total de lugares y grupos de trabajo establecidos.

El regreso en 1983 a la vida democrática mejora la situación, aunque el apoyo a la ciencia y a la tecnología es insuficiente y a veces sólo declamatorio y aparente, como se refleja en la distribución del presupuesto nacional. Argentina dedica para la finalidad Ciencia y Técnica sólo el 0,31% del PBI, mientras que Chile utiliza el 0,78% para el mismo propósito, Canadá el 1,52% y Estados Unidos el 2,40% (datos de 1995). Además, de acuerdo a los datos publicados en *The World Competitiveness Report,* también en 1995, sobre el gasto de crecimiento real en Ciencia y Técnica entre 1989 y 1993, nuestro país figura con un decrecimiento del 76%. Esto contrasta con el ascenso que para esta

finalidad se registra en países desarrollados y aun en algunos otros de América Latina, como por ejemplo Brasil. Bernardo Houssay decía que "el grado de desarrollo de la investigación es un índice seguro de la jerarquía y la posición de un país entre las naciones del mundo moderno. Se puede medir la ilustración y la clarividencia de los gobernantes por la importancia que acuerdan a la investigación científica fundamental, por lo que realmente hacen para ayudarla y por el apoyo y respeto que dispensan a los auténticos hombres de ciencia". Son palabras que tendrían que hacer pensar a los responsables en la necesidad de potenciar ese desarrollo científico, cuyo beneficio alcanza a toda la sociedad.

En el caso específico de CONICET, entre 1994 y 1996 el presupuesto se redujo en un 20%. En este último año esta institución utilizaba el 81% de su presupuesto en salarios y sólo el 2% en subsidios, cuando en 1984 empleaba el 48% en salarios y el 30% en subsidios. Por otra parte, la distribución del presupuesto no siempre ha seguido los criterios más razonables. Como ejemplo podemos mencionar la atribución de cantidades muy elevadas, en diferentes períodos, para realizar construcciones, la mayoría de las veces innecesarias o, por lo menos, no prioritarias. También en este aspecto es oportuno recordar la palabras de Bernardo Houssay cuando decía que "algunos creen que es muy fácil investigar, que basta erigir un bonito edificio, comprar aparatos costosos, tener mucho personal improvisado... (...) no debe olvidarse que la investigación depende ante todo de la calidad de los hombres que la hagan y no de los edificios". Finalmente, creemos que tampoco hay que olvidar el sacrificio personal de los hombres y mujeres dedicados a la investigación, supliendo con su esfuerzo y dedicación, en muchos casos, la escasez de medios con que realizan su trabajo.

Se estima que en Argentina existen actualmente algo más de 18.000 científicos, de los cuales cerca de 5000 son becarios. Alrededor de 1900 becarios y 3500 investigadores dependen del CONICET, con dedicación exclusiva, y de ellos, aproximadamente un 50% desarrollan su investigación en universidades. Un porcentaje importante del resto trabaja en institutos propios de CONICET, como el de Biología y Medicina Experimental, el de Investigaciones Bioquímicas, el Instituto Mercedes y Martín Ferreyra, el de Química y Fisicoquímica Biológicas, y otros. Algunos de estos institutos funcionan en estrecha relación con las universidades.

En el ámbito universitario, el número de investigadores y becarios supera los 15.000, pero de ellos sólo alrededor de 5500 tienen una dedicación a la investigación y docencia equivalente a jornada completa. El resto de los científicos desarrollan su tarea en instituciones como la CNEA y el INTA, ya mencionados, o bien en el Instituto Nacional de Tecnología Industrial, en los Institutos Nacionales de Salud, o en la Academia de Medicina.

A pesar de los problemas que se han expuesto, existen todavía en Argentina grupos competitivos a escala internacional, lo que nos hace mantener esperanzas de poder revertir la situación actual. Muchos de nuestros científicos cuentan con el apoyo de agencias extranjeras o desarrollan proyectos en colaboración con investigadores europeos, de Estados Unidos, Canadá y de otros países de América Latina, especialmente Brasil y Chile. En el ámbito nacional, algunas universidades vienen desarrollando programas de ciencia y tecnología que permiten, mediante becas, la formación de recursos humanos y apoyan con pequeños subsidios proyectos de investigación. También existen algunas fundaciones sin fines de lucro que contribuyen al mantenimiento de la vida académica y científica. Sin embargo, todos estos recursos resultan insuficientes si queremos ser realmente una nación competitiva y es éste el motivo por el que la comunidad científica reclama un justo aumento

de presupuesto destinado a la finalidad Ciencia y Técnica, al mismo tiempo que mayor difusión y transparencia en su ejecución. Se considera necesario, ya que sus efectos redundarían en nuevas y mejores oportunidades, especialmente para los jóvenes, pues si ellos no tienen cabida para acceder y trabajar de forma competitiva en el ámbito científico el futuro de la ciencia en Argentina corre el riesgo de detener su marcha. Y en la situación actual a escala mundial esto es sumamente peligroso para el progreso y frustrante para quienes se dedican con vocación a la ciencia.

Sin ninguna duda, el CONICET es la institución fundamental vinculada al desarrollo de la ciencia en Argentina. En la presente década la institución estuvo presidida hasta muy recientemente por el Secretario de Ciencia y Técnica. Se trataba de un cargo carente de la independencia necesaria para evitar los vaivenes políticos que generan un clima de intranquilidad, ajeno a la estabilidad que se requiere para trabajar en ciencia. En fecha reciente, y después de un período de intervención, la dirección del CONICET ha sido normalizada, separándose la figura del Secretario de Ciencia y Técnica de la del presidente del CONICET y, aunque se cuestionó el procedimiento, culminó con la designación como presidente de un científico de prestigio internacional, Enrico Stefani, formado en la Argentina pero residente en Estados Unidos hasta hace unos meses. Además, cuatro de los ocho miembros del consejo director fueron elegidos por el poder ejecutivo teniendo en cuenta ternas sugeridas por los científicos. Esto parece que indica un cambio, que esperamos prosiga.*

Stella M. González Cappa
Universidad de Buenos Aires

* Los datos para la realización de este estudio provienen de las siguientes referencias:

Pichael, R. H. (1991). Desarrollo sin ciencia: otra fantasía argentina. Propuesta para un diálogo. Fundación Favaloro. Departamento de Investigación y Docencia. Colección Investigación y Docencia. Torres Aguero (ed.). Buenos Aires.

Sonnino, S., Novick, M., Bianchi, E. (1993). La investigación en salud en la Argentina. Publicación núm.36. Organización Panamericana de la Salud.

Secretaría de Ciencia y Tecnología. Ministerio de Cultura y Educación (1996). Bases para la discusión de una política de ciencia y tecnología.

Editorial de *Ciencia Hoy* (1997). ¿La última oportunidad? Vol. 7, núm. 37.

Editorial de *Ciencia Hoy* (1997). La necesaria reforma del CONICET. Vol. 7, núm. 40.

Stella M. González Cappa es Investigadora Principal del CONICET y Profesora titular del Departamento de Microbiología, Parasitología e Inmunología de la Facultad de Medicina de la Universidad de Buenos Aires. Actualmente es directora de dicho Departamento y Presidenta de la Asociación Argentina de Microbiología. Posee una larga experiencia en investigación biomédica y un profundo conocimiento de la problemática general de la ciencia en su país.

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MICROBIOLOGÍA SEM **13** (1997), 271–272

FRONTIERS IN ANTIMICROBIAL RESISTANCE

Foreword

Microorganisms are the most adaptable forms of life, they can respond to a wide variety of stimuli. At the same time, bacteria produce disease in humans and animals, and their role in infectology has been the origin of a human activity focused on the research, discovery, development and application of molecules known as antibiotics able to kill bacteria. The extensive use of antibiotics provides new selective conditions that lead to the rapid evolution of bacteria and to the emergence of resistant strains. Then, at present, we can consider that research and bacterial evolution are involved in a cycle. When humans act on a pathogen by using an antibiotic, the emergence of a new pathogen (which has an enriched genetic background with a new gene encoding for antibiotic resistance) is promoted. This emergence is a stimulus for researchers to search for new antibiotics.

At the beginning of the antibiotic era no resistances were detected, but they appeared very early.Their number and sophistication have been increasing during the last few years in which extensive (and sometimes abusive) use of antibiotics has occurred.

Traditionally, mechanisms of microbial resistance to antimicrobial agents were known as enzymatic functions derived from the presence and expression of genes in plasmids and/or in the bacterial chromosome. In the last few years a great scientific adventure that led to the present knowledge about channels, carriers and pumps took place. Many of these mechanisms had been merely hypothetical until recent years. Today they have been isolated, and found to be proteins, whose genes have been cloned and sequenced. In most cases, even the structures of the proteins have been described in detail. The current state of knowledge in this topic includes plasmid, efflux pumps, outer membrane proteins, and alternative mechanisms of resistance.

This special issue includes contributions from different authors working in laboratories specialized in the topic. Efflux pumps in *Pseudomonas aeruginosa* and other Gram-negative bacteria constitutes the main topic of the minireview authored by Taiji Nakae. *P. aeruginosa* is characterized by its intrinsic resistance to a large variety of antimicrobial agents. The contribution of Nakae summarizes the state of knowledge on the antibiotic machinery extrusion in this species. The second minireview included in this issue is authored by Marshall and Piddock and it deals with the main topics related to extrusion in both Gram-positive and Gram-negative bacteria. Also a view on specific efflux systems is reported. A paper on the efflux systems in *Haemophilus influenzae* (Sánchez et al.) is also included in the issue. The recent publication of the complete chromosome sequence of *Haemophilus* led to the discovery of a region with a high degree of homology with the *acrRAB* of *Escherichia coli*. Surprisingly, the efflux system in *Haemophilus* does not seem to play any role in antibiotic resistance in this species. Outer membrane permeability of Gram-negative bacteria has been described as a major cause of antimicrobial resistance in many species. An article

paper on the outer membrane of *Serratia marcescens* a describes the role of the outer membrane in this intrinsically multiresistant enterobacterial species. Methicillin-resistance in *Staphylococcus aureus* (MRSA) is a cause of major concern in hospital infectology. Domínguez et al. authored a contribution of this special issue which reviews the molecular mechanisms of such a resistance. The laboratory of Biotechnology of the Biozentrum at the University of Würzburg has contributed a research paper on an outer membrane protein from *Vibrio cholerae* and their measurement by reconstitution experiments in planar bilayer. In the last few years the incidence of moderate resistance to penicillin in *Neisseria meningitidis* has been increasing in several countries and especially in Spain. Sáez Nieto and Vázquez authored a work concerning this penicillin-resistance. Segura and Salvadó reported the state of knowledge on β-lactamases in *Mycobacterium,* which constitutes a reactivated field of interest due to the emergence of multiresistant mycobacteria. Finally a contribution concerning the drug-resistance in viruses is included.

I must thank all the authors for their positive answer when I asked them to prepare a contribution for this issue. I am especially thankful to all the people involved in the editing of *Microbiología SEM*. I am indebted to the Editor-in-chief Prof. Ricard Guerrero, who encouraged all of us and played a central role in the preparation of this special issue.

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MICROBIOLOGÍA SEM **13** (1997), 273–284

Multiantibiotic resistance caused by active drug extrusion in *Pseudomonas aeruginosa* and other Gram-negative bacteria

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Summary

All living organisms have been exposed to noxious compounds throughout their long evolutionary history and those surviving have evolved to fabricate devices that detoxicate and extrude these life threatening substances. It is likely, therefore, that all viable organisms, from bacteria to mammals, are equipped with active extrusion machinery. When bacteria are attacked by antibiotics, they use these tactics to combat the drugs and to develop resistance. Drugs extrusion machinery in Gram-negative bacteria is complex, consisting of the inner membrane transporter which acts as an energy-dependent extrusion pump; a binding protein which presumably connect both membranes; and the outer membrane exit channel. The extrusion pump assemblies are often encoded by chromosomal genes and might be expressed by mutation(s) or induced in the presence of drug(s).

Key words: *Pseudomonas,* drug resistance, diffusion, efflux, regulation

Resumen

Todos los organismos vivos se han visto expuestos a compuestos nocivos a lo largo de su larga historia evolutiva. Los que han sobrevivido han evolucionado creando dispositivos para destoxificar y expulsar al exterior estos compuestos. En consecuencia, es razonable pensar que todos los organismos viables, desde las bacterias a los mamíferos, estén equipados con maquinaria especializada en la extrusión. Cuando una bacteria es atacada por antibióticos usa esta táctica para combatir las drogas y desarrollar resistencia. La maquinaria de extrusión de drogas en las bacterias Gram

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negativas es compleja; consiste en un transportador de membrana interna que actúa como una bomba de reflujo dependiente de energía, una proteína de unión, que presumiblemente establece contacto entre las dos membranas, y una proteína que constituye un canal de salida en la membrana externa. Las bombas de reflujo están frecuentemente codificadas por genes cromosómicos y pueden ser expresadas por mutación o inducidas en presencia de fármacos.

Introduction

Infections of low-virulence bacteria in immunocompromised patients with cancers or burns, or subject to dialysis, transplantations, etc., are a major concern. Bacteria such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus*, *Streptococcus* spp., found commonly in hospitals, often have low or no virulence effects in healthy persons whose immune activity has been firmly established. Infections by these bacteria can often be treated successfully with antibiotics. A major drawback of the widespread use of antibiotics is the selection of antibiotic resistant strains that can cause nosocomial infections. Antibiotic resistance may be caused by different mechanisms consisting of alteration of the drug target, enzymatic inactivation of the drugs, low drug penetration across the cell envelope, and active extrusion of drugs across the cell envelope. *P. aeruginosa* has a natural resistance to structurally and functionally different antibiotics; this resistance may be attributed to low outer membrane permeability and active extrusion of antibiotics. Among these resistance factors, low antibiotic permeability and antibiotic extrusion are broad and low stereospecific antibiotic resistance mechanisms. Although low antibiotic permeability across the outer membranes usually confers intrinsic resistance, the antibiotic extrusion systems contribute to both intrinsic and acquired resistance to many structurally unrelated antibiotics. This article reviews the role of drug extrusion proteins in the multiantibiotic resistance of *P. aeruginosa* and other Gram-negative bacteria. The terms antibiotics, drugs and chemotherapeutic agents are used as synonyms, including both natural and synthetic compounds. For comprehensive reviews of antibiotic extrusion in a wide range of bacteria, refer to references 21 and 32.

Multiple drug resistance in *P. aeruginosa*

Rella and Haas (37) isolated a *P. aeruginosa* strain that was moderately resistant to nalidixic acid and showed cross-resistance to β-lactams and noboviocin, but was susceptible to tetracycline, rifampin, polymyxin B and aminoglycoside antibiotics (Table 1). The gene *nalB* that confers the resistance was located at 20 min of the chromosomal map of *P. aeruginosa*. Several investigators have reported *nalB*-type mutants that showed a high level of resistance to meropenem, β-lactam antibiotics including penicillins and cephalosporins, fluoroquinolones, tetracycline, and chloramphenicol (38, 39) (Table 1). These mutants overproduced an outer membrane protein designated as OprM (24), with an apparent molecular weight of about 50 kDa (9). Lei et al. (16) reported other mutants resistant to fluoroquinolones, chloramphenicol, tetracycline, and minocycline, yet susceptible to most β-lactam antibiotics (Table 1).

Fukuda et al. (7) and Hirai et al. (12) isolated a mutant resistant to fluoroquinolones and chloramphenicol, but with wild strains susceptible to tetracycline and chloramphenicol. This mutation, designated as *nfxB,* conferred higher sus-

TABLE 1. Antibiotic resistance profiles of the multidrug resistant mutants of *Pseudomonas aeruginosa*

Mutant	Antibiotics										
	IPM	MPM	PCs	CSs	CPR/ CZOP	TC	CP	QLs	AGs	NAL	Ref.
nalB			R	R		S		R	S	R	37
nalB-type	S	R	R	R	R	R	R	R	SS		24
nalB-type	S		S	S		R	R	R	SS		16
nfxB	S		SS	SS		S	S	R	SS	R	7,12
nfxB-type	S	S		S	R	R	R	R	S		27
nfxB-type A	S	S	SS	SS	R	S	S	R	SS		13
nfxB-type B	SS	S	SS	SS	R	R	R	R	SS		13
nfxC	R		SS	SS		S	R	R	SS		7
nfxC-type	R	R		S	S/R	R	R	R	SS		27

Abbreviations: R, resistant; S, sensitive as the parent; SS, more sensitive than the parent.

Drug abrreviations: IPM, imipenem; MPM, meropenem; PCs, penicillin derivatives; CSs, cephalosporin derivatives; CPR, cefpirome; CZOP, cefzopram; TC, tetracycline; CP, chloramphenicol; QLs, quinolone derivatives; AGs, aminoglycosides; NAL, nalidixilic acid.

ceptibility to β-lactam antibiotics and aminoglycosides than the wild type strain, and was mapped at 0 min of the chromosomal map of *P. aeruginosa* (7, 12). The *nfxB* mutant, which was later designated as OprJ, overexpressed the outer membrane protein with an apparent molecular weight of 54 kDa (26). A similar mutant, *cfxB*, isolated independently, was identified to be the same mutation as *nfxB* (38). Masuda et al. (25) isolated two types of *nfxB* mutants. One type showed four to eight times higher resistance to ofloxacin, erythromycin and the new zwitterionic cephems, cefpirome/cefzopram; unchanged susceptibility to chloramphenicol and tetracycline; and increased susceptibility to the classic β-lactams. Another type of mutant showed resistance to tetracycline, chloramphenicol, ofloxacin erythromycin and cefpirome/cefzopram and increased susceptibility to the classic β-lactams (Table 1). Outer membrane protein analyses demonstrate an overproduction of OprJ in the second group of mutants, whereas the increase in the first group was not so significant. OprJ protein was undetectable (13, 40) in the wild type strain as tested by immunoblotting technique using the monoclonal antibody raised against OprJ. This suggest that the *nfxB*-type extrusion pump may not be produced under normal growth conditions (13). Neither the natural inducer nor the conditions that express this efflux system are known.

Susceptibility to antibiotics of the mutant isolated by Fukuda et al. (7) that carries the *nfxC* mutation is intriguing, since the mutant exhibited 8 to 32, 2, and 8 times fold resistance to fluoroquinolones, to chloramphenicol and to imipenem, respectively. The mutant showed more susceptibility towards most β-lactam antibiotics (except for imipenem) and aminoglycoside antibiotics. Two significant symptoms were noticed in the outer membrane protein profile. One was the lower expression of the OprD porin, which has been well characterized as the imipenem-permeable channel protein; another was the high expression of a novel 50 kDa protein, OprN (7, 26). Imipenem resistance of this mutant may be explained by the decreased permeation of imipenem due to the lack of the

imipenem-specific OprD channel. The mutation was located at 45 min of the *P. aeruginosa* chromosomal map (7), which suggests that the mutation is distinct from *nalB* and *nfxB*.

Since *nalB*, *nfxB* and *nfxC* mutants became cross-resistant to unselected antibiotics, and all these mutants expressed new outer membrane proteins (26), researchers initially tried to explain multiple drug resistance in terms of increased barrier function. However, more recent explanations are based on the increasing ability of mutants to extrude antibiotics.

Antibiotic uptake experiments

Celesk and Robillard (4) reported that the *nalB* (*cfxB*) mutant accumulated 2 to 4-fold less drug than the wild type strain, and stated that ciprofloxacin accumulation in *P. aeruginosa* is a complex phenomenon that may be affected by both energy-dependent efflux of drug and the composition of the outer envelope. Fukuda et al. (7) and Hirai et al. (12) measured fluoroquinolone accumulation in *nfxB* and *nfxC* mutants, respectively, and found that both mutants accumulated low level of norfloxacin compared with the level in the respective parent strains.

The endogenous extrusion of energy-dependent fluoroquinolone was reported by Lei et al. (16) and McCaffrey et al. (27), who found that their mutants accumulated low level of fluoroquinolone, which increased to high levels of accumulation in the presence of proton ionophore, carbonylcyanide m-chlorophenylhydrazone (CCCP) or 2,4-dinitrophenol.

More recently, Li et al. (17, 18) demonstrated that tetracycline, chloramphenicol and β-lactam antibiotics are also the substrates of the *nalB*-type extrusion system. They inferred that fluoroquinolones, tetracycline, chloramphenicol and β-lactams are the substrates of the efflux machinery powered by a proton motive force.

The MexA-MexB-OprM efflux system

Poole et al. (33, 35) cloned an operon that overcomes growth deficiency in the mutant that lacks the ferri-pyoverdin receptor in the presence of the non-metabolizable iron-chelator, 2,2'-dipyridyl. The cloned DNA fragment contained three open reading frames, ORFA, ORFB and ORFC, encoding the 41, 112 and 50 kDa proteins corresponding to late designation MexA, MexB and OprM, respectively (8, 35). The products of *mexA* and *mexB* were found to be very similar to the EnvC and EnvD proteins of *Escherichia coli*.

The *mexB* was similar to membrane-associated efflux protein in other bacteria. The expression of the operon was associated with enhanced resistance to 2,2'-dipyridyl, streptonigrin, ciprofloxacin, nalidixic acid, chloramphenicol and tetracycline, suggesting that this operon may encode the machinery for the antibiotic extrusion. Mutants in which *mexA*, *mexB* or *oprM* were destroyed, became more susceptible to these compounds than the parent strain, suggesting that even the wild type strains might express the *mexA/B-oprM* operon and possibly extrude antibiotics (33).

Independent from this study, Morshed et al. (28) cloned genes that confer multiantibiotic resistance. The nucleotide sequencing of cloned genes revealed that the genes were identical to *mexA/B-oprM*. The strain harboring the plasmid encoding MexA/B-OprM showed *nalB*-type antibiotic resistance. To test whether high antibiotic resistance is attributable to the high expression of the *mexA/B-oprM* operon, Morshed et al. (28) determined the level of the mRNA that hybridizes with the cloned gene, and found that

the *mex* genes were highly expressed in *nalB*, and *nalB*-type mutants. Antibiotic susceptible wild-type strains also expressed low levels of mRNA corresponding to the *mex* genes, which is consistent with the observation that the mutants whose *mex* genes were destroyed become more susceptible to many antibiotics than the wild-type strains (33). The *nfxB* and *nfxC* mutants expressed a level of *mex* mRNA similar to that of the wild type strain.

If the *nalB*-type mutants and the strain harboring the plasmid carrying the *mexA/B-oprM* operon had a high rate of transcription of the *mex* genes, high expression of the corresponding membrane proteins would be expected. The appearance of a novel outer membrane protein with a molecular weight of 50 kDa was recognized in the *nalB*-type mutant (9, 26, 28) and the strains harboring the plasmid carrying the *mexA/B-oprM* operon (28, 33). This protein was located in the outer membrane as determined by the immunoblotting method in purified outer membrane (9). This outer membrane protein was designated as OprM (24) and was identical to the product of former *oprK* gene by transposon mutagenesis and immunoblotting (8).

The *mexA/B-oprM* operon encodes two inner membrane proteins, MexA and MexB, with an apparent molecular weight of 41 and 112 kDa, respectively. Those proteins probably assemble an efflux machinery that extrudes antibiotics across the inner membrane. The amino acids similarity between MexB and the Czca protein of *Alcaligenes eutrophus*, a cytoplasmic protein, suggested that MexB might function as an efflux pump itself (29). This protein probably belongs to a family of membrane proteins called Resistant Nodulator Division (RND) proteins. It was predicted that MexA protein anchored in the cytoplasmic membrane protruded to the periplasmic space, since MexA seems to lack the membrane spanning domain and is very similar to the protein involved in the formation of the septum, EnvC, of *E. coli* (22, 23). The expression of MexA with an apparent molecular weight of 41 kDa was confirmed in the *nalB*-type multiantibiotic resistant mutant and in the strains harboring the plasmid that carries the *mexA/B-oprM* operon (28, 33). The MexB protein with a molecular weight of 112 kDa was difficult to visualize on the electrophoretogram as the sample was heated, but it was resolved in unheated samples (35).

The expression of the *mexA/B-oprM* operon seemed to be regulated by the *nalB* gene, since *nalB* and *nalB*-like mutants overproduce MexA, MexB and OprM. Poole et al. (34) sequenced the *mexR* gene from immediately upstream of the *mexA/B-oprM* operon, encoding a small protein with a molecular weight of 16,964. This protein was similar to MarR in *E. coli*, the repressor of the MarA-dependent multidrug efflux transporter.

The cloned *mexR* lowered expression of the *mex* reporter gene, suggesting that *mexR* might repress the expression of the *mex* genes (Fig. 1). The *mexR* knockout mutant was more resistant to several antibiotics. Interestingly, introduction of the *mexR* knockout gene into the *nalB*-type multidrug resistant mutant suppressed the increased expression of the *mexA/B-oprM* operon-encoded genes, and rendered the mutant susceptible to structurally diverse antibiotics (34). These results suggest that the expression of the *mexA/B-oprM* operon might be negatively regulated by MexR, of whose expression was regulated by the upper regulator, the *nalB* gene product. The *nalB* gene was not cloned, and its product remains to be identified.

The MexC-MexD-OprJ efflux system

The region of the gene coding for OprJ was cloned and the nucleotide sequence analysis re-

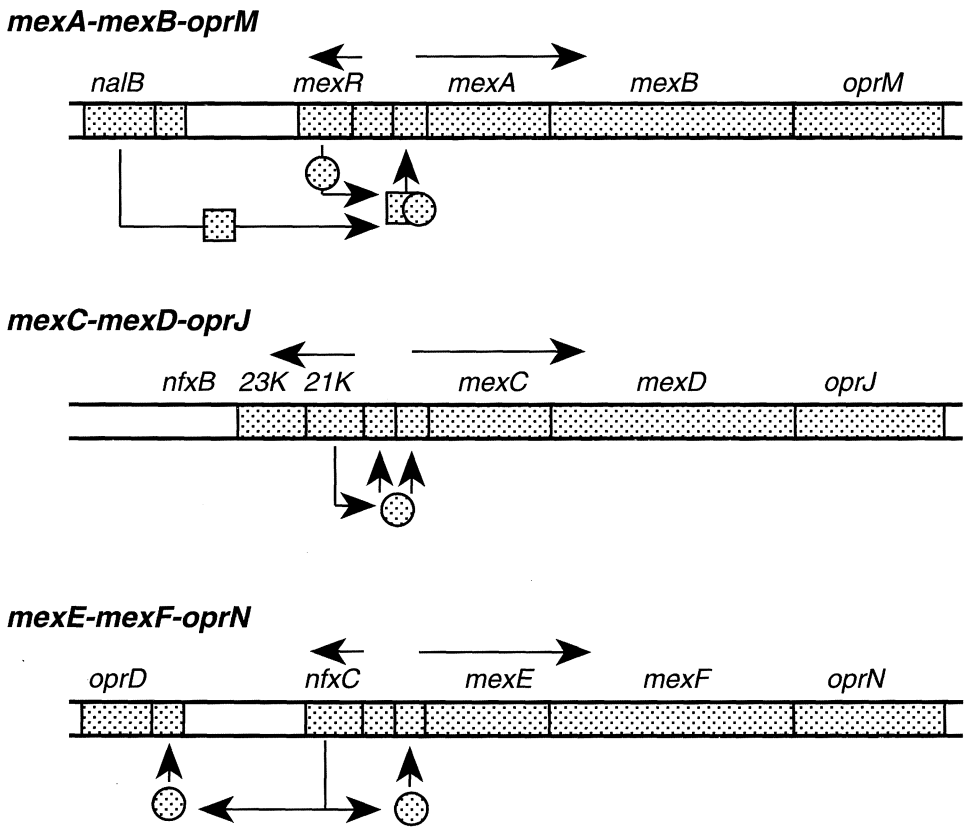

FIG. 1. Highly schematic representation of the *mex* operon organization. The *mexA/B-oprM* operon overexpresses in either *nalB* or *mexR* mutation. Distances between genes and the size do not correspond to physical sizes.

vealed the presence of two additional coding regions upstream of *oprJ* (36). These genes were designated as *mexC* and *mexD* and were similar (43–46% identity) with *mexA* and *mexB*, respectively. Molecular weights of MexC, MexD and OprJ were predicted to be 40, 112 and 51 kDa, respectively. The *nfxB* gene was located upstream of the *mexC/D*-OprJ operon and was transcribed divergently (36).

The *nfxB* gene was cloned from both wild type and antibiotic resistant strains (31). Nucleotide sequencing of the cloned gene predicted that the gene encodes two proteins with a molecular weight of 21 and 23 kDa. Amino acid sequencing suggested that the 21 kDa NfxB might be the DNA binding protein because it contains a helix-turn helix motif of *E. coli*. A point mutation in the coding region at 21 kDa protein was found in the *nfxB* gene of the antibiotic resistant strain. It is likely, therefore, that the NfxB protein regulates the expression of the genes encoding the operon containing the *oprJ* gene. This prediction was substantiated by the demonstration of direct binding of the NfxB protein (21 kDa) to upstream of the *nfxB* coding region by the gel retardation assay (40).

The expression of the *nfxB*-reporter gene in the presence of the *nfxB* gene product was undetectable as examined by the immunoblotting method whereas it is detectable in the antibiotic resistant *nfxB* mutant. This suggests that NfxB might auto-regulate negatively the expression of

nfxB itself, as in the auto-regulation of the *marR* gene expression in *E. coli*.

These results suggest the regulation of the gene coding for the MexC-MexD-OprJ efflux pump as follows: *nfxB* located upstream of the *mexC/D-oprJ* operon is transcribed in the opposite direction to the *mexC/D-oprJ* operon and produces NfxB repressor protein that binds to the regulatory region of the *mexC/D-oprJ* operon.

The antibiotic resistant *nfxB* mutant produces a defective repressor that results in the uncontrolled expression of the *mexC/D-oprJ* operon and, therefore, overproduction of the MexC, MexD, and OprJ proteins.

MexE-MexF-OprN efflux system

An operon encoding OprN was cloned and sequenced (15). The operon consisted of three open reading frames that encoded MexE (45 kDa), MexF (115 kDa) and OprN (51 kDa) and were likely to play a role in the linkage formation between inner and outer membranes, the efflux pump at the inner membrane, and the exit channel at the outer membrane, respectively. MexE, MexF and OprN showed high similarity to MexA, MexB and OprM, respectively.

Overexpression of the operon resulted in lower accumulation of sparfloxacin than the wild type strain. Upstream of this operon there is an open reading frame similar to LysR transcriptional activator. Overexpression of this fragment on the vector raised the levels of antibiotic resistance in the wild type strain. This indicated that the gene positively regulates the MexE/D-OprM efflux system, which would confirm the induction of OprN and reduced expression of OprD. It was suggested that this efflux machinery is involved in the excretion of intermediates for the biosynthesis of pyocyanin.

Model for the antibiotic extrusion machinery

On the basis of the available data, Li et al. (17) proposed a model of the MexA-MexB-OprM pump. The function of this machinery stems from the fact that MexB plays a role as the antibiotic extrusion pump loosely recognizing antibiotics and taking up the substrate antibiotics from the inner surface of the inner membrane and the lipid domain of the inner membrane (Fig. 2). This assumption is based on the sequence similarity of MexB to the CzcA, a metal transporter of *A. eutrophus* (19, 29), NolG, NolH and NolI of the oligosaccharide transporter of *Rhizobium meliloti* (1), and EnvC of *E. coli* (14). Those are collectively called the RND family. It was assumed that the MexA subunit of the Mex pump machinery forms an extension bridge or a linker between inner and outer membranes associated with the MexB, and OprM, respectively (Fig. 2). This assumption is also based on the sequence similarity of MexA to the EnvC polypeptide of *E. coli* involved in septum formation (14). These proteins were referred to as members of Membrane Fusion Protein (MFP) together with AcrA of *E. coli*, CzcB of *Alcaligenes*, MtrA of *Neisseria,* etc. The OprM protein associated with the outer membrane was assumed to play a role in the antibiotic exit channel (Fig. 2).

The role of MexC, MexD and OprJ proteins of the *nfxB*-regulated extrusion machinery and that of the putative MexE, MexF and OprN proteins of the *nfxC* regulated machinery were assumed to be similar to the role of MexA, MexB and OprM of the *nalB*-regulated machinery, respectively. However,the proposed model is totally hypothetical and based on the similarity of the subunit proteins to the protein assemblies of RND and MFP in other bacteria. To assign the roles of these subunit proteins, many questions have to be answered: (i) The topological loca-

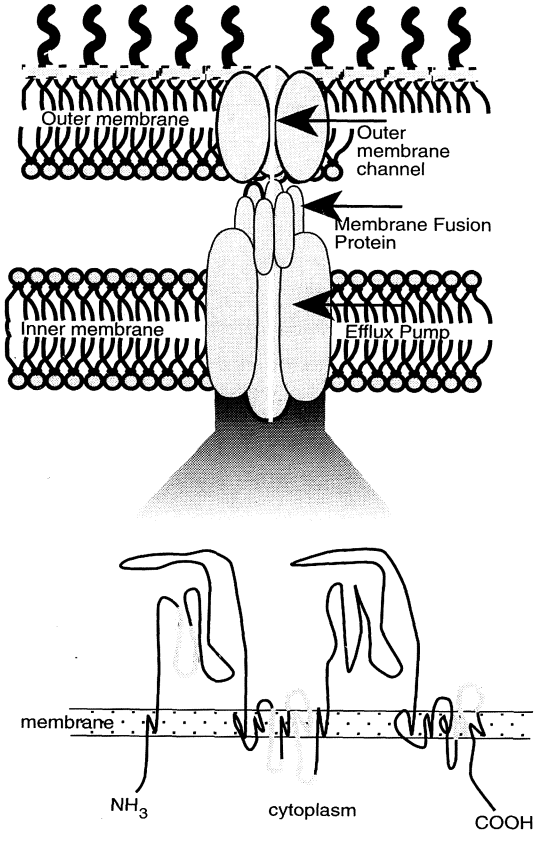

FIG. 2. Highly schematic representation of the antibiotic extrusion machinery assembly in Gram-negative bacteria. The organization of the inner membrane extrusion pump, the periplasmic linker protein and the outer membrane exit channel is purely hypothetical. The size and number of subunits do not correspond to the physical assembly. Lower part of the figure represents deduced transmembrane segments of the MexB protein, adopted from ref. 32.

lization of the subunit proteins. (ii) Interaction of the subunit proteins from each other. (iii) Localization of the site from which antibiotics are taken up or absorbed: Are the antibiotics taken up from the cytoplasm, periplasm or the lipid domain of the membranes? (iv) How is cellular energy transmitted to the transport proteins to drive the extrusion efficiently and which subunit transmits energy? Does the outer membrane protein, OprM, require cellular energy to be functional? (v) Does the outer membrane OprM protein form the channel?

Multiantibiotic extrusion systems in other Gram-negative bacteria

Chromosomal mutation *acr* conferring hypersusceptibility to acriflavine renders *E. coli* more susceptible to hydrophobic dyes and several antibiotics. Cloning and sequencing of the gene responsible for this change revealed that the *acr* operon contained two coding regions, designated as *acrA* and *acrB*, that coded for 397 and 1049 amino acid residues, respectively (21, 22). This suggests that the AcrA and AcrB proteins are likely to be located in the periplasmic space and the inner membrane, respectively, both anchoring at the inner membrane. Two other operons encoding similar proteins to AcrA and AcrB were discovered by the DNA sequencing study and were designated as AcrC/D and AcrE/F. The molecular nature of these gene products is not known.

Studies on the susceptibility of AcrC/D and AcrE/F mutants to dyes, surfactant and antibiotic showed functional or overlapping similarity with the susceptibility of AcrA/B. This functional overlapping is somewhat analogous to that caused by the presence of MexA/B-OprM, MexC/D-OprJ and MexE/F-OprN in *P. aeruginosa*. Unlike the Mex efflux machineries in *P. aeruginosa*, the Acr systems do not appear to encode the outer membrane protein. The function of OprM, OprJ and OprN of *P. aeruginosa* may be taken placed by the *E. coli* TolC protein located at the outer membrane (6). TolC was originally reported to be a component of the protein export machinery at the outer membrane.

Another multiantibiotic transport system in *E. coli* containing two membrane proteins is a single operon encoded EmrA and EmrB with a molecular weight of 43 and 56 kDa (19). Induction of this transport system by drugs renders *E. coli* resistant to several antibiotics and iono-

TABLE 2. Antibiotic extrusion machinery

Inner Membrane Transporter (AA residues/mol. wt)	Outer Membrane Protein (AA residues/mol. wt)	Fusion Protein (AA residues/mol. wt)	Bacterial species	Ref.
Resistant Nodulator Division-Membrane Fusion Protein type				
AcrB (1049 aa)	AcrA (397 aa)	TolC	*E. coli*	21,22
AcrF (1034 aa)	AcrE (384 aa)	TolC?	*E. coli*	21,22
MexB (112 kDa)	MexA (41 kDa)	OprM (50 kDa)	*P. aeruginosa*	16,35
MexD (112 kDa)	MexC (40 kDa)	OprJ (51 kDa)	*P. aeruginosa*	36
MexE (45 kDa)	MexF (115 kDa)	OprN (51 kDa)	*P. aeruginosa*	15
Major Facilitator type				
EmrB (56.2 kDa)	EmrA (42.7)	?	*E. coli*	20
Cmr (411)			*E. coli*	30
MtrC (44 kDa)			*N. gonorrhoeae*	10,11
CzcA (116 kDa)		CzcB/C (54/37 kDa)	*A. eutrophus*	29
CnrA (115 kDa)		CnrB/C (40/44 kDa)	*A. eutrophus*	19
SMR type				
EmrE (12 kDa)			*E. coli*	41
Other transporters (detailed properties are to be investigated)				
CmlA (44 kDa)			*P. aeruginosa*	2
		55 kDa, 39 kDa	*C. jejuni*	5
		OpcM (51kDa)	*B. cepacia*	3

phores. This operon does not encode outer membrane protein either (Table 2).

A single membrane protein that functions as an efflux transporter of cationic noxious compounds exchanged with protons in *E. coli* is EmrE (41), with a molecular weight of 12 kDa. This small multidrug transporter was purified, reconstituted into liposome membrane and shown to catalyze H^+/cation antiport. This suggested that the transporter may not require any other associated protein. This protein spans the membrane only 4 times, and forms the extrusion pump probably by forming an oligomeric aggregate. Thus, it is analogous to the Qac and Ebr exporters of other bacteria.

The soil bacteria *A. eutrophus* harbors a plasmid that confers resistance to metal ions, such as cobalt, zinc and cadmium (19, 29). Nucleotide sequencing of a putative operon revealed four open reading frames, *czcA, B, C* and *D* that encoded four membrane protein of 116, 54.5, 37.3 and 21.2 kDa, respectively. Another plasmid encodes CnrA, B, C which is involved in the heavy metal export much like CzcA, B, C (Table 2).

Neisseria gonorrhoeae became resistant to hydrophobic agents, including detergent-like fatty acids, bile acid, and surfactant (10, 11). The genetic organization of the resistance determinant, the *mtr* genes, encode a transcriptional regulator, *mtrR* and tandemly arranged genes termed *mtrC, D, E* similar to *mexA/B-oprM* of *P. aeruginosa* and *envC, D* of *E. coli*. The inactivation of *mtrC* renders gonococci hypersusceptible to hydrophobic agents, suggesting the significance of this operon for the efflux of the hydrophobic agents (Table 2).

The *E. coli* chromosome at 18.8 min encodes a protein consisting of 411 amino acid residues

that mediate resistance to chloramphenicol when expressing from the multicopy vector (30). Cells expressing this gene accumulate significantly lower levels of chloramphenicol like the wild type strain in the presence of CCCP (Table 2).

An integron of transposon 1696 of *P. aeruginosa* is a DNA element that confers non-enzymatic chloramphenicol resistance (2). The nucleotide sequencing of the *cmlA* gene showed sequence similarity to the bacterial efflux system. The expression of CmlA in *E. coli* promotes a reduction in the content of the major outer membrane proteins, OmpA and OmpF. Resistance conferred by the expression of this gene is limited to chloramphenicol (Table 2).

A mutant strain of *Campylobacter jejuni* expressed two outer membrane proteins with the molecular weights of 55 and 39 kDa, and became resistant to fluoroquinolones, β-lactams, erythromycin, chloramphenicol, and tetracycline (5). The mutants accumulated a significantly low level of fluoroquinolones and minocycline and this low level of antibiotic accumulation was abolished in the presence of CCCP. The nature of the inner membrane transporter remains unknown.

Burkholderia (*Pseudomonas*) *cepacia*, which is resistant to trimethoprim and ciprofloxacin, was isolated, and the resistant determinant gene was sequenced (3). The sequence data revealed that the region of the chromosome contained two open reading frames highly similar to *mexA/B-oprM* of *P. aeruginosa*. The outer membrane protein, OpcM, was labeled with [^{3}H]-palmitate, which suggests that OpcM is most likely the lipoprotein.

References

1. Baev, N., Endre, G., Petrovics, G., Banfalvi, Z., Kondorosi, A. (1991). Six nodulation genes of nod box locus 4 in *Rizobium meliloti* are involved in nodulation signal production: nodM codes for D-glucosamine synthetase. Mol. Gen. Genet. **228**, 113–124.
2. Bissonnette, L., Champetier, S., Buisson, J-P., Roy, P. H. (1991). Characterization of the non-enzymatic chloramphenicol resistance (*cmlA*) gene of the In4 integron of Tn1696: Similarity of the product to transmembrane transport proteins. J. Bacteriol. **173**, 4493–4502.
3. Burns, J., Wadsworth, C. D., Barry, J. J., Goodall, C. P. (1996). Nucleotide sequence analysis of a gene from *Burkholderia* (*Pseudomonas*) *cepacia* encoding an outer membrane lipoprotein involved in multiple antibiotic resistance. Antimicrob. Agents Chemother. **40**, 307–313.
4. Celesk, R. A., Robillard, N. J. (1989). Factors influencing the accumulation of ciprofloxacin in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **33**, 1921–1926.
5. Charvalox, E., Tselentis, Y., Hamzehpour, M. M., Kohler, T., Pechere, J-C. (1995). Evidence for an efflux pump in multidrug-resistant *Campylobacter jejuni*. Antimicrob. Agents Chemother. **39**, 2019–2022.
6. Fralick, J. A. (1996). Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. J. Bacteriol. **178**, 5803–5805.
7. Fukuda, H., Hosaka, M., Hirai, K., Iyobe, S. (1990). New norfloxacin resistance gene in *Pseudomonas aeruginosa* PAO. Antimicrob. Agents Chemother. **34**, 1757–1761.
8. Gotoh, N., Tsujimoto, H., Poole, K., Yamagishi, J-I., Nishino, T. (1995). The outer membrane protein OprM of *Pseudomonas aeruginosa* is encoded by *oprK* of the *mexA-mexB-oprK* multidrug resistance operon. Antimicrob. Agents Chemother. **39**, 2567–2569.
9. Gotoh, N., Itoh, N., Yamada, H., Nishino, T. (1994). Evidence for the location of OprM in the *Pseudomonas aeruginosa* outer membrane. FEMS Microbiol. Lett. **122**, 309–312.
10. Hagman, K. E., Shafer, W. M. (1995). Transcriptional control of *mtr* efflux system of *Neisseria gonorrhoeae*. J. Bacteriol. **177**, 4162–4165.
11. Hagman, K. E., Pan, W., Spratt, B. G., Balthazar, J. T., Judd, R. C., Shafer, W. M. (1995). Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the *mtrRCDE* efflux system. Microbiology **141**, 611–622.
12. Hirai, K., Suzue, S., Irikura, T., Iyobe, S., Mitsuhashi, S. (1987). Mutations producing resistance to norfloxacin *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **31**, 582–586.

13. Hosaka, M., Gotoh, N., Nishino, T. (1995). Purification of a 54-kilodalton protein (OprJ) produced in *nfxB* mutants of *Pseudomonas aeruginosa* and production of monoclonal antibody specific to OprJ. Antimicrob. Agents Chemother. **39**, 1731–1735.
14. Klein, J. R., Henrich, B., Plapp, R. (1991). Molecular analysis and nucleotide sequence of the *envCD* operon of *Escherichia coli*. Mol. Gen. Genet. **230**, 230–240.
15. Köhler, T., Michéa-Hamzehpour, M., Henze, U., Gotoh, N., Curty, L. K. Pechère, J-C. (1997). Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol. Microbiol. **23**, 343–354.
16. Lei, Y., Sato, K., Nakae, T. (1991). Ofloxacin-resistant *Pseudomonas aeruginosa* mutants with elevated drug extrusion across the inner membrane. Biochem. Biophys. Res. Commun. **178**, 1043–1048.
17. Li, X-Z., Ma, D., Livermore, D. M. Nikaido, H. (1994). Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: Active efflux as a contributing factor to β-lactam resistance. Antimicrob. Agents Chemother. **38**, 1742–1752.
18. Li, X-Z., Nikaido, H., Poole, K. (1995). Role of *mexA-mexB-oprM* in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39**, 1948–1953.
19. Liesegang, H., Lemke, K., Siddiqui, R. A., Schlegel, H-G. (1993). Characterization of the inducible nickel and cobalt resistance determinant cnr from pMOL28 of *Alcaligenes eutrophus* CH34. J. Bacteriol. **175**, 767–778.
20. Lomovskaya, O., Lewis, K. (1992). emr, an *Escherichia coli* locus for multidrug resistance. Proc. Natl. Acad. Sci. USA **89**, 8938–8942.
21. Ma, D., Cook, D. N., Hearst, J. E., Nikaido, H. (1994). Efflux pumps and drug resistance in Gram-negative bacteria. Trends Microbiol. **2**, 489–493.
22. Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H., Hearst, J. E. (1993). Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. J. Bacteriol. **175**, 6299–6313.
23. Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H., Hearst, J. E. (1995). Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16**, 45–55.
24. Masuda, N., Ohya, S. (1992). Cross-resistance to meropenem, cephems, and quinolones in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36**, 1847–1851.
25. Masuda, N., Gotoh, N., Ohya, S., Nishino, T. (1996). Quantitative correlation between susceptibility and OprJ production in *nfxB* mutants of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **40**, 909–913.
26. Masuda, N., Sakagawa, E., Ohya, S. (1995). Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39**, 645–649.
27. McCaffrey, C., Bertassa, A., Pace, J., Georgopapadakou, N. H. (1992). Quinolone accumulation in *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Antimicrob. Agents Chemother. **36**, 1601–1605.
28. Morshed, S. R. Md., Lei, Y., Yoneyama, H., Nakae, T. (1995). Expression of genes associated with antibiotic extrusion in *Pseudomonas aeruginosa*. Biochem. Biophys. Res. Commun. **210**, 356–362.
29. Nies, D. H., Nies, A., Chu, L., Silver, S. (1989). Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. Proc. Natl. Acad. Sci. USA **86**, 7351–7355.
30. Nilsen, I. W., Bakke, I., Vader, A., Olsvik, Ø., El-Gewely, M. R. (1996). Isolation of *cmr*, a novel *Escherichia coli* chloramphenicol resistance gene encoding a putative efflux pump. J. Bacteriol. **178**, 3188–3193.
31. Okazaki, T., Hirai, K. (1992). Cloning and nucleotide sequence of the *Pseudomonas aeruginosa nfxB* gene, conferring resistance to new quinolones. FEMS Microbiol. Lett. **97**, 197–202.
32. Paulsen, I. T., Brown, M. H., Skurray, R. A. (1996). Proton-dependent multidrug efflux systems. Microbiol. Rev. **60**, 575–608.
33. Poole, K., Krebes, K., McNally, C., Neshat, S. (1993). Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involvement of an efflux operon. J. Bacteriol. **175**, 7363–7372.
34. Poole, K., Tetro, K., Zhao, Q., Neshat, S., Heinrichs, D. E., Banco, N. (1996). Expression of the multidrug resistance operon *mexA-mexB-oprM* in *Pseudomonas aeruginosa*: *mexR* encodes a regulator of operon expression. Antimicrob. Agents Chemother. **40**, 2021–2028.
35. Poole, K., Heinrichs, D. E., Neshat, S. (1993). Cloning and sequence analysis of an EnvCD homologue in *Pseudomonas aeruginosa*: regulation by iron and possible involvement in the secretion of siderophore pyoverdine. Mol. Microbiol. **10**, 529–554.
36. Poole, K., Gotoh, N., Tsujimoto, H., Zhao, Q., Wada, A., Yamasaki, T., Neshat, S., Yamagishi, J-I, Li, X-Z., Nishino, T. (1996). Overexpression

of the *mexC-mexD-oprJ* efflux operon in *nfxB*-type multidrug-resistant strains of *Pseudomonas aeruginosa*. Mol. Microbiol. **21**, 713–724.

37. Rella, M., Haas, D. (1982). Resistance of *Pseudomonas aeruginosa* PAO1 to nalidixic acid and low levels of β-lactam antibiotics: Mapping of chromosomal genes. Antimicrob. Agents Chemother. **22**, 242–249.
38. Robillard, N. J., Scarpa, A. L. (1988). Genetic and physiological characterization of ciprofloxacin resistance in *Pseudomonas aeruginosa* PAO, Antimicrob. Agents Chemother. **32**, 535–539.
39. Sanders, C. C., Sanders Jr., W. E., Goering, R. V., Werner, V. (1984). Selection of multiple antibiotic resistance by quinolones, β-lactams, and aminoglycosides with special reference to cross-resistance between unrelated drug classes. Antimicrob. Agents Chemother. **26**, 797–801.
40. Shiba, T., Ishiguro, K., Takemoto, N., Koibuchi, H., Sugimoto, K. (1995). Purification and characterization of the *Pseudomonas aeruginosa* NfxB protein, the negative regulator of the *nfxB* gene. J. Bacteriol. **177**, 5872–5877.
41. Yerushalmi, H., Lebendiker, M., Schuldiner, S. (1995). EmrE, an *Escherichia coli* 12 kDa multidrug transporter, exchanges toxic cations and H^+ and is soluble in organic solvents. J. Biol. Chem. **270**, 6856–6863.

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Antibacterial efflux systems

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Summary

Drug efflux, unidirectional pumping of cytotoxic drugs, is a major mechanism of antimicrobial multiresistance in bacteria. Although these efflux systems are usually chromosomally encoded, some are present on plasmids. Some of the efflux pumps are relatively well known: Emr and Acr system in *Escherichia coli,* whose outer membrane protein seems to be the multifunctional TolC; the mex efflux system described in *Pseudomonas aeruginosa* and ABC-type in Gram-negative bacteria. Also the role of efflux in Gram-positive bacteria are reviewed including *Bacillus*, *Staphylococcus* and *Streptomyces*.

Key words: efflux, multidrug resistance, diffusion, outer membrane, cytotoxic drugs

Resumen

El reflujo de drogas, el bombeo unidireccional de drogas citotóxicas, constituye uno de los principales mecanismos que conducen a la multirresistencia bacteriana a antibióticos. Generalmente estos sistemas de reflujo son codificados por el cromosoma, aunque algunos se encuentran en plásmidos. Algunas de las bombas de reflujo son relativamente bien conocidas; tal es el caso de Emr y Acr de *Escherichia coli,* cuya proteína de membrana externa parece ser la multifuncional TolC o el sistema mex de *Pseudomonas aeruginosa* y el tipo ABC de bacterias Gram negativas. A lo largo de este trabajo también se revisa el papel de las bombas de reflujo en bacterias Gram positivas incluyendo *Bacillus*, *Staphylococcus* y *Streptomyces*.

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Introduction

The aim of this review is to acquaint the reader with the multiplicity of bacterial efflux systems, their structural organization, proposed mechanisms and regulation, similarities and differences between them, and their role in resistance to antibacterial agents. Although this area has been reviewed extensively, developments are rapid and unification of accepted dogma is pertinent. Earlier reviews include Levy (34), Lewis (35), Ma et al. (47), Ouellette et al. (71), Poole (77), George (17), Nikaido (67, 68), Miller and Sulavik (57) and Paulsen et al. (75, 76).

Definition of drug efflux and its role in bacterial resistance phenotypes

Drug efflux is the active, unidirectional pumping of cytotoxic drugs from the bacterial cytoplasm into the external medium. Transport is protein-mediated and performed either by a uniport mechanism coupled to ATP hydrolysis or, more commonly, by an antiport mechanism in which drug efflux is linked to proton influx i.e. energized by the proton motive force (pmf). Net efflux of any molecules is the balance between influx (by simple diffusion in the case of most antibiotics) and the gross efflux achieved by these systems. Accordingly, net efflux may drastically underestimate the specific transport activity of an efflux system.

The structure of the cell envelopes in Gram-positive bacteria, requires that their efflux systems be able to transport molecules across both the cytoplasmic and the outer membrane. Efflux simply across the cytoplasmic membrane would be futile. Gram-positive bacteria merely have to ensure that molecules are transported across the cytoplasmic membrane. Therefore, the efflux systems of Gram-negative bacteria tend to have a more complex arrangement comprising a cytoplasmic membrane-located translocase, a periplasmic-located accessory membrane fusion protein (MFP) and an outer membrane protein. This arrangement ensures the efficient efflux of molecules from the cytoplasm across the entire cell envelope. However, there are exceptions to this rule (see Emr system in *E. coli*), and the identity of some MFP and outer membrane proteins remain to be identified for some efflux systems.

The intrinsic resistance of Gram-negative bacteria such as *Pseudomonas aeruginosa* to a variety of antibacterial agents has often been attributed to low outer membrane permeability. The loss or reduction of outer membrane porins can be sufficient to explain the resistance of some strains towards some antibiotics. However, since the discovery of the first drug efflux system [the tetracycline efflux system, *tetA* (7, 56)], it is clear that the reduced influx of an antibacterial agent does not always correlate with the resistance levels observed, and that active efflux must also be considered. Besides, there is also some debate as to whether efflux alone is sufficient to render a given strain resistant to a given antibiotic and, even in strains with greatly reduced outer membrane permeability, it is unclear whether clinically significant levels of resistance (i.e. the MIC of an agent is above the recommended breakpoint concentration) are achieved by efflux alone.

Despite rapid progress in the identification of a variety of efflux systems in different organisms, a number of questions are yet to be satisfactorily resolved. In general, the systems described to date are able to efflux a diverse range of seemingly, structurally unrelated molecules, e.g. dyes, detergents and antibiotics. The fact that many systems be able to efflux antibiotics seems to be fortuitous for the bacterium, and the true, physiological substrates still need to be

determined. For Gram-negative systems, many putative outer membrane components in particular have still to be unambiguously identified. Several species of bacteria e.g. *Escherichia coli*, *P. aeruginosa* and *Staphylococcus aureus* contain more than one efflux system with overlapping specificities. The ecological advantage to the bacterium is that mutational loss of one system will not render the bacterium susceptible to cytotoxic agents.

Efflux systems with broad substrate specificities that efflux molecules also transported by specific efflux systems can be seen as an energetic advantage in that synthesis of a specific system renders resistance to only a single agent. Indeed, there are parallels with bacterial solute uptake systems whereby both specific amino acid permeases and peptide permeases coexist (4, 26).

Families of bacterial efflux systems

Although most systems are chromosomally encoded, some are present on plasmids, notably in the Gram-positive organism *S. aureus*. The cytoplasmic membrane-located translocases of bacterial efflux systems can be grouped into one of four families based upon sequence homology, mechanism and supramolecular assembly:

(i) ATP Binding Cassette (ABC) Transporters (Traffic ATPases)

(ii) Staphylococcal (or Small) Multidrug Resistance (SMR)

(iii) Resistance-Nodulation-Division (RND)

(iv) Major Facilitator (MF)

The ABC transporters are energized by ATP hydrolysis and contain two transmembrane domains (each containing 6 transmembrane α-helices) and two ATP-binding/hydrolysing domains (13, 71). Substrates effluxed by these systems include duanorobucin, tylosin and some macrolides. In contrast, the remaining three families consist of a single cytoplasmic protein that functions as a proton-antiporter.

SMR translocases are small proteins (104–115 amino acyl residues) predicted from hydropathy plots to contain four transmembrane α-helices (75). Because of their small size, it has been suggested that SMR translocases are organized as homotrimers within the cytoplasmic membrane to give the "classical" 12 transmembrane α-helical functional unit. The usual substrates of these systems appear to be lipophilic cations such as ethidium.

RND translocases are much larger proteins (>100 kDa) containing 12 transmembrane α-helices and, hence, are believed to act as monomers (88). Transporters of this family seem to be restricted to Gram-negative bacteria and, in contrast to the MF-proteins, they contain two large (about 300 residues) domains projecting into the periplasm. The substrates effluxed by these systems are very diverse and include basic dyes, detergents and antibiotics.

Most Gram-positive bacterial efflux systems belong to the MF-family (20, 35, 51). These are intermediate-sized proteins (about 400 residues) containing 12–14 transmembrane α-helices (51). These systems efflux either specific substrates such as tetracycline or various substrates as described for the RND-family above.

Major Gram-negative bacterial efflux systems

Gram-negative bacterial efflux systems tend to require the presence of three protein components to permit efficient transfer of toxic moieties from the cytoplasm to the external medium (Fig. 1). The membrane fusion proteins (11) are envisaged to form the "link" between the cytoplasmic membrane-located transporter and the

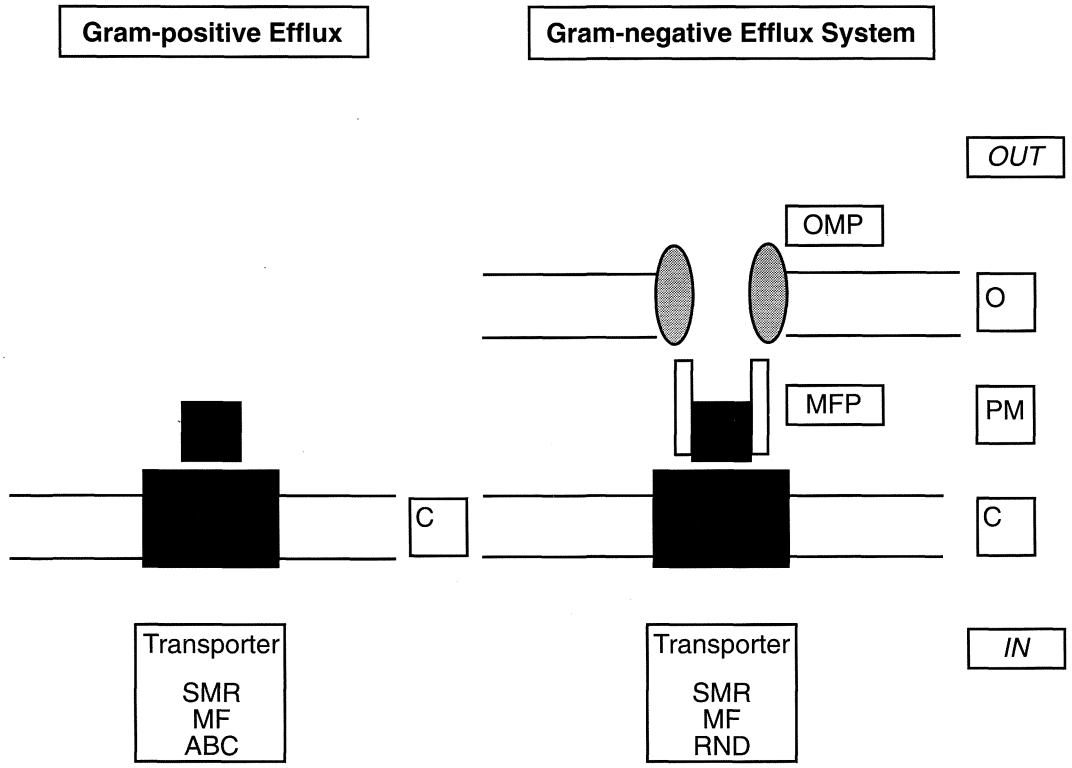

FIG. 1. Molecular architecture of bacterial drug efflux systems.

outer membrane protein. That some Gram-negative systems appear to be able to function without all three components should not be taken as proof that the above generalization is unsound. It remains quite feasible that currently "missing" or "absent" components will be described to substantiate this generalized model. The transporters of the multidrug efflux systems belong mainly to the RND-family of transporters, although there are examples of the SMR- and MF-families.

Emr system in *E. coli*. The *E. coli emr* (*E. coli* multidrug resistance) locus defines three distinct efflux systems, *emrE*, *emrD* and *emrRAB* (43, 60, 95).

EmrE is a small (12 kDa), highly hydrophobic protein of the SMR-family which effluxes toxic cations such as ethidium, methyl viologen and tetraphenylphosphonium (TPP^+) (5, 32). Although the transport activity of EmrE may depend upon the protein forming homo-oligomers (96), Lebendiker and Schuldiner (32) have identified residues involved in the efflux of substrates and they have found that even a decrease in activity by 80% does not correlate with a more susceptible phenotype.

The second system, encoded by *emrD*, is quite poorly characterized (60). EmrD has 397 amino acid residues, shares low homology with EmrB (22% identity), and contains 12 transmembrane α-helices. It is a member of the MF-family of transporters, and is thus energized by the proton motive force. The limited data suggest that it effluxes a restricted range of EmrAB substrates, e.g. carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and phenylmercury acetate.

The third system, encoded by *emrRAB*, was the first report of a multidrug efflux system in a Gram-negative bacterium (43). The *emrRAB* locus (mapping to 57.5 min) comprises a local repressor (EmrR, formerly known as *mprA*)(44), a membrane fusion protein (EmrA) and an MF-type translocase (EmrB) (43). The EmrAB complex interacts with TolC to form the complete efflux machinery. EmrB has 513 residues and contains 14 transmembrane α-helices. EmrA is smaller with 390 amino acids but, it contains a hydrophobic region of 24 residues near the N-terminus that could tether the protein to the cytoplasmic membrane by traversing it once. The substances actively expelled by this system include CCCP, nalidixic acid and thiolactomycin (16, 84) as well as a number of substrates, e.g. CCCP and nalidixic acid have been found to enhance expression of *emrAB*, presumably by binding to EmrR to inhibit its repressor activity (44).

Acr system in *E. coli*. The origins of the identification of the *E. coli* Acr efflux system dates back some 30 years to the original description of *E. coli* mutants that were resistant to the basic dye, acridine. This locus was designated acr (acridine resistance) (58, 59).

In 1993, two teams cloned and sequenced independently the *acr* locus from *E. coli,* and found it to contain at least two genes, *acrA* and *acrB* (formerly called *acrE*) (46). Xu and their colleagues also described in 1993 the cloning and sequencing of a second, unlinked but homologous operon they designated *acrEF* which was found to be identical to a previously characterized operon, *envCD* (29).

Two other unlinked loci were subsequently identified, *orfRAB* (now designated *yhiUV*) (68) and *acrD* (47). OrfR, upstream of *orfAB*, was hypothesized to be a putative regulator of transcription, as were the upstream genes *acrR* and *acrS* of the *acrAB* and *acrEF* operons, respectively. The polypeptides AcrB, AcrF and YhiV (OrfB) contain between 1034 and 1049 amino acid residues, sharing 60–75% sequence identity, and have 12 putative transmembrane α-helices. They belong to the RND-family of transporters. AcrA, AcrE and YhiU (OrfA) are smaller peptides (384–397 residues), sharing 45–60% sequence identity, and are membrane fusion proteins (11). A membrane fusion protein for AcrD, presumably AcrC to maintain nomenclature, has yet to be identified. Similarly, the outer membrane protein components of the transporter complexes are yet to be unambiguously identified. The three proposed regulatory proteins, AcrR, AcrS and OrfR, show the lowest sequence identity (10–20%) and contain 215–242 residues. The accumulated data so far indicates that these systems efflux a very broad range of substrates including antibiotics, dyes and detergents (Table 1).

The most intensively studied Acr system is AcrRAB. Pan and Spratt (72), when discussing the *mtrRCDE* system in *Neisseria gonorrhoeae*, hypothesized that AcrR could be a transcriptional regulator of *acrAB* expression. Further to this, Ma et al. (48) showed that expression of *acrAB* was increased under conditions of stress, e.g. stationary-phase growth or growth in 4% ethanol, 0.5 M NaCl or 5 mM decanoate. Ma et al. (49) further demonstrated that MarA, part of a global stress-responsive regulon (*marRABC*), repressed *acrAB* expression and that the role of the local repressor, AcrR, was to "fine tune" the expression of *acrAB* to prevent wasteful synthesis of AcrAB.

Evidence is mounting that TolC may well be the outer membrane protein associated with AcrAB, as first suggested by Thanassi et al. (93). Fralick (14) has presented further corroborating evidence in support of this proposal, although a physical interaction between AcrA and TolC has yet to be shown. Koronakis et al. (31) have recently obtained 2-D crystals of TolC;

TABLE 1. Efflux systems in Gram-negative bacteria

Locus	Transporter	Membrane Fusion Protein	Outer Membrane Channel	Organism	Substrates	Reference
SMR Family						
emrE	EmrE	–	–	*E. coli*	MeV, Et, Tet, TPP^+, Ery, sulfadiazine	81, 95
qacE	QacE	–	–	*K. aerogenes*	QAC, basic dyes	73
MF Family						
emrRAB	EmrB	EmrA	TolC	*E. coli*	CCCP, Nal, TLM, CPH, PMA	44
RND Family						
acrRAB	AcrB	AcrA	TolC?	*E. coli*	Tet, Chlor, FQ Nov, Ery, Fus, Rif, Et, Acr, CrV, SDS, DOC, Pen G, Mit C, Amp	46, 49
acrSEF	AcrF	AcrE	TolC?	*E. coli*	*acrRAB*	47
yhiUV	YhiV	YhiU	?	*E. coli*	*acrRAB*	47
acrD	AcrD	?	?	*E. coli*	*acrRAB*	47
mexAB-oprM	MexB	MexA	OprM	*P. aeruginosa*	Tet, Chlor, FQ, B-lactams, Nov, Ery, Fus, Rif, Et, Acr, CrV, SDS, DOC	36–38, 78
mexCD-oprJ	MexD	MexC	OprJ	*P. aeruginosa*	Tet, Chlor, FQ, β-lactams (except carbapenems), Nov, Ery, Fus, Rif	25, 54, 55, 79
mexEF-oprN	MexF	MexE	OprN	*P. aeruginosa*	Chlor, FQ, carbapenems (but not conventional β-lactams or 4th generation cephems)	30, 54

Abbreviations: Acr, Acridine; Amp, Ampicillin; CCCP, Carbonyl cyanide *m*-chlorophenylhydrazine; CPH, Chlorophenylhydrazine; CrV, Crystal Violet; DOC, Deoxycholate; Ery, Erythromycin; Et, Ethidium; FQ, Fluoroquinolones; Fus, Fusidic Acid; MeV, Methyl Viologen; MitC, Mitomycin C; Nal, Nalidixic acid; Nov, Novobiocin; Pen G, Penicillin G; PMA, Phenylmercury Acetate; QAC, Quaternary Ammonium Compounds; Rif, Rifampicin; SDS, Sodium dodecyl sulphate; Tet, Tetracycline; TLM, Thiolactomycin; TPP^+, Tetraphenylphosphonium.

TolC appears to exist as trimers within the outer membrane, an identical organization to the OmpC and OmpF porins. Each TolC monomer (molecular weight of about 51.5 kDa) is predicted to contain 18 β-strands forming a transmembrane β-barrel and a C-terminal, 7 kDa domain that is envisaged to form an interaction with a membrane fusion protein such as HlyD or AcrA.

***P. aeruginosa* mex efflux systems**. Earlier work on multidrug resistance in *P. aeruginosa* identified three classes of mutants (*nalB*, *nfxB* and *nfxC*) that displayed cross-resistance phenotypes. These mutants overexpressed outer membrane proteins with a molecular weight of about 50 kDa (54): *nalB* mutants (53, 82) overexpressed OprM [formerly called OprK by Poole et al. (78)]; *nfxB* mutants, OprJ (55); and *nfxC* mutants, OprN (15, 54). However, increased synthesis of outer membrane proteins as a mechanism for resistance appeared to be illogical as accepted dogma suggested that this would lead to an increase, rather than to a decrease, in outer membrane permeability. This anomaly was resolved when OprM was found to be part of an earlier described efflux operon, *mexAB-oprM* (19, 36–38, 78).

The first described *P. aeruginosa* multiple drug efflux operon, *mexAB-oprM*, is known to have at least two other homologues encoded by *mexCD-oprJ* (25, 79) and *mexEF-oprN* (30). The translocases (MexB, MexD and MexF) all belong to the RND family of transporters; MexA, MexC and MexE are the proposed membrane fusion proteins; OprM, OprJ and OprN are the associated outer membrane proteins which complete the efflux complexes. The three genes encoding each efflux system are co-transcribed as single operons. Upstream of *mexAB-oprM* is another divergently transcribed ORF called *mexR* which negatively regulates *mexAB-oprM* expression (79). *nalB* mutations leading to overexpression of *mexAB-oprM* are located within *mexR*. Similarly, the transcriptional repressor of *mexCD-oprJ*, *nfxB* (70), also lies upstream and is transcribed in the opposite direction from *mexCD-oprJ* (80). In contrast, *mexEF-oprN* is positively regulated by an upstream regulator, presumably NfxC, which is transcribed convergently and shows homology with LysR (30).

The three systems have overlapping specificities for the substrates that they are able to efflux (Table 1). MexAB-OprM appears to be the major efflux system and to efflux the widest range of substrates. MexCD-OprJ effluxes a restricted sub-set of MexAB-OprM substrates, whilst MexEF-OprN has the tightest specificity, although it is able to expel carbapenems such as imipenem and also the protonophore, CCCP. That the *mex* systems are able to efflux β-lactams is the cause of some debate. It has been speculated that the β-lactams either enter the efflux system at the level of the outer membrane protein or that they partially partition within the cytoplasmic membrane and get "funnelled" into the efflux process through this route. These mechanistic details and also the identity of the "natural" *mex* substrates await further clarification (68).

Köhler et al. (30) state that MexAB shares greater sequence similarity with *E. coli* AcrAB and AcrEF than with MexCD and MexEF, and speculate as to whether *E. coli* "acquired" its efflux systems from *P. aeruginosa* via a gene transfer event.

ABC-type efflux systems. Bacterial efflux systems containing an ABC-type transporter have been reviewed by Fath and Kolter (13) and Ouellette et al. (71). In Gram-negative bacteria they are responsible for the efflux of proteins rather than antibacterial agents. Examples of ABC-mediated efflux include hemolysin export in *E. coli* performed by the HlyBD-TolC system, alkaline protease export in *P. aeruginosa* by the AprDEF system, and Proteases A and B by the PrtDEF system in *Erwinia chrysanthemi* (47, 68).

However, it would be unwise to speculate that all Gram-negative efflux systems are driven solely by the proton motive force. Firstly, many nutrients uptake systems, e.g. the histidine- and oligopeptide permeases, exist that use ATP hydrolysis to drive influx of histidine and oligo-

peptides, respectively (4, 26). Secondly, a recently described Gram-positive antibiotic efflux system from *Streptomyces peucetius*, *drrAB*, was expressed in a functional form in *E. coli* to give resistance to the "antibiotics" doxorubicin and daunorubicin (28).

Other Gram-negative efflux systems. Efflux systems have also been described in Gram-negative species other than the well-studied organisms *E. coli* and *P. aeruginosa* (Table 2). Of particular relevance are the efflux systems found in the clinically important organisms *N. gonorrhoeae*, *Klebsiella aerogenes* and *Campylobacter jejuni*. Although these systems have not been well characterized, their very existence should be taken as a clear warning that other efflux systems undoubtedly exist in pathogenic bacteria. Furthermore, their ability to efflux a wide range of antibacterial agents should also be considered.

Gram-positive efflux systems

Systems in Gram-positive bacteria, because of their lack of an outer membrane, are composed of only a single, cytoplasmic membrane-located transporters of the SMR, MF or ABC families (Fig. 1).

***S. aureus norA* efflux system**. A *norA* mutation that led to a decrease in susceptibility to hydrophilic fluoroquinolones was identified and originally deemed to be an allele of *gyrA* (94). However, further characterization revealed that *norA* encoded for a protein of the MF family involved in active drug efflux (69, 97). NorA is, in fact, a homologue of Bmr from *Bacillus subtilis* (44% amino acid identity) (62) and similar to tetracycline efflux proteins (34). Although Ohshita et al. (69) reported that a point mutation in the *norA* structural gene resulted in increasing the affinity of the efflux system and, hence, decreasing the susceptibility of *S. aureus* to fluoroquinolones, Kaatz et al. (27) showed that this effect was due to the enhanced expression of *norA* due to increased transcription. This was attributed to either an unidentified change in a putative regulator or to a point mutation that had been found in the *norA* promoter. Besides, Ng et al. (65) showed that this same, single nucleotide change was present in the *norA* promoter of an *flqB* mutant and could lead to increased expression of *norA*. However, gene amplification of *norA* as a mechanism of resistance has not been excluded. Using Southern hybridization analysis, *norA* homologues were found to be present in *S. epidermidis* but not in *E. coli*, *K. pneumoniae* or *Enterococcus faecalis* (27). NorA effluxes basic dyes, TPP^+ and some antibiotics (Table 3).

***Bacillus bmr* and *blt* systems**. The *B. subtilis* *bmr* efflux system was identified in a systematic manner by selecting for step-wise resistance to the cationic dye rhodamine 6G (63). This selec-

TABLE 2. Additional Gram-negative efflux systems

Organism	Locus	Substrates Effluxed	Reference
Neisseria gonorrhoeae	*mtrRCDE*	Antibiotics, detergents, dyes	24, 50
Alcaligenes eutrophus	*czcABC*	Co^{2+}, Zn^{2+}, Cd^{2+}	66
Alcaligenes eutrophus	*cnrABC*	Co^{2+}, Ni^{2+}	39
Klebsiella aerogenes	*qacEΔ1*	Ethidium	73, 90
Campylobacter jejuni	*cje*	Antibiotics	8
Rhizobium melitoti	*nolGHIF*	Nodulation signals	6

TABLE 3. Efflux systems in Gram-positive bacteria

Locus	Transporter	Organism	Substrates	Reference
SMR Family				
smr (*qacC*)	Smr	*S. aureus*	QAC	40, 45
MF Family				
norA	NorA	*S. aureus*	Enox, Nor, Cip, Nal Oxl, Spar	97
bmrR-bmr-bmrU	Bmr	*B. subtilis*	Et, Chlor, Puro TPP^+, CTAB, Nor, Acr Dox	1, 2, 63
bltR-blt-bltD	Blt	*B. subtilis*	As for Bmr	3
qacA	QacA	*S. aureus*	Et, proflavine, CrV, Rho BiG, diamidines, CTAB etc., chlorhexidine	41, 45
qacB	QacB	*S. aureus*	Similar to QacA with reduced efflux of diamidines and chlorhexidine	41

Abbreviations: Acr, Acridine; BiG, Biguanidine; Chlor, Chloramphenicol; Cip, Ciprofloxacin; CrV, Crystal Violet; CTAB, Hexadecyltrimethylammonium bromide; Dox, Doxorubicin; Enox, Enoxacin; Et, Ethidium; Nal, Nalidixic Acid; Nor, Norfloxacin; Oxo, Oxolinic Acid; Pur, Puromycin; QAC, Quaternary Ammonium Compound; Rhod, Rhodamine; Spar, Sparfloxacin.

tion procedure led to the overexpression of *bmr*, a member of the MF family, by the mechanism of gene amplification, also seen in some mammalian MDR systems. Bmr contains 389 residues and has two similar, hydrophobic domains each predicted to contain six, transmembrane α-helices, placing it in the MF-family of transporters. It is a structural and functional homologue of *S. aureus* NorA with which it shares 44% sequence identity at the amino acid level (51% at the nucleotide level) (62, 97). It effluxes a similar range of substrates to NorA (Table 3) and is also inhibited by a lower concentration of reserpine (62, 64). Ahmed et al. (1) found that the sensitivity of Bmr to reserpine could be altered by mutating Val-286 of the putative β-helix IX of the Bmr protein.

Another operon, *blt*, which shares about 50% sequence identity with *bmr* has also been found in *B. subtilis* (3). The Blt protein is slightly larger than Bmr with 400 amino acid residues. Although the proteins efflux similar substrates, their expression levels are different and *blt* is not normally detectable in wild type bacteria (3). Upstream of the *bmr* and *blt* operons are regulatory genes, *bmrR* and *bltR*, respectively, that function as activators, rather than repressors, of their respective operons (2, 3). BmrR (245 residues) appears to bind to its target promoter (the *bmr* promoter) as a dimer to enhance *bmr* expression (2, 52). Although BmrR and BltR display homology at their N-termini, they are completely dissimilar at their C-termini and BltR, in contrast to BmrR, does not bind rhodamine, a known enhancer of *bmr* expression.

A gene co-transcribed with *bmr*, *bmrU*, shares homology with mammalian myosin but has no known function. In contrast, *bltD*, which is co-transcribed with *blt* has a putative acetylation role (3). Both systems efflux similar substrates

which tend to be lipophilic cations (Table 3) although they are expressed differently. Interestingly, norfloxacin (zwitterion, no net charge) is also transported by these two systems but not tetracycline, despite homology of Blt and Bmr with the Tet efflux proteins TetA, B and C (63).

Plasmid-encoded efflux systems of *S. aureus*. *S. aureus* contains three plasmid-encoded pumps that belong to two different transporter families yet efflux quite similar substrates (Table 3) (45). These are the quaternary ammonium compound efflux systems (*qac*).

QacA and QacB belong to the MF-family of transporters but are unusual in that they contain 14 rather than the usual 12 transmembrane α-helices (18, 45, 74, 87, 92). The proteins are virtually identical although QacA (514 residues, 55 kDa) is translated from the less usual start codon, CUG, instead of AUG (87). The efflux systems encoded by *qacA* or *qacB* are both sensitive to the protonophore, CCCP, indicating that they function as proton antiporters (41, 74).

Smr (formerly QacC or Ebr) is a much smaller protein containing 107 amino acid residues and only four putative transmembrane α-helices (40, 89) and is the archetypal member of the SMR family of transporters (22, 75). Another gene, *qacD*, is now considered to be identical to *smr* (except that *qacD* resides on larger, conjugative plasmids) (40, 92). Smr has been reconstituted into proteoliposomes and was found to use the proton motive force to drive drug efflux (21). The C-terminus of Smr appears to be critical for its function because removal of the final 19 residues causes complete loss of function (40). Because of their small size, Smr and its *E. coli* homologue (EmrE) have stimulated much interest and debate concerning the minimal functional unit of a transporter, the seeming ubiquity of the 12 α-helical model, and also their evolutionary origins.

Despite the structural differences described above, these systems efflux similar substances. QacA has the broadest specificity of all, QacB effluxes the same substrates with a reduced affinity for diamidines and chlorhexidine, and Smr usually restricted to quaternary ammonium compounds. Plasmids containing *qacA(B)* or *smr* sequences have also been found in a variety of *S. aureus* clinical isolates and other staphylococci, indicating genetic spread of the system (33, 41).

A possible chromosomal homologue of QacA has been identified in the mycobacterial species *Mycobacterium smegmatis* (91). This gene, *lrfA* (low-level resistance to fluoroquinolones), encodes for a protein of 504 residues with shows 35% sequence identity to *S. aureus* QacA. It is a member of the MF-family of transporters with 14 predicted α-helices. This putative efflux system is sensitive to CCCP and raises the MIC of hydrophilic quinolones such as ciprofloxacin and ofloxacin, but not their more hydrophobic counterparts such as sparfloxacin or nalidixic acid (42, 91). Southern hybridization analysis with cloned *lrfA* has shown that LrfA homologues also exist in *M. tuberculosis* and *M. avium* (12).

Gram-positive ABC-mediated efflux systems. Gram-positive bacteria of the genera *Streptomyces* are especially well known for their ability to synthesize antibiotics. Neal and Chater (61) showed that *Streptomyces coelicolor* was able to protect itself from the antibiotic it produces, methylenomycin, by expelling it actively after synthesis. Subsequently, several antibiotic-producing species of *Streptomyces* have been found that contain efflux systems for their respective antibiotics. These efflux systems belong to either the MF or ABC families and tend to be specific for one or two substances.

Fath and Kolter (13) and Ouellette et al. (71) have reviewed microbial ABC efflux systems and three examples are discussed below. A plas-

mid-encoded system, *msrA*, found in *S. epidermidis* (85), effluxes 14- and 15-membered macrolides, e.g. erythromycin. It is unusual because *msrA* only codes for an ABC protein which must interact with channel-forming proteins, possibly of another pre-existing system to form a complete transport complex. The chromosomally-encoded *drrAB* operon from *Streptomyces peucitius* effluxes the "antibiotics" daunorubicin and doxoribucin (23, 28). The transport complex is hypothesized to comprise two copies of DsrA (the ABC protein) and two copies of DsrB (the channel-forming proteins containing six α-helices). Finally, *Streptomyces fradiae* contains a chromosomal gene, *tlrC*, that is responsible for expelling the antibiotic tylosin (86). This system may also require the presence of a second protein, TlrB, to form the functional transport complex.

Specific antibiotic efflux systems - The tetracycline efflux system

Efflux of antibiotics as a resistance mechanism was first demonstrated in 1980 for the antibiotic tetracycline (7, 56). Since this initial finding, much work has been performed upon tetracycline efflux in particular (9, 34) and has led to the discovery of many closely related tetracycline efflux systems and, more recently, to the discovery of the multidrug efflux pumps described above.

Several tetracycline resistance determinants (*tet*) have been described from both Gram-negative and Gram-positive bacteria (10, 83). Although these may be chromosomally encoded, they are more frequently present on extrachromosomal elements, e.g. plasmids and transposons. The determinants are categorized into various classes on the basis of DNA-DNA hybridization experiments (34). Although some *tet* resistance determinants are expressed constitutively, many of them are regulated and derepressed by nanomolar concentrations of tetracycline.

Tetracycline is a bacteriostatic antibiotic whose primary site of action is the 30S subunit of the bacterial ribosome and thus it inhibits protein synthesis. The resistance determinants confer resistance by one of three mechanisms, the third mechanism being of particular relevance; (i) protection of the ribosome, (ii) enzymatic inactivation of tetracycline, (iii) active efflux of tetracycline. Protection of the ribosome from the action of tetracycline involves the synthesis of proteins of molecular weight about 72.5 kDa which show sequence similarity to the elongation factors Tu and G (83). Enzymatic modification of tetracycline appears to be performed by a 44 kDa protein which is an NADPH-requiring oxidoreductase, although its significance in vivo is questioned (83).

So far, ten resistance determinants encoding efflux systems have been described, three in Gram-positive species [*tetK*, *tetL* and *tetA(P)*], and seven in Gram-negative species (*tetA*, *tetB*, *tetC, tetD, tetE, tetG, tetH*) (83). The *tet* efflux proteins belong to the MF-family of transporters (20, 51). They are all of a similar molecular weight (about 41–51 kDa) with the Gram-negative proteins hypothesized to contain 12 transmembrane α-helices and the Gram-positive members 14, the N- and C-termini of both proteins residing in the cytoplasm. The proteins appear to be split into two functional domains (α and β) which correspond to the N- and C-termini of the protein respectively, separated by a hydrophilic loop which is predicted to project into the cytoplasm (10). Mutations in either domain result in the loss of tetracycline resistance mediated by that particular protein. The Gram-negative Tet efflux proteins appear to belong to two genetically distinct sub-families based on comple-

mentation studies comprising TetA, TetC and TetG as the first, and TetB, TetD, TetE and TetF as the second. Hence, wild-type *tetA* can complement for a mutation in *tetC* and restore partial resistance to tetracycline, but not for a mutation in *tetB*, for example (34). This raises the possibility that *tet* efflux proteins are organized as oligomers within the cytoplasmic membrane.

The *tet* efflux proteins function as tetracycline/proton antiporters and are thus driven by the proton motive force (sensitive to uncouplers such as CCCP or 2,4-dinitrophenol). Tetracycline is effluxed as a complex with chelated, divalent cations, e.g. Mg^{2+}, so that the transmembrane potential is unaltered. Because *tet* determinants are often present on genetically mobile elements, they have been found in a variety of bacterial species and the emergence of tetracycline resistance is particularly widespread and common (83). The sequence similarity between *S. aureus* NorA and *B. subtilis* Bmr and the *tet* efflux systems, despite substrate differences, may indicate that multidrug exporters have arisen by a series of mutational events that removed the unique specificity of a specific transporter.

Concluding remarks and future prospects

As a consequence of both their short generation times and genetic transfer mechanisms, bacteria have developed different antibiotic resistance mechanisms. Even with the current increasing knowledge and experimental sophistication, it is not surprising that bacteria have developed another antibiotic resistance mechanism: efflux systems. Their broad specificity, mechanistic diversity and seeming ubiquity is a serious concern; all efforts to produce agents of increased target potency are fruitless if the bacterium can easily expel the drug. The fact that some efflux systems in Gram-negative bacteria share a common outer membrane component (e.g. TolC for the HlyBD, AcrAB and EmrAB efflux systems) inhibiting either the expression or the function of such a protein may prove to be a useful way of eliminating efflux-mediated multidrug resistance. Much progress has already been made in the identification and characterization of such systems. However, more research is still needed if these systems are to be fully understood and counteracted in the design of future antimicrobial agents.

References

1. Ahmed, M., Borsch, C. M., Neyfakh, A. A., Schuldiner, S. (1993). Mutants of the *Bacillus subtilis* multidrug transporter *bmr* with altered sensitivity to the antihypertensive alkaloid reserpine. J. Biol. Chem. **268**, 11086–11089.
2. Ahmed, M., Borsch, C. M., Taylor, S. S., Vazquez-Laslop, N., Neyfakh, A. A. (1994). A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. J. Biol. Chem. **269**, 28506–28513.
3. Ahmed, M., Lyass, L., Markham, P. N., Taylor, S. S., Vazquez-Laslop, N., Neyfakh, A. A. (1995). 2 highly similar multidrug transporters of *Bacillus subtilis* whose expression is differentially regulated. J. Bacteriol. **177**, 3904–3910.
4. Ames, G. F., Mimura, C. S., Holbrook, S. R., Shyamala, V. (1992). Traffic ATPases - a superfamily of transport proteins operating from *Escherichia coli* to humans. Advances in Enzymology and Related Areas of Molecular Biology **65**, 1–47.
5. Arkin, I. T., Russ, W. P., Lebendiker, M., Schuldiner, S. (1996). Determining the secondary structure and orientation of EmrE, a multidrug transporter, indicates a transmembrane 4-helix bundle. Biochemistry **35**, 7233–7238.
6. Baev, N., Endre, G., Petrovics, G., Banfalvi, Z., Kondorosi, A. (1991). 6 nodulation genes of nod box locus-4 in *Rhizobium melitoti* are involved in nodulation signal production - *nodM* codes for *D*-glucosamine synthetase. Mol. Gen. Genet. **228**, 113–124.
7. Ball, P. R., Shales, S. W., Chopra, I. (1980). Plasmid-mediated tetracycline resistance in

Escherichia coli involves increased efflux of the antibiotic. Biochem. Biophys. Res. Commun. **93**, 74–81.

8. Charvalos, E., Tselentis, Y., Hamzephour, M. M., Köhler, T., Pechere, J.-C. (1995). Evidence for an efflux pump in multidrug-resistant *Campylobacter jejuni*. Antimicrob. Agents Chemother. **39**, 2019–2022.
9. Chopra, I. (1992). Efflux-based antibiotic-resistance mechanisms the evidence for increasing prevalence. J. Antimicrob. Chemother. **30**, 737–739.
10. Chopra, I. (1995). Tetracycline uptake and efflux in bacteria. *In* Georgopapadakou, N. H., (ed.), Drug Transport in Antimicrobial and Anticancer Chemotherapy, pp. 221–243. Marcel Dekker, Inc., New York.
11. Dinh, T., Paulsen, I. T., Saier, M. H. (1994). A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of Gram-negative bacteria. J. Bacteriol. **176**, 3825–3831.
12. Doran, J. L., Pang, Y. J., Mdluli, K. E., Moran, A. J., Victor, T. C., Stokes, R. W., Mahenthiralingam, E., Kreiswirth, B. N., Butt, J. L., Baron, G. S., Treit, J. D., Kerr, V. J., VanHelden, P. D., Roberts, M. C., Nano, F. E. (1997). *Mycobacterium tuberculosis efpA* encodes an efflux protein of the QacA transporter family. Clin. Diagn. Lab. Immunol. **4**, 23–32.
13. Fath, M. J., Kolter, R. (1993). ABC transporters-bacterial exporters. Microbiol. Rev. **57**, 995–1017.
14. Fralick, J. A. (1996). Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. J. Bacteriol. **178**, 5803–5805.
15. Fukuda, H., Hosaka, M., Iyobe, S., Gotoh, N., Nishino, T., Hirai, K. (1995). *nfxC*-type quinolone resistance in a clinical isolate of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39**, 790–792.
16. Furukawa, H., Tsay, J. T., Jackowski, S., Takamura, Y., Rock, C. O. (1993). Thiolactomycin resistance in *Escherichia coli* is associated with the multidrug-resistance efflux pump encoded by *emrAB*. J. Bacteriol. **175**, 3723–3729.
17. George, A. M. (1996). Multidrug resistance in enteric and other Gram-negative bacteria. FEMS Microbiol. Lett. **139**, 1–10.
18. Gillespie, M. T., Skurray, R. A. (1986). Plasmids in multiresistant *Staphylococcus aureus*. Microbiol. Sci. **3**, 53–58.
19. Gotoh, N., Tsujimoto, H., Poole K., Yamagishi, J. I., Nishino, T. (1995). The outer-membrane protein OprM of *Pseudomonas aeruginosa* is encoded by OprK of the MexA-MexB-OprK multidrug-resistance operon. Antimicrob. Agents Chemother. **39**, 2567–2569.
20. Griffith, J. K., Baker, M. E., Rouch, D. A., Page, M. G. P., Skurray, R. A., Paulsen, I. T., Chater, K. F., Baldwin, S. A., Henderson, P. J. F. (1992). Membrane transport proteins: implications of sequence comparisons. Current Opinion in Cell Biology **4**, 684–695.
21. Grinius, L. L., Goldberg, E. B. (1994). Bacterial multidrug resistance is due to a single membrane protein which functions as a drug pump. J. Biol. Chem. **269**, 29998–30004.
22. Grinius, L., Dreganiene, G., Goldberg, E. B., Liao, C. H., Projan, S. J. (1992). A Staphylococcal multidrug resistance gene product is a member of a new protein family. Plasmid **27**, 119–129.
23. Guilfoile, P. G., Hutchinson, C. R. (1991). A bacterial analog of the MDR gene of mammalian tumor cells is present in *Streptomyces peucetius*, the producer of daunorubicin and doxorubicin. Proc. Natl. Acad. Sci. USA **88**, 8553–8557.
24. Hagman, K. E., Pan, W. B., Spratt, B. G., Balthazar, J. T., Judd, R. C., Shafer, W. W. (1995). Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the *mtrRCDE* efflux system. Microbiology **141**, 611–622.
25. Hamzehpour, M. M., Pechere, J. C., Plesiat, P., Kohler, T. (1995). OprK and OprM define 2 genetically distinct multidrug efflux systems in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39**, 2392–2396.
26. Higgins, C. F. (1992). ABC transporters-from microorganisms to man. Annu. Rev. Cell Biol. **8**, 67–113.
27. Kaatz, G. W., Seo, S. M., Ruble, C. A. (1993). Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **37**, 1086–1094.
28. Kaur, P. (1997). Expression and characterization of DrrA and DrrB proteins of *Streptomyces peucetius* in *Escherichia coli*: DrrA is an ATP binding protein. J. Bacteriol. **179**, 569–575.
29. Klein, J. R., Henrich, B., Plapp, R. (1991). Molecular analysis and nucleotide sequence of the *envCD* operon of *Escherichia coli*. Mol. Gen. Genet. **230**, 230–240.
30. Köhler, T., Hamzehpour, M. M., Henze, U., Gotoh, N., Curty, L. K., Pechere, J. C. (1997). Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Mol. Microbiol. **23**, 345–354.
31. Koronakis, V., Li, J., Koronakis, E., Stauffer, K. (1997). Structure of TolC, the outer membrane

component of the bacterial type I efflux system, derived from two-dimensional crystals. Mol. Microbiol. **23**, 617–626.

32. Lebendiker, M., Schuldiner, S. (1996). Identification of residues in the translocation pathway of EmrE, a multidrug antiporter from *Escherichia coli*. J. Biol. Chem. **271**, 21193–21199.
33. Leelaporn, A., Paulsen, I. T., Tennent, J. M., Littlejohn, T. G., Skurray, R. A. (1994). Multidrug-resistance to antiseptics and disinfectants in coagulase-negative staphylococci. J. Med. Microbiol. **40**, 214–220.
34. Levy, S. B. (1992). Active efflux mechanisms for antimicrobial resistance. Antimicrob. Agents Chemother. **36**, 695–703.
35. Lewis, K. (1994). Multidrug resistance pumps in bacteria - variations on a theme. Trends Biochem. Sci. **19**, 119–123.
36. Li, X. Z., Livermore, D. M., Nikaido, H. (1994). Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa* - resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrob. Agents Chemother. **38**, 1732–1741.
37. Li, X. Z., Ma, D., Livermore, D. M., Nikaido, H. (1994). Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*-active efflux as a contributing factor to beta-lactam resistance. Antimicrob. Agents Chemother. **38**, 1742–1752.
38. Li, X. Z., Nikaido, H., Poole, K. (1995). Role of MexA-MexB-OprM in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39**, 1948–1953.
39. Liesegang, H., Lemke, K., Siddiqui, R. A., Schlegel, H. G. (1993). Characterization of the inducible nickel and cobalt resistance determinant cnr from pMOL28 of *Alcaligenes eutrophus* CH34. J. Bacteriol. **175**, 767–778.
40. Littlejohn, T. G., Diberardino, D., Messerotti, I. J., Spiers, S. J., Skurray, R. A. (1991). Structure and evolution of a family of genes encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. Gene **101**, 59–66.
41. Littlejohn, T. G., Paulsen, I. T., Gillespie, M. T., Tennent, J. M., Midgley, M., Jones, I. G., Purewal, A. S., Skurray, R. A. (1992). Substrate specificity and energetics of antiseptic and disinfectant resistance in *Staphylococcus aureus*. FEMS Microbiol. Lett. **95**, 259–266.
42. Liu, J., Takiff, H. E., Nikaido, H. (1996). Active efflux of fluoroquinolones in *Mycobacterium smegmatis* mediated by LfrA, a multidrug efflux pump. J. Bacteriol. **178**, 3791–3795.
43. Lomovskaya, O., Lewis, K. (1992). Emr, an *Escherichia coli* locus for multidrug resistance. Proc. Natl. Acad. Sci. USA **89**, 8938–8942.
44. Lomovskaya, O., Lewis, K., Matin, A. (1995). EmrR is a negative regulator of the *Escherichia coli* multidrug-resistance pump EmrAB. J. Bacteriol. **177**, 2328–2334.
45. Lyon, B. R., Skurray, R. (1987). Antimicrobial resistance of *Staphylococcus aureus* - genetic basis. Microbiol. Rev. **51**, 88–134.
46. Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H., Hearst, J. E. (1993). Molecular cloning and characterization of *acrA* and *acrE* genes of *Escherichia coli*. J. Bacteriol. **175**, 6299–6313.
47. Ma, D., Cook, D. N., Hearst, J. E., Nikaido, H. (1994). Efflux pumps and drug resistance in Gram-negative bacteria. Trends in Microbiology **2**, 489–493.
48. Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H., Hearst, J. E. (1995). Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16**, 45–55.
49. Ma, D., Alberti, M., Lynch, C., Nikaido, H., Hearst, J. E. (1996). The local repressor *acrR* plays a modulating role in the regulation of *acrAB* genes of *Escherichia coli* by global stress signals. Mol. Microbiol. **19**, 101–112.
50. Maness, M., Sparling, P. F. (1973). Multiple antibiotic resistance due to a single mutation in *Neisseria gonorrhoeae*. J. Infect. Dis. **128**, 321–330.
51. Marger, M. D., Saier, M. H. (1993). A major superfamily of transmembrane facilitators that catalyze uniport, symport and antiport. Trends Biochem. Sci. **18**, 13–20.
52. Markham, P. N., Ahmed, M., Neyfakh, A. A. (1996). The drug-binding activity of the multidrug-responding transcriptional regulator BmrR resides in its C-terminal domain. J. Biol. Chem. **178**, 1473–1475.
53. Masuda, N., Ohya, S. (1992). Cross-resistance to meropenem, cephems, and quinolones in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **36**, 1847–1851.
54. Masuda, N., Sakagawa, E., Ohya, S. (1995). Outer membrane proteins responsible for multiple-drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39**, 645–649.
55. Masuda, N., Gotoh, N., Ohya, S., Nishino, T. (1996). Quantitative correlation between susceptibility and OprJ production in *nfxB* mutants of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **40**, 909–913.
56. McMurry, L., Petrucci Jr., R. E., Levy, S. B. (1980). Active efflux of tetracycline encoded by

four genetically different tetracycline resistance determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77**, 3974–3977.
57. Miller, P. F., Sulavik, M. C. (1996). Overlaps and parallels in the regulation of intrinsic multiple-antibiotic resistance in *Escherichia coli*. Mol. Microbiol. **21**, 441–448.
58. Nakamura, H. (1965). Gene-controlled resistance to acriflavin and other basic dyes in *Escherichia coli*. J. Bacteriol. **90**, 8–14.
59. Nakamura, H. (1968). Genetic determination of resistance to acriflavin, phenyl alcohol, and sodium dodecyl sulphate in *Escherichia coli*. J. Bacteriol. **96**, 987–996.
60. Naroditskaya, V., Schlosser, M. J., Fang, N. Y., Lewis, K. (1993). An *Escherichia coli* gene *emrD* is involved in adaptation to low-energy shock. Biochem. Biophys. Res. Commun. **196**, 803–809.
61. Neal, R. J., Chater, K. F. (1987). Nucleotide sequence analysis reveals similarities between proteins determining methylenomycin-A resistance in *Streptomyces* and tetracycline resistance in eubacteria. Gene **58**, 229–241.
62. Neyfakh, A. A. (1992). The multidrug efflux transporter of *Bacillus subtilis* is a structural and functional homolog of the staphylococcus NorA protein. Antimicrob. Agents Chemother. **36**, 484–485.
63. Neyfakh, A. A., Bidnenko,V. E., Chen, L. B. (1991). Efflux-mediated multidrug resistance in *Bacillus subtilis* similarities and dissimilarities with the mammalian system. Proc. Natl. Acad. Sci. USA **88**, 4781–4785.
64. Neyfakh, A. A., Borsch, C. M., Kaatz, G. W. (1993). Fluoroquinolone resistance protein NorAof *Staphylococcus aureus* is a multidrug efflux transporter. Antimicrob. Agents Chemother. **37**, 128–129.
65. Ng, E. Y. W., Trucksis, M., Hooper, D. C. (1994). Quinolone resistance mediated by NorA - physiological characterization and relationship to *flqB*, a quinolone resistance locus on the *Staphylococcus aureus* chromosome. Antimicrob. Agents Chemother. **38**, 1345–1355.
66. Nies, D. H., Nies, A., Chu, L., Silver, S. (1989). Expression and nucleotide sequence of a plasmid encoded divalent cation efflux system from *Alcaligenes eutrophus*. Proc. Natl. Acad. Sci. USA **86**, 7351–7355.
67. Nikaido, H. (1994). Prevention of drug access to bacterial targets - permeability barriers and active efflux. Science **264**, 382–388.
68. Nikaido, H. (1996). Multidrug efflux pumps of Gram-negative bacteria. J. Bacteriol. **178**, 5853–5859.
69. Ohshita, Y., Hiramatsu, K., Yokota, T. (1990). A point mutation in *norA* gene is responsible for quinolone resistance in *Staphylococcus aureus*. Biochem. Biophys. Res. Commun. **172**, 1028–1034.
70. Okazaki, T., Iyobe, S., Hashimoto, H., Hirai, K. (1991). Cloning and characterization of a DNA fragment that complements the *nfxB* mutation in *Pseudomonas aeruginosa* PAO. FEMS Microbiol. Lett. **79**, 31–35.
71. Ouellette, M., Legare, D., Papadopoulou, B. (1994). Microbial multidrug-resistance ABC transporters. Trends in Microbiology **2**, 407–411.
72. Pan, W. B., Spratt, B. G. (1994). Regulation of the permeability of the gonococcal cell-envelope by the *mtr* system. Mol. Microbiol. **11**, 769–775.
73. Paulsen, I. T., Littlejohn, T. G., Radstrom, P., Sundstrom, L., Skold, O., Swedberg, G., Skurray, R. A. (1993). The 3' conserved segment of integrons contains a gene associated with multidrug resistance to antiseptics and disinfectants. Antimicrob. Agents Chemother. **37**, 761–768.
74. Paulsen, I. T., Brown, M. H., Littlejohn, T. G., Mitchell, B. A., Skurray, R. A. (1996). Multidrug-resistance proteins QacA and QacB from *Staphylococcus aureus* - Membrane topology and identification of residues involved in substrate-specificity. Proc. Natl. Acad. Sci. USA **93**, 3630–3635.
75. Paulsen, I. T., Skurray, R. A., Tam, R., Saier Jr., M. H., Turner, R. J., Weiner, J. H., Goldberg, E. B., Grinius, L. L. (1996). The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs. Mol. Microbiol. **19**, 1167–1175.
76. Paulsen, I. T., Brown, M. H., Skurray, R. A. (1996c). Proton-dependent multidrug efflux systems. Microbiol. Rev. **60**, 575–608.
77. Poole, K. (1994). Bacterial multidrug-resistance - emphasis on efflux mechanisms and *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. **34**, 453–456.
78. Poole, K., Krebes, K., McNally, C., Neshat, S. (1993). Multiple antibiotic-resistance in *Pseudomonas aeruginosa* - evidence for involvement of an efflux operon. J. Bacteriol. **175**, 7363–7372.
79. Poole, K., Gotoh, N., Tsujimoto, H., Zhao, Q. X., Wada, A., Yamasaki, T., Neshat, S., Yamagishi, J. I., Li, X. Z., Nishino, T. (1996). Overexpression of the MexC-MexD-OprJ efflux operon in NfxB-type multidrug-resistant strains of *Pseudomonas aeruginosa*. Mol. Microbiol. **21**, 713–724.
80. Poole, K., Tetro, K., Zhao, Q. X., Neshat, S., Heinrichs, D. E., Bianco, N. (1996). Expression

of the multidrug-resistance operon MexA-MexB-OprM in *Pseudomonas aeruginosa* - MexR encodes a regulator of operon expression. Antimicrob. Agents Chemother. **40**, 2021–2028.

81. Purewal, A. S. (1991). Nucleotide sequence of the ethidium efflux gene from *Escherichia coli*. FEMS Microbiol. Lett. **82**, 229–232.
82. Rella, M, Haas, D. (1982). Resistance of *Pseudomonas aeruginosa* PAO to nalidixic acid and low levels of β-lactam antibiotics - mapping of chromosomal genes. Antimicrob. Agents Chemother. **22**, 242–249.
83. Roberts, M. C. (1996). Tetracycline resistance determinants - Mechanism of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev. **19**, 1–24.
84. Rock, C. O., Furukawa, H., Tsay, J. T., Jackowski, S., Takamura, Y. (1993). An altered EmrAB efflux pump imparts resistance to thiolactomycin but not cerulenin in *Escherichia coli*. FASEB Journal **7**, A1310.
85. Ross, J. I., Eady, E. A., Cove, J. H., Cunliffe, W. J., Baumberg, S., Wooton, J. C. (1990). Inducible erythromycin resistance in staphylococci is encoded by a member of the ATP-binding transport supergene family. Mol. Microbiol. **4**, 1207–1214.
86. Rosteck, P. R., Reynolds, P. A., Hershberger, C. L. (1991). Homology between proteins controlling *Streptomyces fradiae* tylosin resistance and ATP-binding transport. Gene **102**, 27–32.
87. Rouch, D. A., Cram, D. S., Diberardino, D., Littlejohn, T. G., Skurray, R. A. (1990). Efflux-mediated antiseptic resistance gene *qacA* from *Staphylococcus aureus*-common ancestry with tetracycline transport and sugar-transport proteins. Mol. Microbiol. **4**, 2051–2062.
88. Saier, M. H., Tam, R., Reizer, A., Reizer, J. (1994). Two novel families of bacterial-membrane proteins concerned with nodulation, cell-division and transport. Mol. Microbiol. **11**, 841–847.
89. Sasatsu, M., Shima, K., Shibata, Y., Kono, M. (1989). Nucleotide sequence of a gene that encodes resistance to ethidium bromide from a transferable plasmid in *Staphylococcus aureus*. Nucleic Acids Res. **17**, 10103.
90. Sundström, L., Rådström, P., Swedberg, G., Sköld, O. (1988). Site-specific recombination promotes linkage between trimethoprim- and sulfonamide resistance genes. Sequence characterization of *dhfrV* and *sulI* and a recombination active locus of Tn21. Mol. Gen. Genet. **213**, 191–201.
91. Takiff, H. E., Cimino, M., Musso, M. C., Weisbrod, T., Martinez, R., Delgado, M. B., Salazar, L., Bloom, B. R., Jacobs, W. R. (1996). Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in *Mycobacterium smegmatis*. Proc. Natl. Acad. Sci. USA **93**, 362–366.
92. Tennent, J. M., Lyon, B. R, Midgley, M., Jones, I. G., Purewal, A. S., Skurray, R. A. (1989). Physical and biochemical-characterization of the *qacA* gene encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. J. Gen. Microbiol. **135**, 1–10.
93. Thanassi, D. G., Suh, G. S. B., Nikaido, H. (1995). Role of outer-membrane barrier in efflux-mediated tetracycline resistance of *Escherichia coli*. J. Bacteriol. **177**, 998–1007.
94. Ubukata, K., Itohyamashita, N., Konno, M. (1989). Cloning and expression of the *norA* gene for fluoroquinolone resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **33**, 1535–1539.
95. Yerushalmi, H., Lebendiker, M., Schuldiner, S. (1995). EmrE, an *Escherichia coli* 12 kDa multidrug transporter, exchanges toxic cations and H^+ and is soluble in organic solvents. J. Biol. Chem. **270**, 6856–6863.
96. Yerushalmi, H., Lebendiker, M., Schuldiner, S. (1996). Negative dominance studies demonstrate the oligomeric structure of EmrE, a multidrug antiporter from *Escherichia coli*. J. Biol. Chem. **271**, 31044–31048.
97. Yoshida, H., Bogaki, M., Nakamura, S., Ubukata, K., Konno, M. (1990). Nucleotide-sequence and characterization of the *Staphylococcus aureus norA* gene, which confers resistance to quinolones. J. Bacteriol. **172**, 6942–6949.

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Molecular mechanisms of methicillin resistance in *Staphylococcus aureus*

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Summary

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are among the most common nosocomial pathogens. The most significant mechanism of resistance to methicillin in this species is the acquisition of a genetic determinant (*mecA* gene). However, resistance seems to have a more complex molecular basis, since additional chromosomal material is involved in such resistance. Besides, overproduction of penicillinase and/or alterations in the PBPs can contribute to the formation of resistance phenotypes. Genetic and environmental factors leading to MRSA are reviewed.

Key words: *Staphylococcus aureus,* methicillin-resistance, *mecA* gene, nosocomial pathogens, heteroresistance

Resumen

Las cepas de *Staphylococcus aureus* meticilin-resistentes (MRSA) constituyen uno de los patógenos nosocomiales más frecuentes. El mecanismo más destacado que conduce a la resistencia a la meticilina en esta especie bacteriana es la adquisición de un elemento genético (*mecA*). Sin embargo, la resistencia parece tener un origen más complejo, dado que hay material cromosómico adicional que interviene en dicha resistencia. Por otra parte la superproducción de penicilinasa o las alteraciones en las proteínas fijadoras de penicilina pueden también contribuir a la aparición de fenotipos resistentes. Se revisan los aspectos genéticos y ambientales que han conducido a la aparición de MRSA.

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Introduction

Methicillin, the first of the penicillinase-resistant semisynthetic penicillins, was introduced into clinical practice in 1960. One year later, the first methicillin-resistant *Staphylococcus aureus* (MRSA) strains were reported in England (22). Since then, numerous medical centers, first in Europe in the 1960's (4, 21) and then in the United States in the 1970's (10, 29), have reported outbreaks of nosocomial infections caused by MRSA. Nowadays, MRSA remains as one of the most common nosocomial pathogens, especially in large tertiary hospitals and in critical care units, where colonized and infected patients are a significant source of infection spreading to other patients (16, 30).

The most significant mechanism of methicillin resistance among *S. aureus* is associated with the acquisition of a genetic determinant, the *mecA* gene, which is included on a 30 to 50 kb DNA element of non-staphylococcal origin (2, 3).

However, methicillin resistance seems to be a more complex mechanism. Several additional chromosomal sites have been identified recently, outside the *mecA* determinant, which contribute to the phenotypic expression of methicillin resistance (7, 11, 13, 35). In addition, borderline methicillin resistance can be achieved by high penicillinase production (6, 25) or by alterations in the penicillin-binding proteins (PBPs) (34).

Resistance to methicillin often implies not only resistance to the penicillinase-resistant penicillins but also to all beta-lactam antibiotics by poor binding to the altered PBPs (8, 11). Furthermore, and for reasons not fully understood, MRSA strains have also become resistant to almost all commonly used antibiotics with the exception of glycopeptides (11), which remain the only antimicrobial agents available for treating serious infections.

The *mecA* gene and its genetic regulation

All MRSA isolates examined so far contain the *mecA* gene, a 2 kb piece of foreign DNA, that appears to be of non-staphylococcal origin (2, 3, 23). The *mecA* gene codes for a novel PBP termed PBP 2A or PBP 2' which has low binding affinity for beta-lactam antibiotics (18). It is generally assumed that this protein acts as a secondary enzyme to catalyze the synthesis of peptidoglycan under conditions when normal staphylococcal PBPs are inactivated by the antibiotic in the medium (11). The *mecA* gene is found in practically all methicillin-resistant staphylococci, both *S. aureus* and coagulase-negative staphylococci, and it is absent in methicillin-susceptible *S. aureus* strains (20). The detection of the *mecA* gene has been considered to be the gold standard for the correct identification of MRSA. Accordingly, several PCR and hybridization detection methods of *mecA* have been developed for this purpose (1, 27).

The *mecA* gene was found as a component of a large DNA fragment of 30 to 50 kb (*mec* DNA) that integrates into a specific site in the chromosome of *S. aureus* (Fig. 1) (2, 19). The junction between *mec* DNA and *S. aureus* chromosome is bound by incomplete inverted repeats of about 20 bases (indicated by asterisks in Fig. 1) sug-

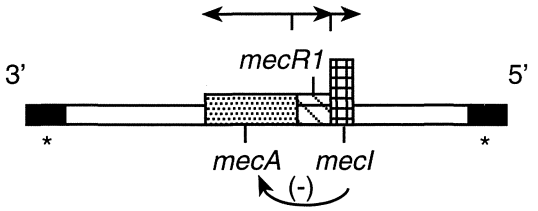

FIG. 1. Schematic representation of *mec* DNA and *mecA* regulation. Open reading frames are indicated by arrow heads. The incomplete inverted repeats flanking *mec* DNA at the junction site of chromosomal integration are indicated by asterisks.

gesting some site specificity for the *mec* DNA location (2, 19).

The length of *mec* DNA is highly variable in *S. aureus*, depending on the number of insertion and deletion events that have occurred (5). In some strains, the *mecA* upstream region contains two regulatory genes (20, 32) that were named *mecR1* and *mecI* based on their strong homology to the *bla* regulatory genes (*blaR1* and *blaI*) which control the inducible production of *S. aureus* penicillinase. The products of *mecR1* and *mecI* genes are a putative transmembrane-signaling protein—MecR1—and a putative repressor—MecI—, respectively (19). These elements are expected to regulate *mecA* gene transcription just as *bla* regulators do on *blaZ* gene (36). Although there has been no direct evidence, the *mecR1* product might act as a transmembrane beta-lactam sensing protein. MecR1 might detect the presence of beta-lactam antibiotics outside the cytoplasmic membrane, and induce the derepression of *mecA* by releasing the MecI repressor protein bound to the operator region of the *mecA* gene (5, 32). When uninduced, i.e. in absence of beta-lactam antibiotics, *mecA* transcription is strongly repressed and methicillin resistance cannot be expressed. Methicillin is a poor inducer of this system, so these strains may appear phenotypically susceptible in routine resistance test (5). However, many clinical *S. aureus* isolates have mutations or deletions that inactivate the *mecI* repressor gene, expressing a high level methicillin-resistance. Furthermore, many MRSA lack the *mecI* gene and have deletions reaching into *mecR1*, thus producing PBP 2A constitutively (5, 20).

Penicillin binding protein 2A and its role in methicillin resistance

PBPs are enzymes that catalyze the cross-linking reactions between peptidoglycan polymers, one of the final steps in bacterial cell wall assembly (5). Beta-lactam antibiotics bind covalently to the active sites of PBPs, leading to a defective, poorly crosslinked cell wall, and finally to cell death and lysis (9). Susceptible strains of *S. aureus* produce at least four PBPs. The enzymatic activities which can be developed by PBPs (as transpeptidases, endopeptidases and carboxypeptidases), have not been defined completely (8, 17). PBPs 2 and 3 have been found to be essential for cell growth and survival (17).

Methicillin resistance is associated with the production of an additional 78 kD PBP, termed PBP 2A or PBP 2'. PBP 2A is encoded by the genetic determinant *mecA*, it has a transpeptidase activity and, characteristically, has low binding affinity for beta-lactam antibiotics (9). In contrast to the normal staphylococcal PBPs which usually bind beta-lactams at low concentrations, PBP 2A binds beta-lactams only at high concentrations. Affinity studies between methicillin and PBPs have shown that methicillin concentrations over 250 μg/ml were capable of saturating only half of the PBP 2A enzymatic activity (9).

When the methicillin level begins to increase in the environment of the bacteria, and the normal set of staphylococcal PBPs become inactivated, it is assumed that PBP 2A takes over the task of cell wall synthesis (10). However, some changes were found in the composition of the peptidoglycan produced under these conditions. De Jonge et al. (14, 15) analyzed the cell wall composition from MRSA strains grown both in drug-free and in methicillin-containing media. Purified peptidoglycan was digested with a muramidase, and the muropeptides obtained were separated by high-pressure liquid chromatography (15). In the wall of cells growing normally , over 60% of the muropeptides were composed of trimers or higher oligomers species (14). In the

peptidoglycan of cells grown in the presence of methicillin (>5 μg/ml), this complex structure was replaced by a simple one in which the proportions of oligomers had been reduced to about 15% and it consisted of monomeric and dimeric units (14). These observations suggest that MRSA may have a second enzymatic system available for peptidoglycan synthesis when methicillin concentration reaches certain level. Under these conditions, PBP 2A may develop a particular transpeptidase activity which would only link two monomers together, thus generating a more simple cell wall structure (11).

Phenotypic expression of methicillin resistance. Heteroresistance

The phenotypic expression of methicillin-resistance presents several interesting features. In spite of the presence of the *mecA* gene and its product PBP 2A, the resistance levels to methicillin and other beta-lactams may vary widely from one strain to another, expressing minimal inhibitory concentration (MIC) values as low as 2 μg/ml, to over 800 μg/ml (33). Some strains exhibit a "heterogeneous" type of resistance: most of the cells in a population show a basal resistance to methicillin, along with a minor subpopulation of cells capable of growing in the presence of higher methicillin concentrations (12, 31, 33). The basal resistance corresponds to the MIC of the strain. For the clinical microbiologist, heterogeneity of MRSA was primarily a problem, making it difficult the identification of MRSA by conventional methods. In addition, methicillin resistance expression was shown to be strongly influenced by external factors such as inoculum, temperature, osmolarity and pH (8, 33). The expression of methicillin resistance is enhanced by antibiotic passage eliminating the susceptible cells and selecting the highly resistant subpopulation. The homogeneous pattern persists in these antibiotic selected cells in the absence of the antibiotic, but it is unstable. By repeated subculturing in a drug-free medium, the culture reverts to its former heterogeneous pattern of heteroresistance (33).

Tomasz et al. (33) classified MRSA clinical strains into four classes depending to the level of basal resistance and the proportion of the highly resistant subpopulation (Table 1): classes I, II and III were heterogeneous strains whose major subpopulations had 1.5–3, 6–12 and 50–200 μg/ml methicillin MICs, respectively, and class IV included homogeneous strains (MIC >800 μg/ml) (12). Classes I and II are methicillin-susceptible according to the National Comittee for Clinical Laboratory Standards (NCCLS) criteria (28). However, methicillin-resistant variants can emerge, capable of growing in the presence of, at least, 100 μg/ml, with frequencies between 10^{-8} and 10^{-6}. These properties (frequency and resistance level) are strain-specific, and highly reproducible under experimental conditions (12, 33).

The genetic and/or physiological basis of this drug-resistance diversity has not been fully understood. One hypothesis suggests that the highly resistant minority consists of mutants that are selected by methicillin pressure (5, 31). Although *mecA* has to be present in the cell population to obtain such mutants, intrinsinc resistance seems to involve other genetic determinants in the *mec* carrier strains. The presence of intrinsic low-level resistance in the background of MRSA was first suggested by De Lencastre et al. (12); following selective inactivation of the *mecA* in both, homogeneous and heterogeneous isolates, a decrease in methicillin MIC was detected. However, the residual methicillin MIC for these inactivated MRSAs was higher than the MIC expected for a truly susceptible strain. Recently, Ryffel et al. (31) suggested that high-

TABLE 1. Expression classes of methicillin-resistant staphylococci

Expression class	MIC (μg/mL) for cell majority[1]	Frequency of highly resistant cells[2]	Phenotypic expression
Class I	1.5–3	10^{-7}–10^{-8}	Heterogeneous
Class II	6–12	10^{-5}–10^{-6}	Heterogeneous
Class III	50–200	10^{-2}–10^{-3}	Heterogeneous
Class IV	> 800		Homogeneous

[1] Methicillin MIC that inhibits the growth of 99% of cells.
[2] Frequency of cells capable of growing in the presence of 100 mg/mL of methicillin.

level methicillin resistance could be due to the presence of chromosomal mutation(s) in undetermined site(s) (*chr**). These *chr** mutation wouls have no effect on susceptible strains and would become apparent only in the presence of *mecA* (5, 31).

Other genes that affect methicillin resistance levels

The level (MIC) and mode of expression (homogeneous vs. heterogeneous) of methicillin resistance can be very variable among MRSA isolates carrying the *mecA* gene and producing comparable amounts of PBP 2A. This fact suggested that other factors of an unknown nature, other than *mecA* gene product, also played an important role in the phenotypic expression of beta-lactam resistance (7, 35).

The role of other genetic determinants in methicillin resistance expression have been described following insertional inactivation studies (6, 7, 13, 35). Transposition experiments were performed on a highly and homogeneously resistant MRSA background using Tn*551*; and the mutants obtained were screened for reduced levels of methicillin resistance (13). Tn*551* insertions in the chromosome of the parental strain led to the identification of at least five different loci named *fem* (factor essential for the expression of methicillin resistance) (6, 7) or *aux* (auxiliary) (35) located outside the *mecA* determinant; they belong to the normal set of *S. aureus* chromosomal DNA genes and their inactivation lead to a decrease in the level of methicillin resistant and/or a change in its phenotypic expression (6, 7).

The exact role of the auxiliary genes (*fem A, B, C, D,* or *E*) is still unknown; they are involved in cell wall metabolism and in the synthesis of cell wall precursors. For example, the *femAB* operon is involved in the production of the pentaglycine interpeptide bridge (6) by catalyzing the addition of glycines in positions 2, 3 (*femA*), 4 and 5 (*fem B*). The *femAB* operon is also involved in the cell division process and in the formation of cell septa, and their inactivation results in poorly crosslinked peptidoglycan and reduced cell-wall turnover (5, 6).

It is not clear how many auxiliary genes exist and how these genes collaborate exactly with the *mecA* determinant in maintaining a high-level beta-lactam resistance. De Lencastre et al. (11) proposed a model to explain how these two models could work together. In the presence of methicillin, the normal set of staphylococcal PBPs become inactivated and the low affinity PBP 2A takes over the task of cell wall synthesis; under these conditions, the complex staphylococcal wall structure is replaced by a simple one. However, the effective functioning of PBP 2A also requires the abundant supply of structurally correct cell wall building blocks

which then successfully compete with the methicillin molecules for the active site of PBP 2A. Altered cell wall precursors (e.g. in *fem* mutants) compete less effectively with the antibiotic molecule, thus the level of methicillin resistance decreases (11).

Borderline resistance and non *mec*-dependant resistance to methicillin

Although the most significant and prevalent mechanism of methicillin resistance in *S. aureus* is associated with the acquisition of the additional *mecA* gene and its product PBP 2A, strains with reduced susceptibility or borderline methicillin resistance (MIC 8–16 μg/ml), lacking this genetic determinant, are commonly found among clinical isolates. Two categories of biological mechanisms could be responsible for borderline methicillin resistance: one depending on beta-lactamase activity; the other relating to alterations of PBPs.

According to an earlier hypothesis (26), the production of large amounts of penicillinase may show borderline resistance to penicillinase-resistant penicillins by partial hydrolysis. However, the assistance of a novel beta-lactamase seems to be essential in the expression of this low level resistance. Massidda et al. (24) have described a methicillin-hydrolyzing beta-lactamase (methicillinase). This plasmid-encoded methicillinase was shown to be produced in addition to the classical penicillinase, and to be necessary for the expression of borderline resistance (25).

Other mechanisms involved in borderline resistance expression are based on PBPs modifications, either by decreasing the affinity of the normally produced staphylococcal PBPs for binding beta-lactam antibiotics, or by changes in the amount of PBP produced. The molecular explanation for that could be the accumulation of multiple mutations on PBP genes (5), which would lead either to structural PBP's modifications (reducing the binding affinity of existing PBPs), or to PBP overproduction, as was shown for PBP 4 (34).

Methicillin-resistant *S. aureus* that do not contain *mecA* are difficult to differentiate by standard procedures from low level resistant true MRSA (carrying the *mecA* gene). The differentiation is important since MRSA, even when of heteroresistance or low level resistance, are able to produce highly resistant subpopulations, which will survive beta-lactam therapy (5).

Conclusions

Methicillin resistance expression is a complex process in which multiple genetic and environmental factors are involved. The acquisition of *mecA* gene provides the prerequisite for resistance, while the genetic background of the strain determines the mode and level of resistance expressed (5, 11).

Intensive genetic, biochemical studies are in progress to better understand the molecular basis of methicillin resistance, which will be of great help to find new targets and to design new antibiotics.

References

1. Archer, G. L., Pennell, E. (1990). Detection of methicillin resistance in staphylococci by using a DNA probe. Antimicrob. Agents Chemother. **34**, 1720–1724.
2. Archer, G. L., Niemeyer, D. M. (1994). Origin and evolution of DNA associated with resistance to methicillin in staphylococci. Trends Microbiol. **2**, 343–347.
3. Beck, W. D., Berger-Bächi, B., Kayser, F. H. (1986). Additional DNA in methicillin-resistant

Staphylococcus aureus and molecular cloning of *mec*-specific DNA. J. Bacteriol. **65**, 373–378.
4. Benner, E. J., Kayser, F. H. (1968). Growing clinical significance of methicillin-resistant *Staphylococcus aureus*. Lancet **ii**, 741–744.
5. Berger-Bächi, B. (1994). Expression of resistance to methicillin. Trends Microbiol. **2**, 389–392.
6. Berger-Bächi, B., Strässle, A., Gustafson, J. E., Kayser, F. H. (1992). Mapping and characterization of multiple chromosomal factors involved in methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **36**, 1367–1373.
7. Berger-Bächi, B., Strässle, A., Kayser, F. H. (1989). *femA*, a host mediated factor essential for methicillin resistance in *Staphylococcus aureus*: molecular cloning and characterization. Mol. Gen. Genet. **219**, 263–269.
8. Chambers, H. F. (1988). Methicillin-resistant staphylococci. Clin. Microbiol. Rev. **1**, 173–186.
9. Chambers, H. F., Sachdeva, M. (1990). Binding of beta-lactam antibiotics to penicillin-binding protein in methicillin-resistant *Staphylococcus aureus*. J. Infect. Dis. **161**, 1170–1176.
10. Crossley, K., Loesch, D., Landesman, B., Mead, B., Mead, K., Chem, M., Strate, R. (1979). An outbreak of infections caused by strains of *Staphylococcus aureus* resistant to methicillin and aminoglycosides. J. Infect. Dis. **139**, 280–287.
11. De Lencastre, J., De Jonge, B. L. M., Matthews, F. R., Tomasz, A. (1994). Molecular aspects of methicillin resistance in *Staphylococcus aureus*. J. Antimicrob. Chemother. **33**, 7–24.
12. De Lencastre, H., Figueiredo, A. M., Urban, C., Rahal, J., Tomasz, A. (1991). Multiple mechanisms of methicillin resistance and improved methods for detection in clinical isolates of *Staphylococcus aureus*. Antimicrob. Agents Chemother. **35**, 632–639.
13. De Lencastre, H., Tomasz, A. (1994). Reassessment of the number of auxiliary genes essential for expression of high-level methicillin resistance in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **38**, 2590–2598.
14. De Jonge, B. L. M., Chang, Y. S., Gage, D., Tomasz, A. (1992). Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain: the role of penicillin binding protein 2A. J. Biol. Chem. **276**, 11248–11254.
15. De Jonge, B. L. M., Tomasz, A. (1993). Abnormal peptidoglycan produced in a methicillin-resistant strain of *Staphylococcus aureus* grown in the presence of methicillin: functional role for penicillin-binding protein 2A in cell wall synthesis. Antimicrob. Agents Chemother. **37**, 342–346.
16. Domínguez, M. A., De Lencastre, H., Liñares, J., Tomasz, A. (1994). Spread and maintenance of a dominant methicillin-resistant *Staphylococcus aureus* (MRSA) clone during an outbreak of MRSA disease in a Spanish hospital. J. Clin. Microbiol. **32**, 2081–2087.
17. Georgopapadakou, N. H., Dix, B. A., Mauriz, Y. R. (1986). Possible physiological functions of penicillin-binding proteins in *Staphylococcus aureus*. Antimicrob. Agents Chemother. **29**, 333–336.
18. Hartman, B. J., Tomasz, A. (1986). Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. J. Bacteriol. **158**, 513–516.
19. Hiramatsu, K. (1995). Molecular evolution of MRSA. Microbiol. Immunol. **39**, 531–543.
20. Hürlimann-Dalel, R. L., Ryffel, C., Kayser, F. J., Berger-Bächi, B. (1992). Survey of the methicillin resistance-associated genes *mecA*, *mecR1-mecI*, and *femA-femB* in clinical isolates of methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **36**, 2617–2621.
21. Jessen, O., Rosendal, K., Bulow, P., Faber, V., Eriksen, K. R. (1969). Changing staphyloccci and staphylococcal infections: a ten year study of bacteria and cases of bacteremia. N. Eng. J. Med. **281**, 627–635.
22. Jevons, M. P. (1961). "Celbenin"-resistant staphylococci. Br. Med. J. **1**, 124–125.
23. Kuhl, S. A., Pattee, P. A., Baldwin, J. N. (1978). Chromosomal map location of the methicillin resistance determinant in *Staphylococcus aureus*. J. Bacteriol. **135**, 460–465.
24. Massidda, O., Montanari, M. P., Varaldo, P. E. (1992). Evidence for a methicillin-hydrolyzing beta-lactamase in *Staphylococcus aureus* strains with borderline susceptibility to this drug. FEMS Microbiol. Lett. **92**, 223–227.
25. Massidda, O., Montanari, M. P., Mingoia, M., Varaldo, P. E. (1996). Borderline methicillin-susceptible *Staphylococcus aureus* strains have more in common than reduced susceptibility to penicillinase-resistant penicillins. Antimicrob. Agents Chemother. **40**, 2769–2774.
26. McDougal, L., Thomsberry, C. (1984). The role of beta-lactamase in staphylococcal resistance to penicillinase-resistant penicillins and cephalosporins. J. Clin. Microbiol. **23**, 832–839.
27. Murakami, K., Minamide, W., Wada, K., Nakamura, E., Teraoka, H., Watanabe, S. (1991). Identification of methicillin-resistant strains of

staphylococci by polymerase chain reaction. J. Clin. Microbiol. **29**, 2240–2244.

28. National Committee for Clinical Laboratory Standards (1997). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. NCCLS document M7-A4. National Committee for Clinical Laboratory Standards. Vilanova, Pa.
29. Peacock, J. R., Marsik, F. J., Wenzel, R. P. (1980). Methicillin-resistant *Staphylococcus aureus*: introduction and spread within a hospital. Ann. Intern. Med. **93**, 526–532.
30. Pujol, M., Peña, C., Pallares, R., Ariza, J., Ayats, J., Domínguez, M. A., Gudiol, F. (1996). Nosocomial *Staphylococcus aureus* bacteremia among nasal carriers of methicillin-resistant and methicillin-susceptible strains. Am. J. Med. **100**, 509–516.
31. Ryffel, C., Strässle, A., Kayser, F. H., Berger-Bächi, B. (1994). Mechanisms of heteroresistance in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **38**, 724–728.
32. Tesch, W., Ryffel, C., Strassle, A., Kayser, F. H., Berger-Bächi, B. (1990). Evidence of a novel staphylococcal *mec*-encoded element (*mecR*) controlling expression of penicillin-binding protein 2'. Antimicrob. Agents Chemother. **32**, 1703–1706.
33. Tomasz, A., Nachman, S., Leaf, H. (1991). Stable classes of phenotypic expression in methicillin-resistant clinical isolates of staphylococci. Antimicrob. Agents Chemother. **35**, 124–129.
34. Tomasz, A., Drugeon, H. B., De Lencastre, H., Jabes, D., McDougal, L., Bille, J. (1989). New mechanism for methicillin resistance in *Staphylococcus aureus*: clinical isolates that lack the PBP2A gene and contain normal penicillin-binding proteins with modified penicillin-binding capacity. Antimicrob. Agents Chemother. **33**, 1869–1874.
35. Tomasz, A. (1990). Auxiliary genes assisting in the expression of methicillin resistance in *Staphylococcus aureus*. *In* Novick, R. P. (ed.), Molecular Biology of the Staphylococci. pp. 565–584, VCH Publishers, New York.
36. Zhu, Y. F., Curran, I. H. A., Joris, B., Ghuysen, J. M., Lampen, J. O. (1990). Identification of BlaR, the signal transducer for beta-lactamase production in *Bacillus licheniformis*, as a penicillin-binding protein with strong homology to the OXA-2 beta-lactamase (classD) of *Salmonella typhymurium*. (1990). J. Bacteriol. **172**, 1137–1141.

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Molecular basis of antimicrobial resistance in non-typable *Haemophilus influenzae*

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Summary

Strains of the facultative anaerobe *Haemophilus influenzae*, both type b and non typable strains, are frequently multiresistant. The measurement of the antibiotic permeability of *Haemophilus influenzae* outer membrane (OM) shows that antibiotics can cross through the OM easily. Thus, enzymatic activity or efflux pumps could be responsible for multiresistance. An efflux system closely related to AcrAB of *Escherichia coli* is present in *Haemophilus influenzae*. However, their role in multiresistance seems irrelevant. Classical mechanisms such as plasmid exchange seems to be playing a major role in the multidrug resistance in *Haemophilus influenzae*.

Key words: *Haemophilus*, outer membrane, efflux pump, multidrug resistance, plasmid exchange

Resumen

Las cepas del anaerobio facultativo *Haemophilus influenzae*, tanto las de tipo b como las no tipables, son con frecuencia multirresistentes. Se ha medido la permeabilidad de la membrana externa de *Haemophilus influenzae* y los resultados demuestran que los antibióticos pasan fácilmente a través de esta membrana. En consecuencia, cabe pensar que la actividad enzimática o las bombas

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de reflujo serían responsables de la multirresistencia. Un sistema de reflujo, estrechamente relacionado con AcrAB de *Escherichia coli* se encuentra activo en las cepas de *Haemophilus influenzae*. Sin embargo, su papel en la multirresistencia parece irrelevante. Son los mecanismos clásicos, tales como el intercambio de plásmidos los que parecen la principal causa de multirresistencia en *Haemophilus influenzae*.

Introduction

The facultative anaerobe *Haemophilus influenzae* includes obligate parasites characteristic of human and other mammalian mucosal surfaces. Most strains can be classified in 7 distinct biotypes on the basis of simple biochemical tests. The species includes both capsulated and unencapsulated strains, and whereas the former exhibit prominent virulence, the latter have been related to several kinds of disease (27). Diseases associated with *H. influenzae* include meningitis, pneumonia, epiglotitis, arthritis, cellulitis, pericarditis, neonatal infections, among others (13).

Several studies reporting antimicrobial resistance in *Haemophilus* have been published. Geographically, major differences can be detected when comparing data from the US and Europe. Spanish data account for the highest percentages of multiple resistance. In the US, 20% of the strains were ampicillin-resistant, most of them owing to the production of β-lactamase (9). Although in the rest of Europe the fraction of ampicillin-resistant strains is only 10.9%, in Spain 63.3% of b-type and 25.8% of non-b strains are ampicillin-resistant (6). Chloramphenicol resistance shows data quite similar : 0.5% of strains in US (9), 4.7% in Europe (18), and 45% in Spain (4). Similar situations can be described concerning other antibiotics (4). Multiple resistance to ampicillin and chloramphenicol was described associated to meningitis. In Spain, such a type of multiple resistance can be detected in approximately 45% of strains (5).

The emergence of resistant bacteria is the result of selective pressure exerted by the continuous presence of antibiotics in the hospital environment. Furthermore, well known pathogenic species which initially were highly susceptible to antibiotics suffered a selection due to the conditions provided by the indiscriminate use of antibiotics in extrahospital media. Then, these strains acquired plasmids and/or transposons encoding for resistance, or some mutations leading to antimicrobial resistance took place.

Despite differences in the susceptibilities to antimicrobial agents, genetic diversity in this species is really low. In a study of a collection of *H. influenzae* strains isolated in a single Spanish hospital by using many biochemical tests, a genetic diversity among non typable strains was as low as H = 0.04. However, if data concerning antimicrobial resistance were used to calculate diversity, a value of 0.28 would be obtained.

The high degree of homogeneity among strains makes the study of the genetic structure of *H. influenzae* populations difficult. The same collection was used to study the clonality by investigating 15 structural loci. The study also included several susceptible strains isolated in the same hospital. All enzymes were polymorphic, and the analysis revealed many electrophoretic types.

When the multilocus linkage disequilibrium was measured, a strong association between alleles was detected, suggesting an unlikely genetic mixing (11).

Outer membrane permeability

As antibiotic resistance has become a major concern, especially in Spain, and outer membrane permeability is frequently a cause of multiple resistance, the knowledge of outer membrane composition is a matter of great interest. The various components of the external leaflet of the species have been studied by several research groups. It is a well-established fact that in certain species lipopolysaccharide (LPS) is an important encoding factor for microbial virulence (7, 19). The LPS of typable *H. influenzae* has been studied by electrophoresis mostly in type b strains (29). Data obtained from the electrophoresis of purified LPS of non-typable strains demonstrated no differences with the LPS obtained from type b or other typable serogroups. In all cases, the LPS had only short O-side chains. Similarly, electrophoretic profiles exhibited no major differences between the LPS of different non-typable strains (Sánchez, submitted). This remarkable similarity in LPS content suggests that no differences in outer membrane permeability between strains are due to this component.

Until recently, little was known about the role of the outer membrane in antibiotic resistance in *H. influenzae*. Over the last few years our group has been carrying out a series of studies in which the outer membrane of *H. influenzae* has been examined. Outer membrane proteins (OMPs) of non-typable *H. influenzae* have previously been studied by electrophoresis by Barenkamp and co-workers (1, 2) using a wide collection of strains different sources. Initially, the interest focused on the identification of certain molecular structures presumably useful in vaccine design, but later attempts were made to provide an epidemiological marker for *Haemophilus*. According to Barenkamp and coworkers (1,2), the electrophoretic profiles of OMP were highly diverse; the same happened when strains were obtained in a single hospital. A total of nine major proteins have been described. Among the OMPs from *H. influenzae,* only Omp2 has been reported to be a true porin (3). Studies in vitro by using artificial bilayer and measuring pore conductance have demonstrated that Omp2 is one of the largest pores known in nature. The pore has an exclusion limit of 1.4 kDa (8, 28), which is in agreement with the high in vivo permeability of the outer membrane to some β-lactams obtained in experiments performed using the Zimmermann and Rosselet technique (25, 30). Initially the major differences in OMP profiles suggested variations in outer membrane permeability to antibiotics in non-typable strains. When the Zimmermann and Rosselet technique was applied to such strains, the values were as high as those presented in Table 1. The calculation of these permeability values was accurate, since no addition of any inducer was necessary and the enzymatic parameters of β-lactamase were low enough to allow good measurements. The addition of inducer in β-lactamase expression tends to damage the bacterial envelopes, and the values of permeability coefficients might be overestimated because of enzyme leakage. P values reported by Sánchez et al. (26) can be regarded as being very high, especially when compared with those obtained in other species. To sum up, antibiotics enter the periplasmic space of *H. influenzae* following

TABLE 1. P coefficients and TAI values of five isolates of *Haemophilus influenzae*

Strain	P (10^{-4} cm/sec)	TAI	AI_{peri}	AI_{OM}
1208	0.37	0.48	1.45	0.33
1373	0.51	0.52	1.16	0.45
1253	0.93	0.42	0.51	0.82
1276	0.6	1.37	2.55	0.54
1220	0.92	0.69	0.84	0.81

kinetics, which is only very slightly affected by the permeability barrier of the outer membrane of *H. influenzae*. In fact, high permeability values are characteristic of this species. When alternative methods, such as Target Index Determination (TAI) (the probability of β-lactam reaching the Penicillin Binding Proteins) (20), were used, very high values were once again obtained. TAI values are determined by two factors: outer membrane permeability and enzymatic activity in the periplasmic space (AI_{OM} and AI_{peri} respectively). We having obtained both values, it became apparent that enzymatic activity is highly "responsible" for antimicrobial resistance of *H. influenzae* (26). If antibiotics entered the cell with such ease, this would suggest the presence of a highly active efflux mechanism that could pump much of the antibiotic out. We, therefore, developed further studies to assess the possible role of efflux pumps in the antibiotic resistance of *H. influenzae*.

Antimicrobial efflux

For many years, the intrinsic resistance to antimicrobial agents was attributed to the presence of the external leaflet, that is the outer membrane, and its narrow pores, which were thought to limit the diffusion of solutes. In recent years, several studies have argued that the membrane barrier cannot fully explain the intrinsic resistance (21, 22). The active efflux of drugs, dyes and detergents seems also to contribute efficiently to the resistance. Two kinds of efflux pump have been described: the "classical" pump, able to secrete only one class of drugs (12); and the multidrug efflux pump, which can secrete a large variety of compounds (16, 22, 23). Among multidrug efflux pumps described, MexAB-OprM in *Pseudomonas aeruginosa* (14, 24) and *acr*RAB in *Escherichia coli* (15, 17) are the most fully characterized. Homologous of these systems have been described in many Gram-negative species.

In 1995, Fleischmann et al. (10) published the complete sequence of the *H. influenzae* Rd chromosome. Three of the sequenced genes exhibited a high degree of homology with those of *acrRAB* system in *E. coli*. A 2.8 kb fragment containing whole *acrR* and *acrA* and part of *acrB H. influenzae* homologous genes was prepared using PCR. This fragment was used to perform insertional mutations in *acrA* and *acrB* genes by using a kanamycin cartridge, and mutations were introduced into Rd wild-type strain by homologous recombination (25). When the susceptibilities to different antimicrobials in the wild-type strain and the mutants were compared, the results were agreed with those obtained in *E. coli* by Ma et al. (15) for their description of *acrAB* system in *E. coli*. This means that the disruption of any of the *acrA* or *acrB* genes makes the mutants hypersusceptible to various agents. Uptake experiments of erythromycin supported these conclusions. However, and possibly due to the high outer membrane permeability of *H. influenzae*, no differences were observed in the MIC levels of ampicillin, tetracycline, chloramphenicol and ciprofloxacine.

Concluding remarks

The role of outer membrane and efflux mechanisms in antimicrobial resistance in *H. influenzae* seems to be negligible, since the high permeability suggest the idea of a vicious circle in which antibiotics pass very efficiently through the outer membrane. This would allow to reach high antibiotic concentration in the periplasmic and cytoplasmic spaces. However, the efflux pump (*acrRAB*) would work properly pumping the antibiotic molecules out. Then, a vicious cycle

of entry and exit of antibiotic molecules would be established.

From a populational point of view, antimicrobial resistance in *H. influenzae*, like in other species, could be regarded as a result of genetic exchange. However, populational studies and analysis of linkage disequilibrium suggested low possibility of chromosomal genetic mixing. This is in agreement with the results observed when unselected characters (like chromosomal encoded enzymes or biochemical trends) are studied (11). The high diversity observed in antimicrobial susceptibility profiles (a strongly selected character) suggest that resistance characters have their origin in genetic exchange between microorganisms. On the other hand, values for outer membrane permeability (high) were not related with the differences in the outer membrane protein or LPS profiles. Efflux pump resulted also with a poor role in determining antimicrobial resistance. Both outer membrane permeability and efflux pumps are chromosomally encoded and submitted to a low selective pressure when bacteria lives in constant habitats. Habitats like vertebrate mucosal surfaces should be regarded as very constant and then, it is feasible that no strong selective pressure acts on the microbiota. The wild-type level expression of one efflux pump was shown to be important in the baseline resistance to erythromycin, novobiocin and rifampicin, but had little effect in the susceptibility to fluoroquinolones, chloranphenicol, tetracycline, and ampicillin. However, it is still possible that overexpression of the pump, either mediated through the putative repressor AcrR or through mutations, could produce low levels of resistance to various agents, so that the bacteria can survive while accumulatting more specific and more effective mechanisms of plasmid-coded resistance. In the final stages, the "classical" mechanisms such as plasmid exchange seems to be playing a predominant role in the multidrug resistance in *H. influenzae*, at least at the present moment.

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References

1. Barenkamp, S. J., Munson, R. S., Granoff, D. M. (1981). Subtyping isolates of *Haemophilus influenzae* type b by outer membrane protein profiles. J. Infect. Dis. **143**, 668–676.
2. Barenkamp, S. J., Munson, R. S., Granoff, D. M. (1982). Outer membrane protein and biotype analysis of pathogenic non-typable *Haemphilus influenzae*. Infect. Immun. **36**, 535–540.
3. Burns, J. L., Smith, A. L. (1987). A major outer membrane protein functions as a porin in *Haemophilus influenzae*. J. Gen. Microbiol. **133**, 1273–1277.
4. Campos, J., García Tornel, S., Sanfeliu, I. (1984). Susceptibility studies of multiple resistant *Haemophilus influenzae* isolated from paediatric patients and contacts. Antimicrob. Agents Chemother. **25**, 706–709.
5. Campos, J., García-Tornel, S., Gairí, J. M., Fabregues, J. (1986). Multiple resistant *Haemophilus influenzae* type b causing meningitis. Comparative clinical and laboratory study. J. Pediatrics. **108**, 897–902.
6. Campos, J. (1988). Resistència antibiòtica en *Haemophilus influenzae*. Enf. Inf. Microbiol. Clin. **6**, 117–119.
7. Cryz, Jr. S. J., Pitt, T. L., Ferer, E., Germanier, R. (1984). Role of lipopolysaccharide in virulence of *Pseudomonas aeruginosa*. Infect. Immun. **44**, 508–513.
8. Dahan, D., Vachon, V., Laprade, R., Coulton, J. W. (1994). Voltage gating of porins from *Haemophilus influenzae* type b. Biochim. Biophys. Acta **1189**, 204–211.
9. Doern, G. V., Jorgensen, J. H., Thornsberry, C., Preston, D. A., Taubert, T., Redding, J. S.,

Maher, L. A. (1988). National collaborative study of the prevalence of antimicrobial resistance among clinical isolates of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **32**, 180–185.

10. Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., Bult, C. J., Tomb, J. F., Dougherty, B. A., Merrick, J. M., McKenney, K., Sutton, G., FitzHugh, W., Fields, C., Gocayne, J. D., Scott, J., Shirley, R., Liu, L. I., Glodeck, A., Kelley, J. M., Weidman, J. F., Philips, C. A., Spriggs, T., Hedblom, E., Cotton, M. D., Utterback, T. R., Hanna, M. C., Nguyen, D. T., Saudeck, D. M., Brandon, R. C., Fine, L. D., Fritchman, J. L., Fuhrmann, J. L., Geoghagen, N. S. M., Gnehm, C. L., McDonald, L. A., Small, K. V., Fraser, C. M., Smith, H. O., Venter, J. C. (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269**, 496–512.
11. Fusté, M. C., Pineda, M. A., Palomar, J., Viñas, M., Loren, J. G. (1996). Clonality of multidrug-resistant nontypeable strains of *Haemophilus influenzae*. J. Clin. Microbiol. **34**, 2760–2765.
12. George, A. M., Levy, S. M. (1983). Amplificable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: Involvement of a non-plasmid-determined efflux of tetracicline. J. Bacteriol. **155**, 531–540.
13. Hoiseth, S. K. (1992). The genus *Haemophilus*. *In* Ballows, A., Trüpper, H. G., Dworkin, M., Tno, W. H., Schleifer, K. H. (ed.), The Prokaryotes, 2nd. Vol. III. pp. 3304–3330, Springer-Verlag, New York.
14. Li, X.-Z., Nikaido, H., Poole, K. (1995). Role of MexA-MexB-OprM in antibiotic efflux in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39**, 1948–1953.
15. Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H., Hearts, J. H. (1993). Molecular cloning and characterization of *acrA* and *acrB* genes from *Escherichia coli*. J. Bacteriol. **175**, 6299–6313.
16. Ma, D., Cook, D. N., Hearts, J. E., Nikaido, H. (1994). Efflux pumps and drug resistance in Gram-negative bacteria. Trends Microbiol. **2**, 489–493.
17. Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H., Hearts, J. H. (1995). Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol. Microbiol. **16**, 45–55.
18. Machka, K., Bravery, Y., Dobemat, M., Dornbusch, K., Van Dych, E., Kayser, F. H., Van Klingeren, B., Perea, E., Powell, M. (1988). Distribution and resistance-patterns of *Haemophilus influenzae*: a european cooperative study. Euro. J. Clin. Microbiol. Infect. Dis. **7**, 14–24.
19. Mäkela, P. H., Valtonen, V. V., Valtonen, M. (1973). Role of O-antigen (lipopolysaccharide) factors in the virulence of *Salmonella*. J. Infect. Dis. Suplement, S81–S85.
20. Nikaido, H., Gehring, K. (1988). Significance of the outer membrane barrier in β-lactam resistance. *In* Actor, P., Daneo-Moore, L., Higgins, M. L., Salton, M. R. J., Shockman, G. D. (ed.), Antibiotic Inhibition of Bacterial Cell Surface Assembly and Function. pp. 419–435, ASM. Washington, DC.
21. Nikaido, H. (1989). Outer membrane barrier as a mechanism of antimicrobial resistance. Antimicrob. Agents Chemother. **33**, 1831–1836.
22. Nikaido, H. (1994). Prevention of drug acces to bacterial targets: permeability barriers and active efflux. Science **264**, 382–388.
23. Nikaido, H. (1996). Multidrug efflux pumps of Gram-negative bacteria. J. Bacteriol. **178**, 5853–5859.
24. Poole, K., Krebes, K., McNally, C., Neshat, S. (1993). Multiple antibiotic resistance in *Pseudomonas aeruginosa*: evidence for involment of an efflux operon. J. Bacteriol. **175**, 7363–7372.
25. Sánchez, L., Pan, W., Viñas, M., Nikaido, H. (1997). The *acrAB* homolog of *Haemophilus influenzae* codes for a functional multidrug efflux pump. J. Bacteriol. (In press).
26. Sánchez, L., Puig, M., Fusté, C., Lorén, J. G., Viñas, M. (1996). Outer membrane permeability of non-typable *Haemophilus influenzae*. J. Antimicrob. Chemother. **37**, 341–344.
27. Stuy, H. J. (1978). On the nature of nontypable *Haemophilus influenzae*. Antonie van Leeuwenhoek **44**, 367–376.
28. Vachon, V., Laprade, R., Coulton, J. W. (1986). Properties of the porin of *Haemophilus influenzae* type b in planar lipid bilayer membranes. Biochim. Biophys. Acta **861**, 74–82.
29. Weiser, J. N. (1992). The oligosaccharide of *Haemophilus influenzae*. Microb. Pathog. **13**, 342–355.
30. Zimmermann, W., Rosselet, A. (1977). Function of the outer membrane of *Escherichia coli* as a permeability barrier to β-lactam antibiotics. Antimicrob. Agents Chemother. **12**, 368–372.

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The role of outer membrane in *Serratia marcescens* intrinsic resistance to antibiotics

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Summary

Three different porins from *Serratia marcescens* were described. They were named Omp1, Omp2 and Omp3 and their molecular weights were 42, 40 and 39 kDa respectively. Omp2 and Omp3 showed osmoregulation and thermoregulation in a similar way to OmpC and OmpF of *Escherichia coli*. Permeability coefficients of the outer membrane of this species were calculated following the Zimmermann and Rosselet method. P values were similar to those obtained in *Escherichia coli*, which suggests that the chromosomal β-lactamase would play a major role in the resistance of *Serratia marcescens* to β-lactam antibiotics. Both MIC values and permeabilities were modified by salycilates and acetylsalycilate. Synergism between the outer membrane and the β-lactamase was also evaluated. When bacteria grew in the presence of a β-lactam in the medium, the β-lactamase accounted for most of the resistance.

Key words: *Serratia marcescens*, intrinsic resistance, porins, synergism, β-lactam

Resumen

Se han descrito tres porinas de *Serratia marcescens* denominadas Omp1, Omp2 y Omp3, con unos pesos moleculares de 42, 40 y 39 kDa respectivamente. Dos de estas porinas (Omp2 y Omp3) muestran osmorregulación y termorregulación, al igual que ocurre con OmpC y OmpF de *Escherichia coli*. Se han calculado los coeficientes de permeabilidad de la membrana externa de esta especie y,

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siguiendo la metodología de Zimmermann y Rosselet, los valores de P son muy similares a los de *Escherichia coli*. Esto indica que la β-lactamasa cromosómica tiene un papel destacado en la resistencia de *Serratia marcescens* a los β-lactámicos. La presencia de salicilatos y ácido acetilsalicílico en el medio de cultivo modifica los valores de CMI así como la permeabilidad de la membrana. Se ha evaluado también el sinergismo entre la membrana externa y la β-lactamasa, mostrando que cuando la bacteria crece en presencia de una β-lactama, la β-lactamasa es la principal responsable de la resistencia.

Introduction

The enterobacterium *Serratia marcescens* had been long considered a harmless microbe; so much, that experiments using pigmented strains were performed even to demonstrate the ascent of bacteria around Foley catheters. However, this bacterium is now considered to be a major nosocomial pathogen. Its ability to act as an opportunistic pathogen and to produce a large variety of infectious diseases, including urinary tract infections, bacteremia, endocarditis, soft tissue infections, arthritis, osteomyelitis, ocular infections, meningitis, abscesses and others, have altered radically the status of the species (23). Moreover, several pathogenicity factors in the species have been described (3, 10, 12, 17, 18).

The fact that *S. marcescens* was not considered a major pathogen until only recently, means there are relatively few data concerning the antimicrobial susceptibility of the bacterium in the past. However, a number of studies were published in the 1960's in which almost all strains exhibited resistance to tetracycline, ampicillin, nitrofurantoin, polymyxin B and cephalothin, even if almost 50% of the strains were resistant to streptomycin and chloramphenicol. The species is now considered intrinsically resistant to a large variety of antimicrobial agents. Additionally, most, if not all, strains of *S. marcescens* elaborate chromosomally encoded β-lactamase. Besides, the transfer of R factors from *Serratia* isolates to *Escherichia coli* was first described in 1969 and in a number of subsequent studies (4).

The intrinsic resistance of *Serratia* to β-lactams and to a large number of other antibiotics suggested that some general resistance mechanisms should be constitutively active in the species. *Serratia*, like all Gram-negative bacteria, is surrounded by an outer membrane which forms a semi permeable barrier to many compounds. The diffusion of hydrophilic nutrients, waste products, antibiotics, etc. through this outer membrane is carried out by means of small trimeric proteins called porins. Several studies have been published dealing with the outer membrane proteins of *Serratia* (7, 8, 11, 21, 22).

Outer membrane proteins of *Serratia*

Malouin et al. (11) reported, in 1990, the presence of a single porin of *Serratia* (strain UOC69, a clinical isolate) with a molecular mass of 41 kDa. Their polyacrylamide gel electrophoresis analysis showed the presence of a unique protein in the porin region together with an OmpA-like protein. However, these authors, in the final sentences of their report, were reluctant to conclude that *S. marcescens* had a unique porin. Puig et al. (21), two years later, reported an electrophoretic study dealing with the outer membrane porins of *Serratia*. Two alternative polyacrylamide gel electrophoresis methods were used; one based on Laemmli (9) and the other

using a very similar method but involving the addition of 4 M urea to the separating gel. In this conditions the 41 kDa porin was resolved into three separate bands, where the molecular masses of the major outer membrane proteins were 42, 40, 39 and 37 kDa. These were named Omp1, Omp2, Omp3 and OmpA, since the 37 kDa protein accomplished the typical feature of OmpA protein (i.e. heat modifiability). Neither osmoregulation nor thermoregulation had previously been demonstrated for the 41 or 40 kDa "porins". However, when Omp1, 2 and 3 were tested for osmoregulation, it was demonstrated that in the absence of added NaCl, Omp1, 2 and 3 could be clearly distinguished. Omp2 and Omp3 seemed to be present at very similar concentrations, whereas Omp1 was the most clearly expressed protein. When the bacteria were incubated in a medium with 600 mM NaCl, Omp3 almost disappeared, while the concentration of Omp2 increased. However, in all cases, the overall concentration of both proteins seemed to remain constant, since when one of them increased, the other decreased; in some cases Omp3 could be hardly detected. Similar results were obtained when glucose concentrations were assayed. A comparison of the thermoregulation revealed no differences in porin expression were detected between 30 and 37°C. However, when lower temperatures were assayed, certain differences appeared, including a decrease in the amount of Omp2 present. This suggested that *E. coli* porins and *S. marcescens* porins behaved differently due to the varying optimal temperature of both species (21, 22). *S. marcescens* optimal temperature is much lower than that of *E. coli*. Therefore, the comparison of their expressions at different temperatures needs to be established at temperatures ranging from 18°C to 30°C.

Hutsul and Worobec (7) and Hutsul et al. (8) undertook the cloning and molecular characterization of a protein of 40 kDa. In at work, a 11 kb EcoRI fragment was subcloned and introduced into *E. coli*. The examination for expression demonstrated that the product was a 40 kDa protein similar in sequence to some *E. coli* porins. However, the physiological behaviour of that protein differed markedly from that of *E. coli* porins, since no osmoregulation or thermoregulation could be demonstrated. The general conclusion reached by the authors was that the 40 kDa protein was a porin similar to the *E. coli* OmpC porin.

Outer membrane permeability

Several attempts to calculate the permeability of the outer membrane of *S. marcescens* to β-lactams have been made (5, 6, 8, 11, 19, 20, 22). An initial hypothesis might be that the multidrug intrinsic resistance to antibiotics in the species is due to the low permeability of its outer membrane. Malouin et al. (11) reported that the 41 kDa protein behaved in a similar way to that of non-selective porin in experiments using black planar lipid bilayer. A pore size of 1.1 nm was estimated for such porin (1.62 nS in 1.0 M KCl). However, the histogram of the single channel conductance values could be interpreted in two ways, (i) where only one porin is acting as a channel or (ii) where several proteins (the mixture of all porins) are being assayed (no major differences in distribution can be expected). The authors reported values of P coefficient for cephaloridine using the Zimmermann and Rosselet technique (24). Values calculated for P reached 30×10^{-5} cm s^{-1}, which were higher than values detected in porin mutants of *E. coli* expressing only OmpC, but smaller than values detected when OmpF alone or both OmpF and OmpC were expressed.

We performed measurements in other strains of *S. marcescens* (Table 1). The values reported

TABLE 1. Values of P (10^{-6} cm s^{-1}) coefficients in strains of *Serratia marcescens*

Antibiotic	NIMA	NR1	NR2	2170
Cephalothin	4.8	4.3	5.4	7.0
Cephaloridine	29.4	6.3	28	11

are in the same range as those obtained in *E. coli* (Nikaido, personal communication). These results suggest that the two dominant factors in β-lactamase resistance in this species are the outer membrane and the β-lactamase. It has often been assumed that a low permeability value for the outer membrane of *Serratia* might explain its intrinsic resistance (13). However, results obtained in our laboratory, and partially reported here, show that β-lactamase (inducible) is responsible for differences between the MIC values of *Serratia* and *Escherichia*.

The effect of salycilates or acetylsalycilate (aspirin) on the outer membrane permeability has been described in various studies.The effectiveness of β-lactamic antibiotics on Gram-negative bacteria depends upon their efficiency in passing through the outer membrane. The increased resistance of bacteria to antibiotics, particularly β-lactams, may occur as a result of decreased outer membrane permeability. Sodium salycilates, as well as aspirin, have been described as inducing a phenotypic resistance in several Gram-negative bacteria such as *E. coli* (2) or *Pseudomonas* (1). It was demonstrated that this increased resistance was due to a decrease in the outer membrane permeability, that it was reversible, and that it required protein synthesis, which affects the outer membrane expression. Similar effects have been described in *Serratia* (20). The MICs of ampicillin, cefotaxime, cefoxitin, chloramphenicol, kanamycin, cephalothin and cephaloridine when bacteria were cultured in the presence of salycilate or acetylsalycilate were modified and quite considerably in some cases (e.g. MIC of cefotaxime increased 20-fold in the presence of therapeutic concentrations of salycilate). In other cases the modification was almost negligible or even undetectable. However, when salycilates were used at therapeutic concentrations outer membrane electrophoretic profiles failed to show any noticeable differences. These data suggest that extreme caution should be exercised when using analgesics and antibiotics simultaneously. When permeability coefficients were calculated in the presence of salycilates a good correlation between the decrease of permeabilities and the increase in resistance was found.

The chromosomal β-lactamase of *Serratia*

S. marcescens produces a chromosomally-encoded β-lactamase (6) which has been characterized by isoelectrofocusing and shown to be a single inducible β-lactamase (Class C) with a pI 8. The kinetic parameters of the enzyme for cephalothin and cephaloridine were calculated and the results are presented in Table 2. Calculations were made following the methods described by Nikaido and Gehring (16). This allows the estimation of the synergism between the outer membrane and β-lactamase activity. As can be seen, the activity of β-lactamase increased markedly when a β-lactam was present during cell growth (Table 2). If we consider the synergism between the outer membrane and the enzyme, in the presence of an inducer (maximal β-lactamase expression), the target access indexes for cephalothin and cephaloridine were lower in *S. marcescens* (Table 2) than indexes found in *E. coli*, which is in agreement with the higher MICs of *Serratia*. The two half indexes indicate that when bacteria grow with a β-lactam in the medium, the β-lactamase accounts for

TABLE 2. Variables related to the interplay between the two barriers in *Serratia marcescens*

	Cephalothin		Cephaloridine	
	Induced	Uninduced	Induced	Uninduced
MIC	10,000		312.5	
Vmax	0.72	0.0051	0.995	0.0103
AI_{OM}	1.61	1.61	2.99	2.99
AI_{peri}	5.1×10^{-4}	4.0×10^{-2}	4.6×10^{-4}	5.6×10^{-2}
TAI	8.4×10^{-4}	6.5×10^{-2}	1.4×10^{-3}	16.8×10^{-2}

MIC: susceptibility in μg/ml. Vmax: nmol × μg $prot^{-1} \times s^{-1}$. AI_{OM} and AI_{peri}: access indexes that reflect the probability of crossing the outer membrane (AI_{OM}) and of diffusing though the periplasm (AI_{peri}). TAI: Target access index.

most of the resistance. In conclusion, although low susceptibility needs to be explained on the basis of both restricted outer membrane permeability and chromosomal inducible β-lactamase, the differences observed between the MICs values of *Serratia* and those of certain other enterobacteria should be regarded as a consequence of the differences between the enzymatic parameters of chromosomally encoded β-lactamase. However, certain aspects of the bacterial resistance remain unknown. In the recent years, mechanisms of antibiotic efflux in bacteria have been described. In this way, bacteria pump out antibiotic molecules, thereby reducing the concentration of the antimicrobial agent in the cytoplasmic and periplasmic spaces (14, 15). Work is currently being carried out in our laboratory to determine the efficiency of the efflux mechanisms in this species.

References

1. Burns, J. L., Clark, D. A. (1992). Salycilate-inducible antibiotic resistance in *Pseudomonas cepacia* associated with absence of a pore-forming outer membrane protein. Antimicrob. Agents Chemother. **36**, 2280–2285.
2. Foulds, J., Murray, D. M., Chai, T., Rosner, J. L. (1989). Decreased permeation of cephalosporins through the outer membrane of *Escherichia coli* grown in salycilates. Antimicrob. Agents Chemother. **33**, 412–417.
3. Franczek, S. P., Williams, R. P., Hull, S. I. (1986). A survey of potential virulence factors in clinical and environmental isolates of *Serratia marcescens*. J. Med. Microbiol. **22**, 151–156.
4. Grimont, P. A. D., Grimont, F. (1978). The genus *Serratia*. Ann. Rev. Microbiol. **32**, 221–248.
5. Hancock, R. E. W. (1987). Role of porins in outer membrane permeability. J. Bacteriol. **169**, 929–933.
6. Hechler, U., van den Weghe, M., Martin, H. H., Frère, J. M. (1989). Overproduced β-lactamase and the outer membrane barrier as resistance factors in *Serratia marcescens* highly resistant to β-lactamase-stable β-lactam antibiotics. J. Gen. Microbiol. **135**, 1275–1290.
7. Hutsul, J. A., Worobec, E. (1994). Molecular characterization of a 40 fDa OmpC-like porin from *Serratia marcescens*. Microbiol. **140**, 379–387.
8. Hutsul, J. A., Worobec, E., Parr, T. R., Becker, G. W. (1993). Comparative analyses of *Serratia* spp. outer membrane porin proteins. Can. J. Microbiol. **39**, 442–447.
9. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680–685.
10. Leranoz, S., Orús, P., Berlanga, M., Dalet, F., Viñas, M. (1997). New fimbrial adhesins of *Serratia marcescens* isolated from urinary tract infections: description and properties. J. Urol. **157**, 694–698.
11. Malouin, F., Campbell, G. D., Halpenny, M., Becker, G. W., Parr, T. R. (1990). Outer membrane and porin characteristics of *Serratia marcescens* grown in vitro and in rat intraperitoneal chambers. Infect. Immun. **58**, 1247–1253.
12. Montilla, R., Williams, R. P., Lorén, J. G., Viñas, M. (1991). Lipopolysaccharide is the receptor for kappa phage in *Serratia marcescens*. Antonie van Leeuwenhoek **59**, 15–18.
13. Neu, H. C. (1992). The crisis in antibiotic resistance. Science **257**, 1064–1073.
14. Nikaido, H. (1994). Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science **264**, 382–387.
15. Nikaido, H. (1992). Porins and specific channels of bacterial outer membrane. Mol. Microbiol. **6**, 435–442.
16. Nikaido, H., Gehring, K. (1988). Significance of the outer membrane barrier in β-lactam resist-

ance. *In* Actor, P., Daneo-Moore, L., Higgins, M. L. et al. (ed.), Antibiotic Inhibition of Bacterial Assembly and Function, pp. 419–435. ASM. Washington, DC.

17. Palomar, J., Leranoz, A. M., Viñas, M. (1995). *Serratia marcescens* adherence: the effect of O-antigen presence. Microbios **81**, 107–113.
18. Palomar, J., Montilla, R., Fusté, C., Viñas, M. (1993). The role of O-antigen in susceptibility of *Serratia marcescens* to non-immune serum. Microbios **76**, 189–196.
19. Palomar, J., Puig, M., Montilla, R., Lorén, J. G., Viñas, M. (1995). Lipopolysaccharide recovery restores susceptibility levels towards β-lactams in *Serratia marcescens*. Microbios **82**, 21–26.
20. Puig, M., Palomar, J., Lorén, J. G., Viñas, M. (1995). Modification by analgesics of the susceptibility to antiobiotics in *Serratia marcescens*. New Microbiol. **18**, 385–390.
21. Puig, M., Fusté, C., Viñas, M. (1993). Outer membrane proteins from *Serratia marcescens*, Can. J. Microbiol. **39**, 108–111.
22. Puig, M. (1994). La membrana externa de *Serratia marcescens* i la resistència als antibiòtics β-lactamics. Tesis Doctoral. Universitat de Barcelona.
23. von Graevenitz, A. (1980). Infection and colonization with *Serratia*. *In* von Gaevenitz, A., Rubin, S. J. (ed.), The Genus *Serratia*, pp. 168–179. CRC press Boca Raton Fla.
24. Zimmermann, W., Rosselet, A. (1977). Function of the outer membrane of *Escherichia coli* as a permeability barrier to β-lactam antibiotics. Antimicrob. Agents Chemother. **12**, 368–372.

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Purification of OmpU from *Vibrio cholerae* classical strain 569B: evidence for the formation of large cation-selective ion-permeable channels by OmpU

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Summary

The outer membrane of the classical *Vibrio cholerae* strain 569B was isolated by sucrose density centrifugation. The simple treatment of the isolated outer membrane or the cell envelopes with different detergents allowed the purification of two outer membrane proteins, the 38 kDa OmpU and the 25 kDa OmpV. Furthermore, a 35 kDa outer membrane protein (probably the 35 kDa OmpA-like protein) was purified by two-fold treatment of the cell envelope with 2% SDS solution. A subsequent wash of the SDS-pellet with 2% Genapol buffer yielded in the 38 kDa OmpU protein, which formed SDS-resistant oligomers (66 kDa). The Genapol pellet contained OmpV. Reconstitution experiments with lipid bilayer membranes demonstrated that OmpU was a channel-forming component, whereas OmpV had a small channel-forming ability if any. The OmpU channels appeared to be large and water-filled and had a single-channel conductance of about 2 nS in 1 M KCl for the monomer in a trimer, which means that they have a larger cross-section than enterobacterial porins. The channels showed rapid switching between open and closed configuration. They were slightly cation-selective, which suggests that they contain an excess of negatively charged amino groups.

Key words: *Vibrio cholerae* classical strain, porin, outer membrane, OmpU, lipid bilayer membrane

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Resumen

Se ha aislado la membrana externa de la cepa clásica 569B de *Vibrio cholerae* mediante centrifugación en gradiente de sacarosa. Un sencillo tratamiento de la membrana externa aislada o de las envueltas celulares con distintos detergentes, permitió la purificación de dos proteínas de membrana externa de 38 kDa (OmpU) y de 25 kDa (OmpV). Además, mediante tratamiento de las envueltas celulares con 2% de SDS por duplicado, se aisló una proteína de 35 kDa (probablemente la proteína homóloga a OmpA). Lavados subsiguientes con Genapol al 2% permitieron recuperar OmpU (38 kDa), que forma oligómeros resistentes al SDS (66 kDa). El sedimento obtenido a partir del Genapol contenía la proteína OmpV. Los experimentos de reconstitución con bicapas lipídicas demostraron que OmpU era un componente formador de poros. En cambio, OmpV tiene, en el mejor de los casos, una débil actividad formadora de poros. Los canales de OmpU son de gran tamaño, están rellenos de agua y presentan una conductancia, para cada monómero en el trímero, de 2nS en KCl 1M. Esto significa que presenta una sección mucho mas ancha que las porinas de las enterobacterias. Los canales muestran una tendencia al cambio rápido entre la configuración abierta y cerrada. Finalmente, presentan una escasa selectividad catiónica, lo que sugiere que contienen un exceso de grupos amino cargados negativamente.

Introduction

The outer membrane of Gram-negative bacteria contains a small number of major proteins, which are present in high copy number (3, 20). Some of these proteins, termed porins, form channels in the outer membrane that are responsible for the uptake of nutrients. Other proteins such as OmpA of *Escherichia coli* and the 35 kDa outer membrane protein of *Vibrio cholerae* do not form channels but are responsible for outer membrane stability and adherence to the peptidoglycan layer (1, 3). Porins can be divided in two groups: the general diffusion porins that form water-filled channels, and the specific porins that are specific channels for one class of solutes (3, 20). The structural analysis of porins shows that they are formed by 16–18 stranded antiparallel β-barrel cylinders as the main structural motif of these membrane proteins (25, 29). Porins usually consist of three identical subunits each containing one channel, and are very stable towards denaturing conditions like heat and detergents.

The Gram-negative *V. cholerae* is an intestinal pathogen that is non-invasive and adheres to the apical side of the small intestine (2, 24). The epidemic qualities of this pathogen have recently been dramatically underscore by the reappearance of cholera in the Western Hemisphere after more than 100 years and by the emergence of a new strain, the *V. cholerae* O-139 Bengal strain, that appeared in India and spread rapidly to countries surrounding the Indian Ocean (15). To establish an infection, *V. cholerae* colonizes the intestinal surface and releases cholera toxin composed of two subunits (12, 23). The binding component (5 B subunits) binds to the surface and facilitates the entry of the A subunit into the epithelial cells. The A subunit effects an increase of the intracellular level of cyclic AMP. The high level of intracellular AMP stimulates the secretion of chloride and bicarbonate out of the cell and leads to a diarrhoea because of the severe loss of isotonic body fluid (2, 24).

The outer membrane of *V. cholerae* contains five to six major proteins. One of them, OmpS (45 kDa) is induced upon growth on maltose and

has a similar function as LamB of *E. coli* and *Salmonella typhimurium* (16). OmpV (25 kDa) is resistant to trypsin and is associated with the peptidoglycan layer (27). A 35 kDa outer membrane protein resembles probably the function of OmpA in *E. coli* (1). Two other major outer membrane proteins, OmpT and OmpU appear to be osmoregulated, and it has been suggested that they could form outer membrane channels similar to the osmoregulated OmpF and OmpC channels of certain enteric bacteria (17, 18, 19). Recently, it has been demonstrated that the 38 kDa OmpU and the 40 kDa OmpT proteins, possess channel-forming ability in proteoliposomes (10). Here we describe a simple method for the isolation and purification of OmpU and OmpV from a *V. cholerae* classic strain (569B). Furthermore, we purified a 35 kDa outer membrane protein to homogeneity. The trimeric protein OmpU but not OmpV or the 35 kDa protein formed ion-permeable channels with a moderate cation-selectivity when reconstituted into lipid bilayer membranes. The high single-channel conductance in 1 M KCl suggested that the channels formed by OmpU are large and water-filled.

Materials and methods

Bacterial strains and growth conditions. The classical strain 569B (Δtox S) of *V. cholerae* (22) (biotype classical; serotype, Inaba) was grown overnight at 37°C in DYT media, with shaking.

Isolation of bacterial cell envelopes. The cells were harvested by centrifugation (1000 × *g*, 10 min), washed once with a 50 mM tris-hidroxy-aminomethane HCl (Tris-HCl) solution pH 7.2, and resuspended in the same buffer. The suspension was passed three times through a French pressure cell (900 psi), unbroken cells were removed by centrifugation (1000 × *g*, 10 min), and the supernatant was centrifuged (100,000 × *g*, 60 min).

Preparation of the outer membrane. The envelope fraction was layered on top of a five step sucrose gradient (35, 40, 45, 50 and 55%; each 2.3 ml volume). The gradient was centrifuged for 36 h in a SW 41 rotor (Beckman) at 36,000 rpm. The gradient was divided in fractions of 500 µl and their content was checked by sodium dodecylsulfate-polyacrylamide-gelelectrophoresis (SDS-PAGE). The bottom fractions (fraction 1 to 4) of the gradient contained the outer membrane, which was pelleted by centrifugation (100,000 × *g*, 60 min). The inner membrane was present in fractions 11 to 13.

Preparation of the peptidoglycan associated proteins and SDS-PAGE. The cell envelopes or the outer membranes were resuspended in a buffer containing 2% SDS, 10 mM Tris-HCl pH 8 and 2 mM $MgSO_4$. The peptidoglycan layer and the associated proteins were centrifuged at 100,000 × *g* for 30 min. The different steps were checked on a 12% SDS-PAGE stained with Coomassie Brilliant Blue.

Purification of a 35 kDa outer membrane protein and OmpU. The final pellet (either outer membranes or peptidoglycan-associated proteins) was subjected to a second SDS wash. The supernatant of this wash contained a pure outer membrane protein when cell envelopes were used (see Results section). The pellet was suspended in a buffer containing 2% Genapol, 10 mM Tris-HCl pH 8 and 2 mM ethylenediamine tetraacetic acid (EDTA). The supernatant of the subsequent centrifugation (100,000 × *g*, 30 min) contained pure OmpU porin as judged from 12% SDS-PAGE stained with Coomassie Brilliant Blue. The pellet contained OmpV.

Proteolytic digest and peptide sequencing. Purified 35 kDa outer membrane protein or OmpU was precipitated using trichloroacetic acid to remove the detergent. Then 100 µg of the protein

was digested with 2.5 μg trypsin at 37°C for 8 h. After addition of another 2.5 μg trypsin the digestion was continued for 12 h. Proteolytic peptides were separated by reversed phase high-performance liquid chromatography (HPLC) using a 250 × 4 mm Vydac 2181P column equilibrated with 0.12% trifluoroacetic acid. The peptides were eluted over 90 min by a linear gradient of 0–35% acetonitrile containing 0.1% trifluoroacetic acid (flow rate 0.7 ml/min). The effluent was monitored at 215 nm. The amino acid sequence of two peptides of interest was determined by the Edman degradation method using a gas phase sequenator (470A, Applied Biosystems) with on-line detection of the amino acids.

Membrane experiments. The methods used for black lipid bilayer experiments have been described previously (4). The instrumentation consisted of a Teflon chamber with two compartments separated by a thin wall. The membranes were formed from a 1% (w/v) solution of diphytanoyl phosphatidylcholine (Avanti Biochemicals, Birmingham, AL) in n-decane. The protein was added from the concentrated protein solutions (concentration 1 mg/ml) either immediately before membrane formation or after the membrane had turned black. The single-channel conductance of the pores was measured after application of a fixed membrane potential with a pair of calomel electrodes with salt bridges inserted into the aqueous solutions on both sides of the membrane.

The zero-current membrane potentials were measured as previously described (5). The membranes were formed in a 10 mM KCl solution containing a predetermined protein concentration so that the membrane conductance increased about 100- to 1000-fold within 10 to 20 min after membrane formation. At this time the instrumentation was switched to the measurements of the zero-current potentials and the salt concentration on one side of the membrane was raised by adding small amounts of concentrated salt solutions. The zero-current membrane potential reached its final value within 2 to 5 min.

Results

Purification of a 35 kDa outer membrane protein and OmpU. Fig. 1 shows the protocol of the purification of a 35 kDa outer membrane protein and OmpU. Lane 1 corresponds to the marker enzymes and lane 2 to the inner membrane (fraction 12) obtained from the sucrose density gradient. Lanes 3 and 4 show the composition of the outer membrane (fraction 2 of the sucrose density gradient centrifugation) and of the cell envelope, respectively. Proteins from the outer membrane or the cell envelope were isolated and purified in a two step procedure. First all SDS-soluble material was removed from either the outer membranes or the cell envelope by a SDS-wash (lane 5, first SDS-supernatant). Then this SDS-wash was repeated once. The composition of the supernatant contains only one protein when outer membranes were used (data not shown) or in the case of the cell envelopes (lane 6), which had the same mobility when it was not heated (data not shown). In a following step, part of the peptidoglycan-associated proteins was removed by treatment with Genapol in the presence of EDTA. This procedure was very efficient, yielding in pure oligomeric OmpU as judged from SDS-PAGE (66 kDa; lane 8), which dissociated in monomers when the protein was heated (38 kDa; lane 7). The pellet of the Genapol treatment contained only small traces of OmpU, if any. It was enriched in OmpV (25 kDa, data not shown). OmpT, which has probably also channel function, was not detected under our growth conditions as checked by SDS-PAGE containing urea. It is only expressed in large

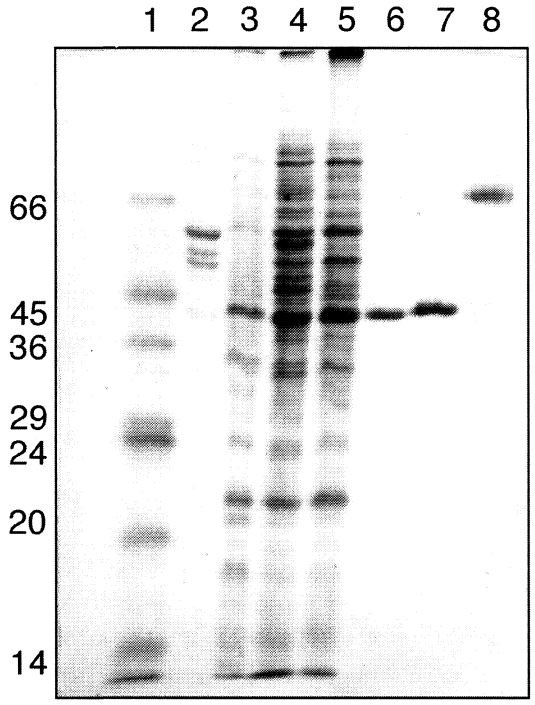

FIG. 1. 12% SDS-PAGE of different steps of the purification of OmpU from *Vibrio cholerae* strain 569B ΔtoxS stained with Coomassie blue. Lane 1: Molecular mass markers 66, 45, 36, 29, 24, 20 and 14 kDa. Lane 2: Inner membrane (fraction 12 of the sucrose density gradient), 100 μl were precipitated and solubilized at 100°C in sample buffer. Lane 3: Outer membrane (fraction 2 of the sucrose density gradient), 100 μl were precipitated and solubilized at 100°C in sample buffer. Lane 4: 15 μl of the cell envelope, solubilized at 100°C in sample buffer. Lane 5: 15 μl of the supernatant of the first SDS-wash of the cell envelope, solubilized at 100°C in sample buffer. Lane 6: 20 μl of the supernatant of the second SDS-wash of the cell envelope, solubilized at 100°C in sample buffer. Lane 7: 20 μl of the supernatant of the first Genapol-wash, solubilized at 100°C in sample buffer. Lane 8: 20 μl of the supernatant of the first Genapol-wash, solubilized at 20°C in sample buffer.

quantities, when *V. cholerae* is grown in high osmolarity media (10, 18).

Reconstitution of *V. cholerae* proteins in lipid bilayer membranes. We checked the channel-forming ability of the different proteins in experiments with lipid bilayer membranes. When OmpU from *V. cholerae* was added in small concentrations (around 100 ng/ml) to the aqueous phase bathing a black lipid bilayer membrane from diphytanoyl phosphatidylcholine/n-decane, the specific membrane conductance increased several orders of magnitude. The time course was similar to that described earlier for bacterial porins from *E. coli* and *Rhodobacter capsulatus* (7, 8). After an initial rapid increase for 15 to 20 min, the membrane conductance increased at a much slower rate. The addition of the detergent alone at the same concentrations did not lead to any significant increase of the membrane conductance. The result of these experiments defined OmpU as a channel-forming protein. We made a second set of similar experiments with the 35 kDa outer membrane protein. After its addition in a concentration of 100 ng/ml to the aqueous phase we observed only a increase of the membrane conductance lower than 2% of the increase with OmpU. When we added protein to the lipid bilayer membranes that contained essentially OmpV (from the pellet of the Genapol treatment), the conductance increase was even lower (less than 0.2% of that observed with OmpU).

Single-channel analysis of channel formed by OmpU. The addition of small amounts of OmpU porin to lipid bilayer membranes allowed the resolution of single-channel. Fig. 2 shows a single-channel record observed with 10 ng/ml of OmpU using a diphytanoyl phosphatidylcholine membrane in the presence of 1 M KCl. A few min after the addition of the protein we observed steps of about 6 nS. It is noteworthy that these channels were very noisy (A). This was caused by the rapid opening and closing of the channels (see inset in A). In fact, the channels formed by OmpU showed rapid opening and closing as indicated in Fig. 2B. The lifetime of the open state was only about 100 ms. Otherwise the conductance fluctuations were fairly homogeneous as derived from histograms (data not shown). Only a few channels were observed that had

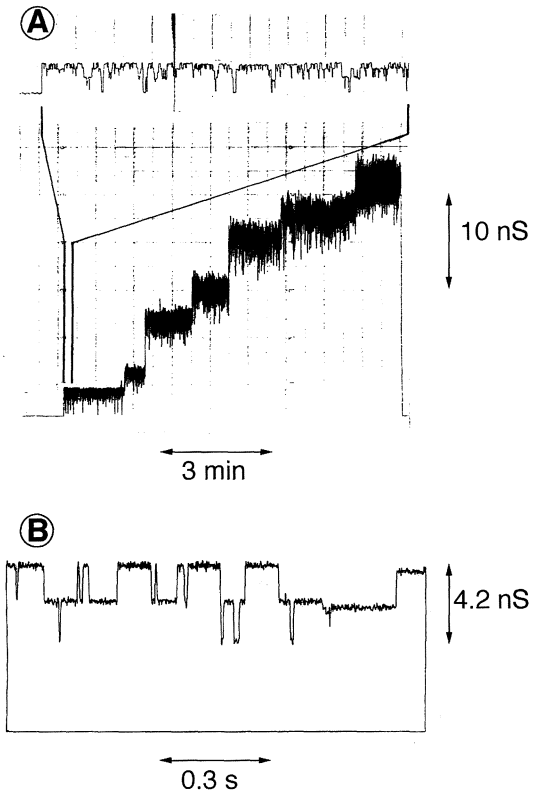

FIG. 2. Single-channel recording of a membrane made of diphytanoylphosphoglycerocholine/n-decane after the addition of 10 ng/ml OmpU from *Vibrio cholerae* strain 569B to the aqueous 1 M KCl solution. The applied potential was 20 mV; T = 25°C. Panel A shows the insertion of OmpU oligomers in a membrane at low time resolution. Note the high current noise due to rapid opening and closing of single conductive units in an oligomer (see inset, which represents a tenfold magnification of the time axe). Panel B shows the rapid opening and closing of the OmpU channels at high time and current resolution.

twice the conductance of 2.1 nS indicating the opening of two channels at the same time, which could not be separated at the time resolution of our instrumentation. It is noteworthy that the 2.1 nS fluctuations corresponded to conductance of a monomeric channel in a trimer, i.e. the trimers had a single-channel conductance of about 6 nS.

OmpU from *V. cholerae* was permeable to a variety of different ions. Table 1 shows the average single-channel conductance, G, as a function of different salt solutions. The single-channel conductance was a linear function of the KCl concentration of the aqueous phase. This may be explained by the assumption that the ions move inside the pore in a manner similar to the way they move in an aqueous environment, i.e. the OmpU channel is wide and water-filled. Nevertheless, the OmpU channels exhibit a certain specificity for charged solutes, because the single-channel conductance was larger in potassium acetate than it was in LiCl. Both salts have the same specific conductivity in the aqueous phase, which suggests that the channels have a limited selectivity for cations (6, 9). Experiments at different voltages suggested that OmpU was voltage-dependent in a slightly asymmetric manner, when it was added to one side of the membrane only. Starting with about 80 mV, the channels began to close, when the voltage was negative at the cis-side, the side of addition of protein. For positive voltages at the cis-side, the channels remained open until 150 mV.

Channel formation by the 35 kDa outer membrane protein and OmpV. The 35 kDa outer membrane protein had a very limited channel-forming activity in multi-channel experiments (less than 2% as compared to that of OmpU under otherwise identical conditions). To check whether they form also single channels in lipid bilayer membranes, we performed reconstitution experiments. At small concentrations of the 35 kDa outer membrane protein (10 ng/ml), we observed no current fluctuations. When we increased the protein concentration in the aqueous phase to more than 500 ng/ml we noticed the same channels than those measured with OmpU, indicating that it contained very small impurities of OmpU that could not be detected on SDS-PAGE. These results make it questionable that the 35 kDa outer membrane protein be a channel-forming component. It has been suggested that

TABLE 1. Average single-channel conductance, G, of OmpU of *Vibrio cholerae* strain 569 ΔtoxS in different salt solutions of concentration c

Salt	c (M)	G (nS)
KCl	0.03	0.090
	0.1	0.20
	0.3	0.80
	1.0	2.1
	3.0	4.5
KCH_3COO	1.0	1.3
LiCl	1.0	0.90

The aqueous salt solutions contained about 10 ng/ml OmpU; the pH was between 6.0 and 7.0. The membranes were made from diphytanoylphosphoglycerocholine/n-decane; t = 25°C; V_m = 20 mV. G was determined by recording at least 100 conductance steps and averaging over the distribution of the values.

OmpV could be a porin (27). Experiments with high concentrations of OmpV (1 μg/ml protein from the Genapol pellet) did not show any channel-forming ability of this protein. Only occasionally did we observe OmpU-like current fluctuations under these conditions.

Selectivity of OmpU. Further information about the structure of OmpU was obtained by zero-current membrane measurements in the presence of salt gradients. Table 2 shows the results of the measurements for ten-fold gradients of KCl, LiCl, and potassium acetate. The potential was found to be positive on the more dilute side of the membrane for all salts, indicating that OmpU was cation-selective. The potentials varied for the different salts indicating that they are influenced by the aqueous mobility of the ions. They were analyzed using the Goldman-Hodgkin-Katz equation (5). The ratio of the cation permeability, P_{cation} divided by the anion permeability P_{anion}, suggested that potassium a five-fold higher permeability than chloride inside the pore despite the same mobility of both ions in the aqueous phase (9); we conclude that the net charge inside the pore is negative at neutral pH. The selectivity of the other two salts is consistent with the mobility sequences of the cations and the anions in the aqueous phase.

Partial sequencing of the 35 kDa outer membrane protein and OmpU. We subjected both proteins to partial sequencing following tryptic digestion and HPLC-purification. We examined several peptides with similar retention times. A tryptic peptide of the 35 kDa outer membrane protein had the sequence FVTDVTETXIP (X unidentified amino acid). In the case of OmpU we obtained two partial sequences NSGDMYVR and YVYEADSFWVK.

Discussion

Purification of a 35 kDa outer membrane protein and OmpU. In this study we developed a simple method for the isolation and purification of OmpU of *V. cholerae*. This method consisted in two-fold SDS-wash of the cell envelope fraction or of the outer membranes followed by Genapol treatment of the final SDS-pellet. The supernatant of the second SDS-wash yielded in a pure 35 kDa outer membrane protein, which is probably identical to the OmpA-like protein of *V. cholerae* (1), because it is not heat-modifiable. Similar to OmpA of *E. coli* it is no channel-forming component as judged from single-channel experiments (3). OmpU was also obtained in pure form by treatment of the second SDS-pellet with the detergent Genapol. There was no indication that OmpT was obtained together with OmpU, probably because we used a growth medium with relatively low osmolarity and OmpT is only expressed in large quantities when the growth media were supplemented with 0.4 M NaCl (10, 18). OmpU formed SDS-resistant oligomers on SDS-PAGE, which are probably trimers in analogy to the formation of trimers of

TABLE 2. Zero-current membrane potentials, V_m, measured with membranes containing OmpU of strain 569B Δ1toxS in the presence of a ten-fold concentration gradient of different salts

Salt	V_m (mV)	P_c/P_a
KCl	30 ± 2.5	5.0 ± 1.0
LiCl	15.5 ± 1.8	2.2 ± 0.5
KCH_3COO	48 ± 1.5	15 ± 2.0

The permeability ratio, P_c/P_a, was calculated from the Goldman-Hodgkin-Katz equation (11). V_m is the electrical potential on the dilute side minus the potential of the concentrated side. The membranes were formed from diphytanoylphosphoglycerocholine/n-decane. The values represent the mean SD obtained from at least three membranes. The aqueous salt solutions were unbuffered and had a pH between 6 and 7.

most known bacterial porins. Upon heating, the oligomers dissociated into monomers that were inactive in the lipid bilayer assay. We performed partial sequencing of the 35 kDa outer membrane protein and of OmpU, and obtained one partial sequence for the 35 kDa protein and two sequences for OmpU. The partial sequences did not show major identity to known proteins, which means that both proteins have not been sequenced to date.

OmpU forms larger channels than enterobacterial porins. Lipid bilayer experiments define OmpU as a channel-forming protein in agreement to a previous study (10). The channel shows rapid fluctuations between the open and the closed state. The initial onset of the channel was about 6 to 7 nS in 1 M KCl. It subsequently switched to substates of multiples with about 2 nS, indicating that the channel-forming unit was not stable and the single-channel in a trimer closed and opened again separately for unknown reasons at low voltages, at which enterobacterial porins normally form open channel with long lifetimes (4, 7). It is noteworthy that protein II (31.5 kDa) from the related *Aeromonas hydrophila* showed a similar behavior with steps of 830 pS in 1 M NaCl, which represents probably also monomeric channels of trimers (13). The monomeric steps of enterobacterial porins are about 500 pS in 1 M KCl, one third of the 1.5 nS single-channel conductance of porin trimers (Table 3). It is even larger than that of *R. capsulatus*, which have on average a single-channel conductance of about 1.2 nS, one third of 3.5 nS (6, 8). This means that proteobacterial porins have an effective diameter larger than that of enterobacterial porins as judged from the liposome-swelling assay (21, 28). This result agrees with the 3D-structure of porins as derived from X-ray analysis of porin crystals, indeed has demonstrated that the diameter of porins from *R. capsulatus* (29) and *R. blastica* (14) is somewhat larger than that of OmpF and PhoE of *E. coli* K12 (11). This means also that the diameter of OmpU porin channels is larger than that of enterobacterial and proteobacterial porins. A larger pore diameter allows a higher influx of nutrients in general and also under starving conditions, typically found in the biotope of most *Vibrio* species. Many of these bacteria live in

TABLE 3. Average single-channel conductance G, of different porins and from OmpU of *Vibrio Cholerae* in 1 M KCl solution

Porin	G/nS (trimer)	G/ns (monomer)	Reference
OmpC, *Escherichia coli* K12	1.5	0.5	6
Rhodobacter capsulatus	3.5	1.2	8
Vibrio cholerae OmpU	6.0	2.1	this study

aqueous environments and do not have to cope with bile salts and proteases, like e.g. the Enterobacteriaceae. It is noteworthy that these considerations agree well with recent results of liposome swelling experiments with OmpU, which have demonstrated that raffinose and stachyose permeate reasonably well through OmpU (10) but thus suggesting that not through OmpF of *E. coli* (21) the diameter of OmpU is larger than that of OmpF, which is consistent with the observation that *V. cholerae* is more susceptible towards certain antibiotics than enteric bacteria (26).

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References

1. Alm, R. A., Braun, G., Morona, R., Manning, P. A. (1986). Detection of an OmpA like protein in *Vibrio cholerae*. FEMS Microbiol. Lett. **37**, 99–104.
2. Attridge, S. R., Rowley, D. (1990). Cholera. *In* Smith, G. R., Easmon, C. S. F. (ed.), Principles of Bacteriology, Virology and Immunity. Vol. 3. Bacterial Diseases. pp. 460–468, Edward Arnold, London.
3. Benz, R. (1994). Permeation of hydrophilic molecules through the mitochondrial outer membrane: review on mitochondrial porins, Biochim. Biophys. Acta **1197**, 167–196.
4. Benz, R., Janko, K., Boos, W., Läuger, P. (1978). Formation of large, ion-permeable membrane channels by the matrix protein (porin) of *Escherichia coli*. Biochim. Biophys. Acta **511**, 305–319.
5. Benz, R., Janko, K., Läuger, P. (1979). Ionic selectivity of pores formed by the matrix protein (porin) of *Escherichia coli*. Biochim. Biophys. Acta **551**, 238–247.
6. Benz, R., Schmid, A., Hancock, R. E. W. (1985). Ion selectivity of Gram-negative bacterial porins. J. Bacteriol. **162**, 722–727.
7. Benz, R., Schmid, A., Nakae, T., Vos-Scheperkeuter, G. (1986). Pore formation by LamB of *Escherichia coli* in lipid bilayer membranes. J. Bacteriol. **165**, 978–986.
8. Benz, R., Woitzik, D., Flammann, H. T., Weckesser, J. (1987). Pore forming activity of the major outer membrane protein of *Rhodobacter capsulatus* in lipid bilayer membranes. Arch. Microbiol. **14**, 226–230.
9. Castellan, G. W. (1983). Physical Chemistry: The electrical current in ionic solutions, pp. 769–780, Addison-Wesley Publishing Co., Reading, MA.
10. Chakrabarti, S. R., Chaudhuri, K., Sen, K., Das, J. (1996). Porins of *Vibrio cholerae*: purification and characterization of OmpU. J. Bacteriol. **178**, 524–530.
11. Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N., Rosenbusch, J. P. (1992). Crystal structures explain functional properties of two *E. coli* porins. Nature **358**, 727–733.
12. Gill, D. M. (1976). The arrangement of subunits in cholera toxin. Biochemistry **15**,1242–1248.
13. Janteur, D., Gletsu, N., Pattus, F., Buckley, J. T. (1992). Purification of *Aeromonas hydrophila* major outer membrane protein: N-terminal sequence analysis and channel-forming properties. Mol. Microbiol. **6**, 3355–3362.
14. Kreusch, A., Neubüser, A., Schiltz, E., Weckesser, J., Schulz, G. E. (1994). Structure of the membrane channel porin from *Rhodopseudomonas blastica* at 2.0 Å resolution. Prot. Sci. **3**, 58–63.
15. Lang, D. R., Guerrant, R. L. (1995). Summary of the 29th United States - Japan Joint Conference on cholera and related diarrheal diseases. J. Infect. Dis. **171**, 8–12.
16. Lang, H., Palva, E. T. (1993). The ompS gene of *Vibrio cholerae* encodes a growth-phase-dependent maltoporin. Mol. Microbiol. **10**, 891–901.
17. Lohia, A., Chatterje, A. N., Das, J. (1984). Lysis of *Vibrio cholerae* cells: direct isolation of the outer membrane from whole cells by treatment with urea. J. Gen. Microbiol. **130**, 2027–2033.
18. Lohia, A., Majumdar, S., Chatterje, A. N., Das, J. (1985). Effect of changes in the osmolarity of the growth medium of *Vibrio cholerae* cells. J. Bacteriol. **163**, 1158–1165.
19. Manning, P. A., Imbesi, F., Haynes, D. R. (1982). Cell envelope proteins in *Vibrio cholerae*. FEMS Microbiol. Lett. **14**, 159–166.
20. Nikaido, H. (1994). Porins and specific diffusion

channels in bacterial outer membranes. J. Biol. Chem. **269**, 3905–3908.

21. Nikaido, H., Rosenberg, E. Y. (1983). Porin channels in *Escherichia coli*: studies with liposomes reconstituted from purified proteins. J. Bacteriol. **153**, 241–252.
22. Parker, C., Richardson, S. H., Romig, W. R. (1970). Production of bacteriophage-associated materials by *Vibrio cholerae*: possible correlation with pathogenicity. Inf. Immun. **1**, 417–420.
23. Pearson, G. D. N., Mekalanos, J. J. (1982). Molecular cloning of *Vibrio cholerae* enterotoxin genes in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **79**, 2976–2980.
24. Salyers, A. A., Whitt, D. D. (1994). Bacterial Pathogenesis A Molecular Approach 141–156. ASM Press Washington, DC.
25. Schirmer, T., Keller, T. A., Wang, Y. F., Rosenbusch, J. P. (1994). Structural basis for sugar translocation through maltoporin channels at 3.1 Å resolution. Science **267**, 512–514.
26. Sengupta, T. K., Chaudhuri, K., Majumdar, S., Lohia, A., Chatterjee, A. N., Das, J. (1992). Interaction of *Vibrio cholerae* cells with -lactam antibiotics: emergence of resistant cells at high frequency. Antimicrob. Agents Chemother. **36**, 788–795.
27. Stevenson, G., Leaveslay, D. I., Lagnado, C. A., Heuzenroeder, M. W., Manning, P. A. (1985). Purification of the 25-kDa *Vibrio cholerae* major outer membrane protein and the molecular cloning of its gene: ompV. Eur. J. Biochem. **148**, 385–390.
28. Weckesser, J., Zalman, L. S., Nikaido, H. (1984). Porin from *Rhodopseudomonas sphaeroides*. J. Bacteriol. **159**, 199–205.
29. Weiss, M. S., Kreusch, A., Schiltz, E., Nestel, U., Welte, W., Weckesser, J., Schulz, G. E. (1991). The structure of porin from *Rhodobacter capsulatus* at 1.8 Å resolution. FEBS Lett. **280**, 379–382.

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β-Lactamases of *Mycobacterium tuberculosis* and *Mycobacterium kansasii*

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Summary

Re-emergence of infectious diseases caused by mycobacteria as well as the emergence of multiresistant strains of *Mycobacterium* has promoted the research on the use of β-lactames in the treatment of such diseases. Mycobacteria produce β-lactamases: *M. tuberculosis* produces a wide-spectrum β-lactamase whose behaviour mimicks those of Gram-negative bacteria. *M. kansasii* produces also β-lactamase which can be inhibited by clavulanic acid. An overview on β-lactamases from both species is reported.

Key words: *Mycobacterium tuberculosis*, *Mycobacterium kansasii*, β-lactamases, clavulanate, multiresistance

Resumen

La reaparición de enfermedades producidas por micobacterias, así como la emergencia de cepas multirresistentes de *Mycobacterium*, ha revitalizado la investigación acerca de la utilización de β-lactamas en el tratamiento de las micobacteriosis. Las micobacterias producen β-lactamasas: *M. tuberculosis* produce una β-lactamasa de amplio espectro cuyo comportamiento se asemeja al de las bacterias Gram negativas. *M. kansasii* produce también β-lactamasa que se inhibe por ácido clavulánico. Se aporta una revisión sobre las β-lactamasas de ambas especies.

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Introduction

β-lactamases (EC 3.5.2.6), penicillin (or cephalosporin) and amido-β-lactam hydrolases hydrolyse the amide bond of the β-lactamic nucleus (37). This produces a loss of activity of β-lactamic antibiotics, since they become unable to bind their target (penicillin binding proteins or PBPs).

β-lactamases constitute a diverse group of enzymes widely spread among bacteria. More than 200 β-lactamases have been described, either plasmid or chromosomally encoded. They can act on a wide variety of β-lactamic antibiotics. These can react with β-lactamases either as substrates or as inhibitors (4). β-lactamase production is an efficient mechanism of bacterial protection against the antimicrobial effect of β-lactams and this can be considered to be the most frequent mechanism of resistance in bacteria (24). According to Tipper and Stromminger (35), β-Lactamases have evolved from the enzymes involved in peptidoglycan synthesis. They may have had an active role in the bacterial physiology, although they have also been interpreted as a mechanism of resistance to protect bacteria against β-lactams produced by other bacteria (24).

Mycobacteria are slender, curved rods whose cell wall is unique. It is composed of mycolic acids, complex waxes and glycolipids. The mycobacterial cell wall also includes peptidoglycan. Penicillinasic activity in mycobacteria was demonstrated early (1949 and 1952), when Iland and Baines (14) and Soltys (30) reported the hydrolytic action in *Mycobacterium tuberculosis*. Some years before Abraham et al. (1) reported that this species was not inhibited by high concentrations of penicillin in vitro. Furthermore, β-lactamasic activity in mycobacteria is described not only in *M. tuberculosis* (3,19), but also in *M. bovis* (3, 30), *M. kansasii* (10, 23), *M. phlei* (30), *M. smegmatis* (18, 20, 25, 39), *M. butyricum* (8), *M. fortuitum* (2, 10, 20, 26, 34), *M. chelonei* (32) and *M. leprae* (27). The ability to produce β-lactamase was interpreted as the cause of mycobacterial resistance to penicillin. Several reports deal with the susceptibility of mycobacteria to β-lactam antibiotics (5–7, 9, 12, 15, 38, 39). The interest in antimycobacterial activity of β-lactam antibiotics, β-lactamases, PBPs and permeability has increased in the last few years, due in part to the increase of incidence of infections caused by mycobacteria, particularly in HIV-infected patients. Moreover, although the genus has been historically considered to be resistant to β-lactams, the emergence of multiresistant isolates of *M. tuberculosis* has promoted the research of new therapeutical alternatives and the re-evaluation of "old" antimicrobial compounds.

The cell wall of mycobacteria was shown to be an effective permeability barrier to hydrophilic compounds. Cell wall proteins from different mycobacteria have channel-forming activity in reconstituted lipid bilayers forming water-filled channels. The permeability properties of these porin-like proteins are similar to those of some Gram-negative porins. Negatively charged β-lactamic antibiotics diffused approximately at a 20-fold lower rate than zwitterionic β-lactames. Research on the pore forming activity of cell-wall proteins from mycobacteria will constitute in the future one of the main fields of research to supply bases for future therapies.

Mycobacterium tuberculosis

Infections produced by *M. tuberculosis* is a growing problem, and the emergence of multiresistant strains is a source of major concern. Penicillinasic activity in *M. tuberculosis* was demonstrated as early as 1949 (14). Further-

more, when the spectrum of this enzymatic activity was studied, the β-lactamase from the tubercle bacilli was also active on cephalosporins. Activity on cephalosporins was demonstrated much later in strains isolated before the discovery of this group of antibiotics. Then, β-lactamase from *M. tuberculosis* should be considered as a wide spectrum β-lactamase. Its biosynthesis is constitutive. In general β-lactamases from Gram-positive bacteria are secreted to the external medium, whereas those from Gram-negative bacteria remain in the periplasm. Due to the special features of the mycobacterial cell wall, β-lactamases produced by these bacteria remain in inner locations mimicking the behaviour of Gram-negative bacteria. The study of mycobacterial β-lactamases by analytic isoelectrofocusing demonstrates a high degree of similarity between all strains studied, since their pIs were always 4.9 and 5.1 (23, 29, 40). The genetic diversity of *M. tuberculosis* populations has been studied by bacteriophage typing, multilocus enzyme electrophoresis, serological properties, analysis of DNA fragment polymorphism and others; in all cases calculated values for genetic diversity were low (16, 17). This is consistent with the high homogeneity detected when β-lactamases were characterized.

The antimycobacterial activity of different β-lactamic antibiotics and combinations of β-lactams and β-lactamase inhibitors have been studied extensively (5–7, 9, 29, 31, 38). *M. tuberculosis* is naturally resistant to all β-lactams but Imipenem. This natural resistance is probably due to several mechanisms such as β-lactamase production, low permeability, low affinity of PBPs for the antibiotic molecules or a combination of various mechanisms. The use of compounds able to inhibit β-lactamases is a strategy to analyse the contribution of the β-lactamase to antimicrobial resistance. The most widely used compound among β-lactamase inhibitors is clavulanic acid. Clavulanate is a potent inhibitor of a wide variety of β-lactamases (33), including those of mycobacteria (7, 31), and its action is non-reversible. Clavulanate has no antibacterial effect on mycobacteria, since it has a very low affinity for the PBP3 of *M. tuberculosis*, although it binds PBP1 and PBP2. However, it seems that binding to PBP3 is necessary for the antimycobacterial effect (7). When different antibiotics were assayed in the presence of clavulanic acid, a decrease in antimicrobial resistance was detected. This was demonstrated for amoxicilin, ampicillin, cephaloridine, ticarcilline, carbenicilline, cefoxitine, ceftriaxone and cefotaxime. Furthermore, this decrease was not a consequence of additive effect unrelated with β-lactamase activity, since these compounds bound the mycobacterial PBPs at therapeutical concentrations (7). On the other hand the resistance to nafcillin, cefoperazone, cephalothin and probably azthreonam is due to the low affinity of these compounds for the critical mycobacterial PBPs (7, 29). The activity of β-lactamase of *M. tuberculosis* is also inhibited by sulbactam (31, 38, 40) and by penicilinase-resistant penicillins (21, 22). The only compound that shows activity alone is Imipenem. *M. tuberculosis* is susceptible to Imipenem, with MIC values about 4 μg/ml (7). Imipenem is a β-lactam antibiotic (Carbapenem) with special molecular features (small size, zwitterionic), which allow it to penetrate much easier than any other β-lactam. In addition, Imipenem shows a high affinity for the mycobacterial PBPs (7), and is stable to the hydrolysis by penicillinases and cephalosporinases.

From a molecular point of view, the β-lactamase of *M. tuberculosis* has been classified in the class A (13). Its phenotypical resistance, the ability of clavulanic acid and sulbactam to inhibit the enzymatic activity, the qualitative spectrum of hydrolysis and the ability of crude ex-

tracts to hydrolise cefotaxime (Segura et al. unpublished) suggest that β-lactamase produced by the bacterium should be included in the class 2be (wide spectrum β-lactamase). However further studies using purified enzymes will be required to test this suggestion. Additionally, one more β-lactamase from the strain H37Ra, a BlaC (β-lactamase class C gene) enzyme was described, although the presence of genes encoding for this enzyme is inconsistent with phenotypical characteristics, lack of inducibility, pIs described (Class C pIs are basic and never detected in *M. tuberculosis*) and inhibition by clavulanic acid (13).

Mycobacterium kansasii

M. kansasii produces infections in which patients exhibit lower respiratory diseases similar to tuberculosis, or diseminated disease in persons infected with HIV. Many of non-tuberculous mycobacteria are resistant to usual antituberculosis drugs, then, different drugs are often necessary; additionally, no vaccines are available. The possible use of β-lactamic antibiotics to threat *M. kansasii* infections has been evaluated recently, although the presence of β-lactamase in this species was described in 1966 (10). It has a wide spectrum of activity on penicillins and cephalosporins (29). Their pI were 5.2 (23) and 5.1 and 5.6 (29). There are only a few reports on the activity of β-lactams and the combination of antibiotics and β-lactamase inhibitors in this species (36, 38). *M. kansasii* is intrinsically resistant to amoxicillin, carbenicillin, ceftriaxone and cefotaxime. The contribution of the β-lactamase to the amoxicilin resistance has been demonstrated, since significant reduction in MIC values have been described when 1-clavulanate is added (29, 36, 38). Decrease in the carbenicillin and cefotaxime resistance have also been described when inhibitors are used, although the levels of reduction did not indicate a relevant role of β-lactamase. In the case of ceftriaxone, it can be concluded that some mechanism other than β-lactamase must be responsible for resistance, since the addition of β-lactamase did not modify the susceptibility (29).

Concluding remarks

Multiple-drug-resistance (MDR) has become a particulary threatening aspect of the current epidemics of tuberculosis and other mycobacterial diseases, particularly in AIDS patients. Additionally, MDR strains are frequently associated with rapid progression and high mortality. β-Lactamic antibiotics constitute a possible alternative in the treatment of these infectious diseases, this has estimulated the research on β-lactamases, as well as on the porin-like proteins of the mycobacterial cell-wall that prevent the drug access to the target. The knowledge in this field could supply the basis for a future use of combinations of antibiotics (including small molecules, which can pass through the porin-like molecules and β-lactamase inhibitors), that can substitute the traditional chemoterapeutical protocoles used up to now, to threat mycobacteriosis.

References

1. Abraham, E. P., Chain, E., Fletcher, C. M., et al. (1941). Further obervations on penicillin. Lancet **ii**, 177.
2. Amicosante, G., Franceschini, N., Segatore, B., Oratore, A., Fattorini, L., Orefici, G., Van Beeeumen, J., Frére, J. M. (1990). Characterization of a β-lactamase produced in *Mycobacterium fortuitum* D16. Biochem. J. **271**, 729–734.

3. Bonicke, R., Dittmar, W. (1957). Uber das Vorkommen von Cycloamidasen in Mycobacterien. Zentralbl.. Bakteriol. I. Orig. **170**, 366–376.
4. Bush, K., Jacoby, G. A., Medeiros, A. A. (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. **39**, 1211–1233.
5. Casal, M., Rodriguez, F., Benavente, M. (1986). In vitro susceptibility of *Mycobacterium tuberculosis*, *Mycobacterium fortuitum* and *Mycobacterium chelonei* to amoxycillin/clavulanic acid. Eur. J. Clin. Microbiol. **5**, 453–454.
6. Casal, M., Rodriguez, F., Luna, M. D., Benavente, M. C. (1987). In vitro susceptibility of *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium avium*, *Mycobacterium fortuitum* and *Mycobacterium chelonae* to ticarcillin in combination with clavulanic acid. Antimicrob. Agents Chemother. **31**, 132–133.
7. Chambers, H. F., Moreau, D., Yajko, D., et. al. (1995). Can penicillins and other betalactam antibiotics be used to tract tuberculosis? Antimicrob. Agents Chemother. **39**, 2620–2624.
8. Choubey, D., Gopinathan, K. P. (1986). Characterization of β-lactamase from *Mycobacterium butyricum* ATCC 19979. Biochem. Int. **12**, 207–214.
9. Cynamon, M. H., Palmer, G. S. (1983). In vitro activity of amoxycillin in combination with clavulanic acid against *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **24**, 429–431.
10. Durfor, A. P., Knight, R. A., Harris, H. W. (1966). Am. Rev. Respir. Dis. **94**, 965.
11. Eisenach, K. D., Crawford, J. T., Bates, B. H. (1986). Genetic relatdness among strains of *Mycobacterium tuberculosis* complex. Am. Rev. Respir. Dis. **133**, 1065–1068.
12. Fattorini, L., Orefici, G., Jin, S. H., et al. (1992). Resistance to betalactams in *Mycobacterium fortuitum*. Antimicrob. Agents Chemother. **36**, 1068–1072.
13. Hackbarth, C. J., Unsal, I., Chambers, H. F. (1997). Cloning and sequence analysis of a class A β-lactamase from *Mycobacterium tuberculosis* H37Ra. Antimicrob. Agents Chemother. **41**, 1182–1185.
14. Iland, C. N., Baines, S. (1949). The effect of penicillin on the tubercle bacillus: tubercle penicillinase. J. Path. Bact. **61**, 329–335.
15. Jarlier, V., Gutmann, L., Nikaido, H. (1991). Interplay of cell wall barrier and β-lactamase activity determines high resistance to β-lactam antibiotics in *Mycobacterium chelonae*. Antimicrob. Agents Chemother. **35**, 1937–1939.
16. Jones, W. D., Good, R. C., Thompson, N. J., Kely, G. D. (1982). Bacteriophage types of *Mycobacterium tuberculosis* in the United States. Am. Rev. Respir. Dis. **125**, 640–643.
17. Jones, W. D., Kubica, G. P. (1968). Fluorescent antibody techniques with mycobacteria III. Investigation of five serologically homogeneous groups of mycobacteria. Zentralbl. Bakteriol. (Orig A). **207**, 58–62.
18. Kaneda, S., Yabu, K. (1983). Purification and some properties of β-lactamase from *Mycobacterium smegmatis*. Microbiol. Immunol. **27**, 191–193.
19. Kasik, J. E. (1965). The nature of mycobacterial penicillinase. Am. Rev. Respir. Dis. **91**, 117–119.
20. Kasik, J. E., Peacham, L. (1968). Properties of betalactamases produced by three species of mycobacteria. Biochem. J. **107**, 675–682.
21. Kasik, J., Weber, M., Freehill, P. (1967). The effect of the penicillinase-resistant penicillins and other chemotherapeutic substances on the penicilinase of the R1Rv strain of *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **95**, 12–19.
22. Kasik, J. M., Weber, M., Winberg, E., Barclay, W. (1966). The synergistic effect of dicloxacillin and penicillin G on murine tuberculosis. Am. Rev. Respir. Dis. **94**, 260–261.
23. Kwon, H. H., Tomioka, H., Saito, H. (1995). Distribution and characterization of betalactamases of mycobacteria and related organisms. Tub. Lung Dis. **76**, 141–148.
24. Livermore, D. M. (1995). β-Lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev. **8**, 557–584.
25. Mishra, R. K., Kasik, J. E. (1970). The mechanism of mycobacterial resistance to penicillins and cephalosporins. Int. J. Clin. Pharmacol. **3**, 73–77.
26. Nash, D. R, Wallace, R. J., Steingrube, V. A. et. al. (1986). Characterization of betalactamases in *Mycobacterium fortuitum* including a role in betalactam resistance and evidence of partial inducibility. Am. Rev. Respir. Dis. **134**, 1276–1282.
27. Prabhakaran, K., Harris, E. B., Sanchez, R. M., Hastings, R. C. (1987). Betalactamase synthesis in *Mycobacterium leprae*. Microbios **49**, 183–188.
28. Román, M. C., Sicilia, L. M. J. (1984). Preliminary investigation of *Mycobacterium tuberculosis* biovars. J. Clin. Microbiol. **20**, 1015–1016.
29. Salvadó, M. (1997). β-lactamasas en el género Mycobacterium. PhD Thesis. Autonomous University of Barcelona.

30. Soltys, M. A. (1952). The effect of penicillin on Mycobacteria in vitro and in vivo. Tubercle **33**, 120–125.
31. Sorg, T. B., Cynamon, M. H. (1987). Comparison of four betalactamase inhibitors in combination with ampicillin against *Mycobacterium tuberculosis*. J. Antimicrob. Chemother. **19**, 59–64.
32. Sparks, J., Ross, G. W. (1981). Isolectric focusing studies on *Mycobacterium chelonei*. Tubercle **62**, 289–293.
33. Sutherland, R. (1995). β-Lactam/β-lactamase inhibitor combinations: Development, antibacterial activity and clinical applications. Infection **23**, 191–200.
34. Timm, J., Perilli, M. G., Duez, C., Trias, J., Orefici, G., Fattorini, L., Amicosante, G., Oratore, A., Joris, B., Frére, J. M., Pugsley, A. P., Gicquel, B. (1994). Transcription and expression analysis, using lacZ and phoA gene fusions, of *Mycobacterium fortuitum* β-lactamase genes cloned from a natural isolate and a high-level β-lactamase producer. Mol. Microbiol. **12**, 491–504.
35. Tipper, D. J., Stromminger, J. L. (1965). Mechanisms of action of penicillins; a proposed based on their structural similary to acyl-D-alanyl-D-alanine. Proc. Natl. Acad. Sci. USA **54**, 1133–1141.
36. Utrup, L. J., Moore, T. D., Actor, P., Poupard, J. A. (1995). Susceptibilities of nontuberculosis species to amoxicillin-clavulanic acid alone and in combination with antimycobacterial agents. Antimicrob. Agents Chemother. **39**, 1454–1457.
37. Webb, E. C. (1984). Enzyme nomenclature. Academic Press. vol. **1**, London, 366–374.
38. Wong, C. S., Palmer, G. S., Cynamon, M. H. (1988). In vitro susceptibility of *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *M. kansasii* to amoxycillin and ticarcillin in combination with clavulanic acid. J. Antimicrob. Chemother. **22**, 863–866.
39. Yabu, K., Kaneda, S., Ochiai, T. (1985). Relationship between β-lactamase activity and resistance to β-lactam antibiotics in *Mycobacterium smegmatis*. Microbiol. Immunol. **29**, 803–809.
40. Zhang, Y., Steingrube, V., Wallace, R. J. (1992). Beta-lactamase inhibitors and the inducibility of the beta-lactamase of *Mycobacterium tuberculosis*. Am. Rev. Respir. Dis. **145**, 657–660.

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Moderate resistance to penicillin in *Neisseria meningitidis*

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Summary

Meningococcal moderate resistance to penicillin (MICs 0.12 to 1 mg/l) was rarely reported before the 1980's in Spain. The frequency of isolation of resistant strains increased from 0.4% in 1985 to 42.6% in 1990. In the last few years, these strains have been reported in several countries, which suggests a change in the meningococcal response to penicillin. The resistance is due, at least in part, to a decreased affinity of penicillin binding protein 2 (PBP2) for penicillin. This decreased affinity has also been found in commensal Neisseriae. Population genetic studies demonstrate that recombinational events, replacing parts of the PBP2 gene by the corresponding regions of commensal species, followed by a rapid spread of the clones could be the origin of such resistant strains.

Key words: *Neisseria meningitidis*, penicillin, moderate resistance, pen^r, commensal species

Resumen

Antes de la década de 1980 apenas se había observado resistencia meningocócica moderada a la penicilina (CMIs de 0,12 a 1 mg/l). Tras la detección de la primera cepa en España, se pasó del 0,4% en 1985 al 42,6% en 1990. En los últimos años estas cepas se han aislado en muchos países, lo que indica un cambio de respuesta del meningococo a la penicilina. La resistencia se debe en parte a la disminución de afinidad por la penicilina de la proteína fijadora de penicilina 2 (PBP2). Ese descenso de afinidad también se encuentra en neisserias saprofitas. Estudios de genética de poblaciones atribuyen la aparición de esas cepas a procesos de recombinación que sustituyeron partes del gen de la PBP2 por las correspondientes regiones del gen de neisserias comensales.

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Introduction

Neisseria meningitidis has been described as a microbe extremely susceptible to penicillin. However, in the last few years clinical isolates of meningococcocci with decreased susceptibility to penicillin have been reported in several countries (Table 1). Several studies described these strains (MICs >0.1 mg/l) (13–15, 20, 28, 42, 54). Cases of meningococcal meningitis that did not respond to standard penicillin treatment with no MICs determinations have been also published (12, 17). The failure treatment was reported in the United Kingdom, using a low dosage of penicillin against a strain having a MIC of 0.64 mg/l (47). Other authors have not found significant differences in the clinical symptomatology and prognostic between the cases produced by pen^r strains and pen^s strains (33, 49).

The mechanism of resistance to penicillin in meningococci appears to be similar to that already documented in gonococci. Non-β-lactamase mediated resistance in gonococci has gradually increased MIC values up to 500 (or even 1000) fold higher than those for susceptible strains. Then, it seems likely that further increases in the MICs of meningococci and subsequent treatment failures will occur in the future.

Penicillin-binding proteins (PBP)

The production of a β-lactamase as a mechanism of high level penicillin resistance in *N. meningitidis* has been reported in only five isolates (6, 16, 22, 53). Mendelman et al. (29) showed that the affinity for penicillin of the 63 kD PBP3 (now called PBP2) was reduced in penicillin-resistant meningococci.

Reduction in the affinity of PBP2 for penicillin was also found in non-beta-lactamase producing isolates of gonococci, *N. lactamica, N. polysaccharea* (19, 27, 41).

Transformation experiments by using pen^r meningococcal strains as receptors and chromosomal DNA obtained from pen^r meningococci or commensal species yield transformants expressing low-affinity forms of PBP2 (27, 32, 40). The decrease in the affinity of PBP2 for penicillin in *N. gonorrhoeae* and *N. perflava/sicca* produces a moderate increase in the MIC of penicillin (6–10-fold)(32). By contrast, gonococci with high levels of penicillin resistance (MIC >1 mg/l) produce low-affinity forms of both PBP1 and PBP2 and outer membranes with decreased permeability (19). Unlike pen^r gonococci, no pen^r meningococci with reduction in the affinity of PBP1 have been isolated. Recently, Ropp and Nicholas (36) have cloned and sequenced the *ponA* (PBP1) gene from *N. gonorrhoeae* and *N. meningitidis*, and a comparison between both sequences showed a high degree of identity. Susceptibility studies of pen^r strains of meningococci against several cephalosporins showed significant increases in MIC values against cephalotin and cefuroxime, like in *N. lactamica* and *N. polysaccharea* (40). These data and the similarity between resistance mechanisms might suggest a recruitment of antibiotic resistance in meningococci.

Genetic basis of low-level resistance to penicillin in meningococci and other *Neisseria* species

Sequences of *penA* (PBP2) genes from *N. gonorrhoeae, N. meningitidis*, and most of the commensal species have been determined. Comparison of the *penA* genes of pen^s and pen^r meningococci shows that altered *penA* genes were originated by a novel genetic mechanism, involving the replacement of parts of the gene by

TABLE 1. Studies on the prevalence of pen^r *Neisseria meningitidis* strains

Country	Year	n° pen^r / total strains	MIC (mg/l) (range)	Origin*	Year of isolation	Ref.
U.S.A.	1954	15/54	(0.1–0.2)	ND	1945–1954	14
U.S.A.	1964	11/27	(0.12–0.18)	C	ND	28
U.S.A.	1965	7/56	(0.1–0.2)	P, C	1963–1964	20
U.S.A.	1970	15/40	(0.12–0.5)	C	1969	15
U.S.A.	1970	54/225	(0.1–0.4)	C	1970	54
U.S.A.	1982	5/30	(0.12–0.25)	P	1976–1981	42
France	1984	22/92	(0.12–0.25)	P, C	ND	13
Spain	1987	10/3432	(0.2–0.4)	P	1978–1986	37
South. Africa	1988	1/1	(0.25)	P	1987	6
U. K.	1988	54/3646	(0.16–1.28)	P	1983–1987	46
U. K.	1988	65/4157	(0.16–0.64)	C	1983–1987	46
Spain	1988	44/927	(0.1–0.4)	P	1985–1987	38
Romania	1990	6/499	(> 0.1)	P, C	1986–1989	18
U. K.	1990	139/5465	(0.16–1.28)	P	1985–1989	25
Spain	1990	206/1573	(0.1–0.8)	P	1985–1990	39
Canada	1991	17/373	(> 0.25)	ND	1989–1990	34
Greece	1992	15/31	(> 0.1)	P	1989–1991	48
Greece	1992	182/519	(> 0.1)	C	1989–1991	48
Switzerland	1992	1/177	(0.25)	P	1988–1990	35
Israel	1993	11/122	(0.12–0.25)	P, C	1992	4
Argentina	1993	3/54	(0.12–0.5)	P	1991–1992	26
Belgium	1993	1/1	(0.5)	P	1993	9
Sweden	1993	ND/307	(0.25)	P, C	1981–1990	1
Spain	1993	340/986	(0.12–0.5)	P	1989–1992	50
U.S.A.	1994	1/1	(0.5)	P	1994	10
U.S.A.	1994	3/101	(0.12)	P, C	1991	24
U.S.A.	1994	1/1	(0.25)	P	1992	55
Canada	1995	11/11	(0.12–0.25)	P	1993	5
Netherlands	1996	37/125	(0.1–1)	P	1993–1994	21
Spain	1996	219/859	(0.12–0.5)	P	1993–1995	52

* ND, not done; C, carriers; P, patients.

the corresponding regions from the *penA* genes from *N. flavescens* (44), *N. lactamica* (27, 45), and *N. cinerea* (7, 4 1). The extensive differences between the sequences of the *penA* genes of resistant and sensitive strains result in major differences in their restriction maps, which can be readily observed by RFLP (Restriction Fragments Length Polymorphism)(11, 56).

In most cases, parts of the region encoding the transpeptidase domain of PBP2 have been replaced by regions from *penA* gene of *N. flavescens* (41, 44). The analysis of sequences of pen^r from *N. meningitidis, N. gonorrhoeae* and other *Neisseria* species revealed an insertion codon in the position 573 (Asp). This insertion codon does not exist in the sequences from sensitive strains (30, 43, 45). Moreover, this Asp-573 codon has been found recently in the *penA* genes from *N. perflava/sicca* (31). Transformation experiments of pen^s strains of meningococci

with DNA from pen^r strains of *N. perflava/sicca* showed that the insertion codon Asp-573 appears in the first generation of transformants (32). These results suggest that the reduction in the affinity of PBP2 is therefore largely, although not exclusively, due to the insertion of this codon. This fact, has been also detected in gonococci with an insertion Asp-345 (45). The role of this codon in the resistance has been demonstrated by mutagenesis (8).

Population structure of pen^r strains in Spain

The emergence and spread (due to the selective conditions provided by the selective pressure of penicillin) of pen^r strains in Spain is a cause of major concern. The analysis of pen^r and pen^s strains by multilocus enzyme electrophoresis (2) showed that the moderate resistant isolates were not significantly less diverse that those of the total meningococcal population. In a previous study, serogroup C was about three times more common among the pen^r strains than in the total meningococcal population (41). This serogroup has been increasing over the last few years in Spain. However, no clear relationship between both events can be established, since the increase of serogroup C took place in the same proportion among pen^s meningococci (3). No differences in the sero/subtype distribution have been found, the B:4 P1.15 and C:2b:NT strains being the major meningococcal lines in Spain before 1994 (51) both in pen^r and in pen^s isolates.

We conclude that moderate resistance to penicillin did not appear in a new clone distinct from those already established. It is possible that the resistance appeared in more than one clone and further spreading took place by genetic exchange.

The frequency of pen^r strains increased rapidly from 0.4% in 1985 to 42.6% in 1990, and it went down to 23% during 1995 (52). Then, the incidence increased because most of the meningococcal cases in Spain were produced by a new C:2b:P1.2,5 epidemic strain which is moderately resistant to penicillin.

References

1. Bäckmann, A., Danielsson, D., Olcen, P. (1993). Plasmid carriage and antibiotic susceptibility of *Neisseria meningitidis* strains isolated in Sweden 1981–1990. Eur. J. Clin. Microbiol. Infect. Dis. **12**, 683–689.
2. Berrón, S., Sáez Nieto, J. A., Vázquez, J. A. (1992). Aplicación de marcadores epidemiológicos en cepas de *Neisseria meningitidis* con sensibilidad disminuida a penicilina aisladas en España (1985–1990). Rev. Españ. Quimioter. **5**, 35–41.
3. Berrón, S., Vázquez, J. A. (1994). Increase in moderately penicillin resistance and serogroup C in meningococcal strains isolated in Spain. Is there any relationship ? Clin. Infect. Dis. **18**, 161–165.
4. Block, C., Davidson, Y., Melamed, E., Keller, N. (1993). Susceptibility of *Neisseria meningitidis* in Israel to penicillin and other drugs of interest. J. Antimicrob. Chemother. **32**, 166–168.
5. Blondeau, J. M., Yaschuk, Y. (1995). In vitro activities orf ciprofloxacin, cefotaxime, ceftriaxone, chloramphenicol, and rifampin against fully susceptible and moderately penicillin-resistant *Neisseria meningitidis*. Antimicrob. Agents Chemother. **39**, 2577–2579.
6. Botha, P. (1988). Penicillin resistant *Neisseria meningitidis* in Southern Africa. Lancet **i**, 54.
7. Bowler, L. D., Zhang, Q., Riou, J. Y., Spratt, B. G. (1994). Interspecies recombination between the *penA* genes of *Neisseria meningitidis* and commensal Neisseria species during the emergence of penicillin resistance in *Neisseria meningitidis*: natural events and laboratory simulation. J. Bacteriol. **176**, 333–337.
8. Brannigan, J. A., Tirodinos, J. A., Zhang, Q. Y., Dowson, C. G., Spratt, B. G. (1990). Insertion of an extraminoacid is the main cause of the low affinity of penicillin binding protein 2 in penicillin-resistant strains of *Neisseria gonorrhoeae*. Mol. Microbiol. **4**, 913–919.
9. Brunen, A., Peeterman, W., Verhaegen, J., Robberecht, W. (1993). Meningitis due to *Neisseria meningitidis* with intermediate suscep-

tibility to penicillin. Eur. J. Clin. Microbiol. Infect. Dis. **12**, 969–970.
10. Buck, G. E., Adams, M. (1994). Meningococcus with reduced susceptibility to penicillin in the United States. Pediatr. Infect. Dis. **13**, 156–158.
11. Campos, J., Fusté, C., Trujillo, G., Sáez Nieto, J., Vázquez, J., Lorén, J. G., Viñas, M., Spratt, B. G. (1992). Genetic diversity of penicillin resistant *Neisseria meningitidis*. J. Infect. Dis. **166**, 173–177.
12. Contoyiannis, P,. Adamopoulos, D. A. (1974). Penicillin resistant *Neisseria meningitidis*. Lancet **i**, 462.
13. Dabernat, H., Delmas, C., Lareng, M. B. (1984). Sensibilité aux antibiotiques de méningocoques isolés chez del malades et chez porteurs. Pathol. Biol. **32**, 532–535.
14. Del Love, B., Finland, M. (1954). In vitro susceptibility of meningococci to eleven antibiotics and sulfadiazine. Amer. J. Med. Sci. **228**, 534–539.
15. Devine, L. F., Hagerman, C. R. (1970). Spectra of susceptibility of *Neisseria meningitidis* to antimicrobial agents in vitro. Appl. Microbiol. **19**, 329–334.
16. Dillon, J. R., Pauze, M., Yeung, K. H. (1983). Spread of penicillin producing and transfer plasmids from gonococcus to *Neisseria meningitidis*. Lancet **i**, 779–781.
17. Dominguez, P., Minguella, I. (1985). Existencia de cepas de meningococo resistentes a penicilina. Med. Clin. (Barcelona) **85**, 517–518.
18. Dorobat, O., Levenet, I. Pasolescu, O. Lazaroar, D. (1990). Characteristics of *Neisseria meningitidis* strains isolated in Romania between 1986–1989. Arch. Roum. Pathol. Exp. Microb. **49**, 215–221.
19. Dougherty, T. J., Koller, A. E., Tomasz, A. (1980). Penicillin binding proteins of penicillin susceptible and intrinsically resistant *Neisseria gonorrhoeae*. Antimicrob. Agents Chemother. **18**, 730–737.
20. Eickhoff, T. C., Finland, M. (1965). Changing susceptibility of meningococci to antimicrobial agents. N. Engl. J. Med. **272**, 395–397.
21. Enting, R. H., Spanjaard, L., Van de Beck, D., Hensen, E. F., De Gans, J., Dankert, J. (1996). Antimicrobial susceptibility of *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* isolates causing meninigitis in the Netherlands, 1993–1994. J. Antimicrob. Chemother. **38**, 777–786.
22. Fontanals, D., Pineda, V., Pons, I., Rojo, J. C. (1989). Penicillin-resistant beta-lactamase producing *Neisseria meningitidis* in Spain. Eur. J. Clin. Microbiol. Infect. Dis. **8**, 90–91.
23. Hieber, J. P., Nelson, J. D. (1977). A pharmacologic evaluation of penicillin in children with purulent meninigitidis. N. Engl. J. Med. **297**, 410–413.
24. Jackson, L. A., Tenover, F. C., Baker, C., Plikaytis, B. D., Reeves, M. W., Stocker, S. A., Weaver, R. E., Wenger, J. D. (1994). Prevalence of *Neisseria meningitidis* relatively resistant to penicillin in the United States, 1991. J. Infect. Dis. **169**, 438–441.
25. Jones, D. M., Sutcliffe, E. M. (1990). Meningococci with reduced susceptibility to penicillin. Lancet **i**, 863–864.
26. Lopardo, H. A., Santander, C., Ceinos, M. C., Rubeglio, E. A. (1993). Isolation of moderately penicillin-susceptible strains of *Neisseria meningitidis* in Argentina. Antimicrob. Agents Chemother. **37**, 1728–1729.
27. Lujan, R., Zhang, Q. Y., Sáez Nieto, J. A., Jones, D. M., Spratt, B. G. (1991). Penicillin resistant isolates of *Neisseria lactamica* produced altered forms of penicillin binding protein 2 that arose by interspecies gene transfer. Antimicrob. Agents Chemother. **35**, 300–304.
28. Martin, J. E., Sammuels, S. B., Peacock, W. L., Thayer, J. D. (1964). *Neisseria gonorrhoeae*, and *Neisseria meningitidis* sensitivity to spectinomycin, lincomycin, and penicillin G. Antimicrob. Agents Chemother. **5**, 366–368.
29. Mendelman, P. M., Campos, J., Chaffin, D. O., Serffass, D. A., Smith, A. L., Sáez Nieto, J. A. (1988). Relatively penicillin G resistance in *Neisseria meningitidis* and reduced affinity of penicillin binding protein 3. Antimicrob. Agents Chemother. **32**, 706–709.
30. Pérez Castillo, A., Pérez Castillo, A. M., Sáez Nieto, J. A. (1994). Sequence of the penicillin binding protein 2-encoding gene (penA) of *Neisseria sicca/ perflava*. Gene **146**, 91–93.
31. Pérez Castillo, A., Pérez Castillo, A. M., Lujan, R., Sáez Nieto, J. A. (1994). Comparison of *penA* (PBP2) gene sequences of *Neisseria mucosa* moderately resistant to penicillin (pen^r) and *Neisseria meningitidis* (pen^r and pen^s) strains. Rev. Españ. Quimioter. **7**, 228–237.
32. Pérez Castillo, A., Pérez Castillo, A. M., Sáez Nieto, J. A. (1997). Genetic transformation of penicillin susceptible *Neisseria meningitidis* with *Neisseria perflava/sicca* moderately resistant to penicillin and *penA* gene sequences of transformants. Rev. Españ. Quimioter. (In press).
33. Pérez Trallero, E., Aldamiz-Echevarría, L., Pérez Yarza, E-G. (1990). Meningococci with increased resistance to penicillin. Lancet **i**, 1096–1097.

34. Riley, G., Brown, S. Krishnam, C. (1991). Penicillin resistance in *Neisseria meningitidis*. N. Engl. J. Med. **324**, 997.
35. Rohner, P., Pepey, B., Hirschel, B., Auckenthaler, R. (1992). Typing and sensitivity of meningococci isolated in Switzerland 1988–1990. Schweiz. Med. Wochenschr. **122**, 224–228.
36. Ropp, P. A., Nicholas, R. A. (1997). Cloning and characterization of the ponA gene encoding penicillin-binding protein from *Neisseria gonorrhoeae* and *Neisseria meningitidis*. J. Bacteriol. **179**, 2783–2787.
37. Sáez Nieto, J. A., Fontanals, D., García de Jalón, J., Martínez de Artola, V., Pena, P., Morena, M. A., Verdaguer, R., Sanfeliu, I., Belio Blasco, C., Pérez Sáenz, J. L. (1987). Isolation of *Neisseria meningitidis* strains with increase of penicillin minimal inhibitory concentrations. Epidemiol. Infect. **99**, 463–469.
38. Sáez Nieto, J. A., Campos, J. (1988). Penicillin-resistant strains of *Neisseria meningitidis* in Spain. Lancet **i**, 1452–1453.
39. Sáez Nieto, J. A., Vazquez, J. A., Marcos, C. (1990). Meningococci moderately resistant to penicillin. Lancet **ii**, 54.
40. Sáez Nieto, J. A., Lujan, R., Martínez Suárez, J. V. Berrón, S., Vázquez, J. A., Viñas, M., Campos, J. (1990). *Neisseria lactamica* and *Neisseria polysaccharea* as possible sources of meningococcal beta-lactam resistance by genetic transformation. Antimicrob. Agents Chemother. **34**, 2269–2272.
41. Sáez Nieto, J. A., Lujan, R., Berrón, S., Campos, J., Viñas, M., Fuste, C., Vázquez, J. A., Zhang, Q. Y. Bowler, L. D., Martínez Suárez, J. V., Spratt, B. G. (1992). Epidemiological and molecular basis of penicillin resistant *Neisseria meningitidis* in Spain: a five year history (1985–1989). Clin. Infect. Dis. **14**, 394–402.
42. Scribner, R. K., Wedro, B. C., Weber, A. H., Marks, M. I. (1982). Activities of eight new beta-lactam antibiotics and seven antibiotics combinations against *Neisseria meningitidis*. Antimicrob. Agents Chemother. **21**, 678–680.
43. Spratt, B. G. (1988). Hybrid penicillin binding proteins in penicillin resistant strains of *Neisseria gonorrhoeae*. Nature **332**, 173–176.
44. Spratt, B. G., Zhang, Q. Y., Jones, D. M., Hutchinson, A., Brannigan, J. A., Dowson, C. G. (1989). Recruiments of a penicillin-binding protein gene from *Neisseria flavescens* during the emergence of penicillin resistance in *Neisseria meningitidis*. Proc. Natl. Acad. Sci. USA **86**, 8988–8992.
45. Spratt, B. G., Bowler, J. D., Zhang, Q. Y., Zhou, J., Maynard Smith, J. (1992). Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal *Neisseria* species. J. Mol. Evol. **34**, 115–125.
46. Sutcliffe, E. M., Jones, D. M., El-Sheikh, S., Percival, A. (1988). Penicillin insensitive meningococci in the UK. Lancet **i**, 657–658.
47. Turner, P. C., Suthern, K. W., Spencer, N. J., Pullen, H. (1990). Treatment failure in meningococcal meningitis. Lancet **335**, 732–733.
48. Tzanakaki, G., Blackwell, C. C., Kremastinou, J., Kallergi, C., Kouppari, G., Weir, D. M. (1992). Antibiotic sensitivities of *Neisseria meningitidis* isolates from patients and carriers in Greece. Epidemiol. Infect. **108**, 449–455.
49. Uriz, S., Pineda, V., Grau, M., Nava, J. M., Bella, F., Morera, M. A., Fontanals, D., Font, B., Martí, C., Deulofeu, F., Calderón, A., Duran, P., Matas, E., Garau, J. (1991). *Neisseria meningitidis* with reduced sensitivity to penicillin: observation in 10 children. Scand. J. Infec. Dis. **23**, 171–174.
50. Vázquez, J. A. (1993). Infección meningocócica en España (1989–1992). Bol. Epidemiol. Sem. 11/93.
51. Vázquez, J. A., Marcos, C., Berrón, S. (1994). Sero/subtyping of *Neisseria meningitidis* isolated from patients in Spain. Epidemiol. Infect. **113**, 267–274.
52. Vázquez, J. A., Marcos, C., De la Fuente, L., Berrón, S. (1996). Descenso de la incidencia de meningococos con resistencia moderada a penicilina. Enf. Infec. Microbiol. Clin. **14**, 273.
53. Vázquez, J. A., Enriquez, A. M., de la Fuente, L., Berrón, S., Baquero, M. (1996). Isolation of a strain of beta-lactamase producing *N. meningitidis* in Spain. Eur. J. Clin. Microbiol. Clin. **15**, 181–182.
54. Wiggins, G. L., Mc Laughlin, J. V., Bickman, S. T., Jones, W. L., Balows, A. (1970). Susceptibility of *Neisseria meningitidis* strains from the civilian population to sulfadiazinem penicillin and rifampin. Appl. Microbiol. **20**, 893–898.
55. Woods, C. R., Smith, A. L. Wasilauskas, B. L., Campos, J. Givner, L. B. (1994). Invasive disease caused by *Neisseria meningitidis* relatively resistant to penicillin in North Carolina. J. Infect. Dis. **170**, 453–456.
56. Zhang, Q. Y., Jones, D. M., Sáez Nieto, J. A., Pérez Trallero, E., Spratt, B. G. (1990). Genetic diversity of penicillin binding protein 2 genes of penicillin resistant strains of *Neisseria meningitidis* revealed by fingerprinting of amplified DNA. Antimicrob. Agents Chemother. **34**, 1523–1528.

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Resistance to antivirals in human cytomegalovirus: mechanisms and clinical significance

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Summary

Long term therapies needed for managing human cytomegalovirus (HCMV) infections in immunosupressed patients provided the background for the emergence of the resistance to antivirals active against HCMV. In addition, laboratory selected mutants have also been readily achieved. Both clinical and laboratory resistant strains share the same determinants of resistance. Ganciclovir resistance may be due to a few mutations in the HCMV UL97 gene and/or viral DNA pol gene, the former being responsible for about 70% of clinical resistant isolates. Among them, V464, V594, S595 and F595 are the most frequent mutations. Because of their less extensive clinical use, much less is known about resistance to foscarnet and cidofovir (formerly, HPMPC) but, in both cases, it has been associated to mutations in the DNA pol. Ganciclovir resistant strains showing DNA pol mutations are cross-resistant to cidofovir and their corresponding IC50 are normally higher than those from strains harboring only mutations at the UL97 gene. To date, foscarnet resistance seems to be independent of both ganciclovir and cidofovir resistance.

Key words: cytomegalovirus, antiviral resistance, ganciclovir, foscarnet, cidofovir

Resumen

La larga duración de los regímenes terapéuticos utilizados para el tratamiento de las infecciones por el citomegalovirus humano (HCMV) en los pacientes inmunodeprimidos, es el origen de la aparición de resistencia a los antivíricos más comunes. Además, ha sido fácil la selección de mutantes de laboratorio. Tanto las cepas resistentes obtenidas en clínica como las seleccionadas in

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vitro comparten los mismos determinantes de resistencia. La resistencia al ganciclovir puede originarse en unas pocas mutaciones del gen UL97 del HCMV y/o en el gen de la DNA pol viral, siendo las primeras los determinantes responsables de la resistencia en alrededor del 70% de las cepas resistentes aisladas en la clínica. En particular, las mutaciones V464, V594, S595y F595 del gen UL97 son las más frecuentes. Debido a su menor uso en la práctica clínica, se conoce bastante menos sobre la resistencia a foscarnet y cidofovir (antes HPMPC), si bien en ambos casos está restringida a mutaciones en el gen de la DNA pol. Las cepas resistentes a ganciclovir que son portadoras de mutaciones en este último gen muestran la resistencia cruzada con el cidofovir y suelen presentar unas CI50 más elevadas que las cepas cuya resistencia se debe a mutaciones del gen UL97. Por el momento, la resistencia a foscarnet parece originarse en determinantes diferentes a los del ganciclovir y del cidofovir.

Introduction

Human cytomegalovirus (HCMV) is a prevalent pathogen distributed world-wide. Following the initial (primary) infection, the organism establishes latency, and is capable of reactivating like the other members of the *Herpesviridae* family. Primary infection occurs mostly during childhood. HCMV is excreted through many body fluids (blood, urine, saliva, genital secretions, etc.) and is transmitted by close contact, organ transplantation or, parenterally, with blood products. As a consequence, a substantial proportion of the population in the adulthood becomes infected, as seroepidemiological surveys show. In normal hosts, the proportion of infected adults ranges from 50 to 80%, depending on age and socioeconomic factors (24). However, in selected groups with high-risk practices for transmission, such as human immunodeficiency virus (HIV)-infected patients, virtually 100% of them are seropositive for HCMV, and are at highest risk for developing active HCMV infections by reactivating the latent virus.

In a normal population, the vast majority of active infections (those with viral replication), either primary or reactivations, are assymptomatic. In immunosupressed patients, however, HCMV is a major opportunistic pathogen that causes a wide variety of clinical syndromes ranging from mild to severe life-threatening infections. In bone marrow and solid organ transplant recipients, pneumonitis and hepatitis are the most serious clinical syndromes caused by HCMV (23). In AIDS patients, the infection is associated with retinitis, gastrointestinal disease and encephalitis. Furthermore, retinitis is the most common late-stage manifestation of HCMV in severely CD4+ depressed AIDS patients and is one of the leading causes of opportunistic infections in this group of patients (25).

Recent advances in rapid diagnosis and the availability of active drugs against HCMV have provided the basis for effective therapies, but most of these therapeutical strategies are suppressive rather than curative approaches. Consequently, long-time prophylaxis or maintenance treatments are required for managing clinical situations such as interstitial pneumonitis in bone-marrow transplant recipients and retinitis in patients with AIDS, respectively. In addition, drug toxicity lead to frequent discontinuation and suboptimal dosage of the antivirals currently used against HCMV. Selective pressure in this context provides the background for the emergence of resistant mutants to antivirals.

Drug-resistant strains to the three antivirals used for treating HCMV infections (ganciclovir,

foscarnet and cidofovir) as well as to other investigational drugs have been readily achieved in vitro in the laboratory (4, 11, 28, 40; Cherrington, J. M., Mulato, A. S., Fuller, M., Chen, M. S. [1995]. 35th Intersci. Confer. Antimicrob. Agents Chemother., p. 200). It has been also possible to select in vivo for resistant strains associated with therapeutical failures to antivirals, as it has been demonstrated in clinical practice (2, 16, 19, 22, 37). In vivo, selected mutants share mechanisms and determinants of resistance similar to those described for the laboratory strains, thus allowing to characterize the resistance patterns both phenotypically and genotypically. In the present article, we revised the genetics and resistance mechanisms of antivirals in HCMV as well as the clinical significance of the emergence of such a resistance.

Resistance to ganciclovir in cytomegalovirus

Ganciclovir, (9-[1-3-dihydroxy-2-propoxymethyl] guanine; formerly known as DHPG) is an acyclic nucleoside analogue that is a potent inhibitor of the HCMV DNA polymerase (DNA pol). Although the compound is active also against other members of the herpes family, its primary therapeutical interest has been focused on HCMV (32). It has a unique activity profile and a higher in vitro potency against HCMV than other nucleoside analogues. The drug inhibits the viral enzyme by competition with its natural substrate, deoxy-guanosine (5, 19, 20). Both ganciclovir and deoxy-guanosine must be converted to their respective triphosphate derivatives for interacting with the viral DNA pol. So, it shares with other antivirals the general mechanism of action (32).

Ganciclovir has also a selective activity for the viral enzyme in comparison with cellular polymerases. For instance, ganciclovir triphosphate is able to inhibit the HCMV DNA pol with a K_i of 1.7 μM, while the K_i value for the corresponding cellular enzymes is about 10 times higher (20, 30). Nevertheless, this selective activity does not fully account for the exceptional in vitro activity of ganciclovir. In fact, other nucleoside analogues, such as acyclovir-TP, are also highly inhibitory for the viral enzyme but they show a moderate activity against HCMV. These differences may be explained by the differential cellular metabolism of ganciclovir. Studies on cellular uptake of radiometric ganciclovir have shown intracellular levels of this drug 10-fold higher than those of other nucleoside analogues (5, 20). In conclusion, the selective action is enhanced by a favourable metabolism which leads to the accumulation of ganciclovir triphosphate in the infected cells. Once phosporilated, ganciclovir is incorporated competitively into the DNA chain causing a drastic supression of its elongation (5, 31, 37). The inhibitory effect is reversible, and DNA elongation resumes after the removal of ganciclovir from the medium (32). As a consequence, ganciclovir appears as a virustatic agent rather than a viricidal one; thus the need for prolonged therapies when treating severe infections.

Taking into acount the mechanisms of action of ganciclovir, there are three theoretical possibilities for the development of resistance: (i) a viral-mediated blockage of the intracellular uptake of ganciclovir, (ii) the supression (or lower efficiency) of ganciclovir phosphorilation, and (iii) selection for mutants with alterations in the viral DNA pol. The two later possibilities have been confirmed by current experience.

It has been difficult to define ganciclovir resistante in vitro because of the above mentioned factors influencing susceptibility assays. Data from "wild" pretherapy isolates from patients with HCMV active infections have shown IC_{50} ranging from 0.5 to 10 μM (32, 34), depend-

ing on the type of cell line used for susceptibility testing. In contrast, IC_{50} values for isolates obtained from cases of therapy failures are normally 5 to 10 times higher than their respective pretherapy viruses (16). Taking into account the cumulative experience, a concentration above 12 μM is currently accepted as the IC_{50} breakpoint for resistance (13, 14). Because of the broad range in susceptibility values of both susceptible and resistant groups of strains, it is advisable to assay in parallel reference strains (such as AD169) and, if available, the corresponding pretherapy isolates when testing clinical strains.

Biochemical, genetic and molecular studies with both clinical and laboratory-selected resistant strains have provided a remarkable knowledge of the mechanisms of resistance to ganciclovir in HCMV. Early investigations on drug metabolism in resistant strains showed their inability to induce ganciclovir phosphorilation in HCMV-infected cells systems. In contrast, pretherapy susceptible strains retained their phosphorilative activity (37). No differences were shown when comparing susceptible and resistant strains regarding viral DNA pol activity. These early results suggested the existence of a thymidine kinase-defficient phenotype as occur in herpes simplex virus resistance to acyclovir (4, 32, 37). However, efforts to detect such an enzyme were unsuccessful, as were attempts to identify a viral gene analogous to that coding for thymidine kinase in herpes simplex viruses. In addition, there were several indirect evidencies of the existence of a viral enzyme, not a cellular one, responsible for ganciclovir phosphorilation (5, 20, 37). Genetic and biochemical approaches studying a laboratory-selected, phosphorilation-defective HCMV strain provided the demonstration that mutations in the UL97 HCMV gene are responsible for ganciclovir resistance (27, 40). These mutations have been detected in a significant proportion of laboratory and clinical resistant strains (1, 2, 9, 10, 21, 28, 33, 43). Gene sequencing has shown point mutations or short deletions at very specific sites of the UL97 gene, suggesting the existence of a catalytic pocket associated to the corresponding amino acid residues.

Table 1 summarizes the most frequent UL97 gene mutations responsible for ganciclovir resistance. Note that most of these mutants, but not all, retain an intact DNA pol activity and do not show any cross resistance with other antivirals, such as foscarnet and cidofovir, which primarily inhibit this enzyme. Little is known about the

TABLE 1. Mutations at the UL97 gene conferring resistance to ganciclovir in HCMV

Mutation	Codon	Result	Remarks
ATG→ATT	460	Methionine^{460}→Isoleucine	Clinical and laboratory[a] strains
ATG→GTG	460	Methionine^{460}→Valine	Clincal strains
CAC→CAA	520	Hystidine^{520}→Valine	Clinical strains
GCG→GTG	594	Alanine^{594}→Valine	Clinical strains
TTG→TCG	595	Leucine^{595}→Serine	Clinical strains
TTG→TTT	595	Leucine^{595}→Phenylalanine	Clinical strains
3 bp deletion	595	Lost of Leucine^{595}	Clinical strains
12 bp deletion	638–641	Lost of four amino acids	Laboratory[a] strains
12 bp deletion	591–594	Lost of four amino acids	Clinical strains

[a] Double mutants at UL97 and DNA pol genes.

characteristics of the UL97 gene product, except for an estimated molecular weight of 78 kDa and the homology with several naturally-occurring protein (not nucleotide) kinases (19). This protein has neither been isolated nor has been its biological significance ellucidated, although there is some evidence supporting its relationship with the HCMV replicative cycle.

As noted above, most of the resistant strains can be linked to mutations in the UL97 gene (11, 17). Besides this predominant mechanism, resistance to ganciclovir in HCMV associated to mutations in the viral DNA pol gene has been demonstrated (3, 17, 29, 38); as expected, these two resistance mechanisms are not mutually exclusive (3, 26, 41). However, there is little information regarding genetic determinants and biological functions of DNA pol mutations. Although sequence analysis frequently denotes many different point mutation in the viral DNA pol gene of both susceptible and resistant strains, resistance to ganciclovir seems to be confined to a few codons located at specific sites of conserved regions, namely regions II, IV and V (Table 2) (17, 19, 38). Interestingly, ganciclovir resistant strains harboring such DNA pol mutations, show cross-resistance to cidofovir, but not to foscarnet (17). This suggests that the former has a different active site to that of ganciclovir and cidofovir, and has evident consequences from the therapeutical point of view.

Regarding the clinical significance of resistant mutants, from the literature, it is clear the association between resistance and therapy failures in a significant proportion of cases (16). Moreover, the detection of UL97 resistant mutants may preceed the emergence of a clinically apparent disease during maintenance therapy with ganciclovir (Pérez, J. L, Erice, A., Niubò, J., García, A., Podzamczer, D., Martín, R. [1996]. VI Congr. Nac. Enferm. Infec. Microbiol. Clin., pp. 120). In addition, increasing viral load both in plasma and leukocytes can be demonstrated during breaktrough HCMV infections (7), which offers the possibility of monitoring antiviral treatment with this laboratory marker. However, the frequency of selecting resistant strains during therapy has not been completely defined in different groups of patients at risk. Drew et al. (13) described up to 7.8% of resistant strains among AIDS patients treated for retinitis. The proportion of resistant strains raised to 38% in patients subjected to long-term (more than three months) treatment. Thus, prolonged ganciclovir therapy appears to be the major risk factor for the development of clinical and virological resistance. In contrast, intensive and short courses of therapy, as in the treatment of HCMV infections in solid organ transplant patients, has not been associated with the emergence of resistant strains (6). As it could be expected, most cases of resistance have been described in AIDS and

TABLE 2. Mutations in the DNA pol gene conferring resistance to ganciclovir

Mutation	Codon	Result	Remarks
TTT → GTT	412	Phenylalanine^{412}→Valine	Laboratory strains[a]
GAC→ GAA	413	Aspartate^{413}→Glutamate	Clinical strains
CTC→ ATC	501	Leucine^{501}→Isoleucine	Laboratory strains[a]
ACG→ GCG	700	Threonine^{700}→Glutamate	Clinical strains[a]
GTG→ ATG	715	Valine^{715}→Methionine	Clinical strains[a]
GCC→ GGC	987	Alanine^{987}→Glycine	Laboratory strains[a]

[a] Double mutants at DNA pol and UL97 genes.

bone marrow transplant patients, whereas only sporadically virologically-confirmed cases from solid organ recipients have been reported.

Resistance to foscarnet

Foscarnet is a pyrophosphate analogue with antiviral in vitro activity against members of the herpes family and some other viruses including retroviruses (42). It shows a virustatic activity against HCMV, as the other drugs used in the treatment of the infections caused by this virus. Once again, long-term foscarnet therapies, necessary for managing some of these patients, could lead to the selection of resistant mutants. However, due to its limited clinical use in comparison with ganciclovir, resistant strains to foscarnet has been less frequently reported.

Foscarnet is also a potent, but reversible, inhibitor of the viral DNA pol. Although the precise mechanism of action has not been yet fully understood, foscarnet seems to block the pyrophosphate binding site of the HCMV DNA pol, thus preventing the pyrophosphate cleavage. A complex enzyme-substrate is subsequently formed, leading to the interruption of the enzymatic activity and, eventually, to stop chain elongation (42). Foscarnet also interacts with the cellular enzymes but to a lesser extent than with the HCMV DNA pol (18).

The less frequent use of foscarnet in the clinical practice has hampered the real knowledge of the resistance. In addition, technical factors such as cell type, multiplicity of infection, and method used for susceptibility testing have also influenced the consistency of test results. Nevertheless, by studying reference strains and pretherapy clinical isolates, a temptative breakpoint of >400 μM has been proposed for resistance (14).

The mechanisms and the determinants of resistance to foscarnet are poorly understood and some of the current knowledge comes from a generalization of parallel experiences with other viral pathogens (e.g. herpes simplex virus). Foscarnet resistant strains have been selected both in vitro (11, 39), and from clinical specimens (3, 15, 26, 41). Preliminary data suggest an association of mutations at codons 700 and 715 in a conserved region of the HCMV DNA pol gene with emergence of foscarnet resistance (3). A more extensive characterization of the mutations conferring resistance to foscarnet faces both practical (reduced use) and technical (lenght of the viral DNA pol gene). Besides, it could clarify some concerns about cross-resistance with other DNA pol inhibitors. HCMV strains showing resistance to both ganciclovir and foscarnet have been isolated from patients treated sequently and for long periods of time with these drugs (26, 41). Fortunately, recent data on ganciclovir and cidofovir resistant mutants of the DNA pol gene have shown no cross-resistance with foscarnet (17), thus supporting that these two antiviral do not share the same binding site of that of foscarnet. In addition, the good clinical experiences in patients switched from ganciclovir to foscarnet therapy (and vice versa) also allows us to be optimistic for the future.

Resistance to cidofovir

Cidofovir (S-4-[3-hydroxy-2-phosphonylmethoxypropyl]cytosine), formerly known as HPMPC, is an acyclic phosphonate nucleotide analogue of 2'-deoxyciytidine monophosphate exhibiting a high in vitro activity against a wide variety of DNA viruses of different families. Particularly, it has a potent, long-lasting activity against HCMV and other herpesviruses (12).

The compound has also proven to be effective in vivo in animal models for HCMV infections. Although promising by its pharmacokinetic and virological features, there is a limited experience on clinical use and, consequently, a small knowledge on the mechanisms and of resistance significance. Cidofovir-resistant mutants, either from laboratory or from clinical origin, have been described (8, 17, 35).

Cidofovir and other phosphonate derivatives differ from typical nucleoside analogues (e.g. ganciclovir) in that they present a phosphonate residue. Thus, they only need for two additional phosphorilation steps to be active against viral DNA pol, the ultimate target for the antivirals. Cellular enzymes are able to phosphorilate cidofovir, thus bypassing the requeriment for the initial phosphorilation associated to the HCMV-specific UL97 gene product.

Cidofovir is an inhibitor of the viral DNA pol, but it is also active against cellular polymerases. As in other antivirals, the affinity for the cellular enzymes is much lower than that for the viral DNA pol. As a consequence, IC_{50} for inhibiting viral replication is 1000-fold lower than for cell proliferation (36). Cidofovir and its metabolites tend to accumulate in infected cells for a long period of time, which explains the long-lasting inhibitory effect on HCMV. Whether DNA pol inhibition by cidofovir is due to (i) a competitive inhibition of the natural substrate, (ii) an alternate substrate acting a chain terminator, or (iii) an alternate substrate being incorporated to the new DNA chain, remains to be ellucidated.

As expected because of its mechanism of action, ganciclovir-resistant mutants harboring mutations only in the UL97 gene do not show cross-resistance neither with cidofovir nor with other phosphonate nucleoside derivatives. Then, cidofovir resistant mutants should map to the viral DNA pol gene. In one study with a laboratory resistant strain, mutations at codons 412 (conserved region IV) and 501 (between conserved region IV and A) were associated with cidofovir and ganciclovir resistance (29). Similarly, mutations at codons 408 and 413 of the conserved region IV were responsible of resistance to both drugs in clinical strains (17). It seems that resistance to cidofovir is confined to a specific region of the HCMV DNA pol gene, thus suggesting that the corresponding amino acids are essential components of an active enzymatic pocket. It has been proposed that the nucleoside binding site is altered when these mutations occur. No specific mutations conferring resistance to cidofovir alone have been described to date, nor has been cross-resistance between this drug and foscarnet demonstrated. In addition, no resistance mechanisms other than that associated to DNA pol mutations have been described for cidofovir. This is of significance for the clinical practice, because it confirms the hypothesis of divergent mechanisms of action in nucleoside derivatives and pyrophosphate analogues.

As for the significance of the emergence of cidofovir-resistant strains during therapy for HCMV infections, little is known because of the limited clinical use of this drug. Short treatments do not select for resistant mutants to cidofovir (8). However, cidofovir resistance has been associated to prolonged ganciclovir therapy. In general, isolates showing high level of resistance to the latter compound (above 30 μM) are cross-resistant to cidofovir and, as expected, they harbor point mutations at the DNA pol gene, no matter the presence of mutations at the UL97 gene. On the other hand, strains with IC_{50} for ganciclovir within 12 and 30 μM (low level resistance) do not normally show cross-resistance to cidofovir and do not harbor mutations at the DNA pol gene. To sum up, it should be assumed that resistance to ganciclovir, does not

necessarily mean cross-resistance to cidofovir. On the other hand, cidofovir-resistant strains should be considered to be resistant also to ganciclovir and susceptible to foscarnet.

References

1. Alain, S., Mazeron, M. C., Pépin, J. M., Bergman, J. F., Narwa, R., Raskine, L., Sanson-Le Pors, M. J. (1995). Value of a new rapid non-radiactive sequencing method for analysis of the cytomegalovirus UL97 gene in ganciclovir-resistant strains. J. Virol. Methods. **51**, 241–252.
2. Baldanti, F., Silini, E., Sarasini, A., Talarico, C. L., Stanat, S. C., Biron, K. K., Furione, M., Bono, F., Palú, G., Gerna, G. (1995). A three nucleotide deletion in the UL97 open reading frame is responsible for the ganciclovir resistance of a human cytomegalovirus clinical isolate. J. Virol. **69**, 796–800.
3. Baldanti, F., Underwood, M. R., Stanat, S. C., Biron, K. K., Chou, S., Sarasini, A., Silini, E., Gerna, G. (1996). Single amino acid changes in the DNA polymerase confer foscarnet resistance and slow-growth phenotype, while mutations in the UL97-encoded phosphotransferase confer ganciclovir resistance in three double-resistant human cytomegalovirus strains recovered from patients with AIDS. J. Virol. **70**, 1390–1395.
4. Biron, K. K., Fyfe, J. A., Stanat, S. C., Leslie, L. K., Sorrell, J. B., Lambe, C. U., Coen, D. M. (1986). A human cytomegalovirus mutant resistant to the nucleoside analog 9-{[2-hydroxy-1-(hydroxymethyl)ethoxy]methyl}guanine (BW B759U) induces reduced levels of BW B759U triphosphate. Proc. Natl. Acad. Sci. USA **83**, 8769–8773.
5. Biron, K. K., Stanat, S. C., Sorrell, J. B., Fyfe, J. A., Keller, P. M., Lambe, C. U., Nelson, D. J. (1985). Metabolic activation of the nucleoside analog 9-{2-hydroxy-1-(hydroxymethyl) ethoxy]methyl} guanine in human diploid fibroblasts infected with human cytomegalovirus. Proc. Natl. Acad. Sci. USA **82**, 2473–2477.
6. Boivin, G., Erice, A., Crane, D. D., Dunn, D. L., Balfour, H. H. (1993). Ganciclovir susceptibilities of cytomegalovirus (CMV) isolates from solid organ transplant recipients with CMV viremia after antiviral prophylaxis. J. Infect. Dis. **168**, 332–335.
7. Boivin, G., Chou, S., Quirk, M. R., Erice, A., Jordan, M. C. (1996). Detection of ganciclovir resistance mutations and quantification of cytomegalovirus (CMV) DNA in leukocytes of patients with fatal disseminated CMV disease. J. Infect. Dis. **173**, 523–528.
8. Cherrington, J. M., Miner, R., Hitchcock, M. J. M., Lalezari, J. P., Drew, W. L. (1996). Susceptibility of human cytomegalovirus to cidofovir is unchanged after limited in vivo exposure to various clinical regimens of drug. J. Infect. Dis. **173**, 987–992.
9. Chou, S., Erice, A., Jordan, M. C., Vercellotti, G. M., Michels, K. R., Talarico, C. L., Stanat, S. C., Biron, K. K. (1995). Analysis of the UL97 phosphonotransferase coding sequence in clinical cytomegalovirus isolates and identification of mutations conferring ganciclovir resistance. J. Infect. Dis. **171**, 576–583.
10. Chou, S., Guentzel, S., Michels, K. R., Miner, R. C., Drew, W. L. (1995). Frequency of UL97 phosphonotransferase mutant related to ganciclovir resistance in clinical cytomegalovirus isolates. J. Infect. Dis. **172**, 239–242.
11. D'Aquila, R. T., Summers, W. C. (1987). Isolation and characterization of phosphonoacetic-acid resistant mutant of human cytomegalovirus. J. Virol. **61**, 1291–1295.
12. De Clercq, E. (1993). Therapeutic potential of HPMPC as an antiviral drug. Rev. Med. Virol. **3**, 85–96.
13. Drew, W. L., Miner, R. C., Busch, D. F., Follansbee, S. E., Gullett, J., Mehalko, S. G., Gordon, S. M., Owen, W. F., Matthews, T. R., Buhles, W. C., de Armond, B. (1991). Prevalence of resistance in patients receiving ganciclovir for serious cytomegalovirus infection. J. Infect. Dis. **163**, 716–719.
14. Drew, W. L., Miner, R. C., Saleh, E. (1993). Antiviral susceptibility testing of cytomegalovirus: criteria for detecting resistance to antivirals. Clin. Diagn. Microbiol. **1**, 179–185.
15. Dunn, J. P., MacCumber, M. W., Forman, M. S., Charache, P., Apuzzo, L., Jabs, D. A. (1995). Viral sensitivity testing in patients with cytomegalovirus retinitis clinically resistant to foscarnet or ganciclovir. Am. J. Opthalmol. **119**, 587–596.
16. Erice, A., Chou, S., Biron, K. K., Stanat, S. C., Balfour, H. H., Jordan, M. C. (1989). Progressive disease due to ganciclovir-resistant cytomegalovirus in immunocompromised patients. N. Eng. J. Med. **320**, 289–293.

17. Erice, A., Gil-Roda, C., Pérez, J. L., Balfour, H. H., Sannerud K. J., Hanson, M. N., Boivin, G., Chou, S. (1997). Antiviral susceptibilities and analysis of UL97 and DNA polymerase sequences of clinical cytomegalovirus isolates from immunocompromised patients. J. Infect. Dis. **175**, 1087–1092.
18. Ericksson, B., Öberg, B., Wahren, B. (1982). Pyrophosphate analogues as inhibitors of DNA polymerases of cytomegalovirus, herpes simplex virus and cellular origin. Biochim. Biophys. Acta **696**, 115–123.
19. Field, A. K., Biron, K. K. (1994). "The end of innocence" revisited: resistance of herpesvirus to antiviral drugs. Clin. Microbiol. Rev. **7**, 1–13.
20. Freitas, V. R., Smee, D. F., Chernow, M., Boehme, R., Matthews, T. R. (1985). Activity of 9(1,3-dihydroxy-2-propoxymethyl)guanine compared with that of acyclovir against human, monkey and rodent cytomegaloviruses. Antimicrob. Agents Chemother. **28**, 240–245.
21. Hanson, M. N., Preheim, L. C., Chou, S., Talarico, C. L., Biron, K. K., Erice, A. (1995). Novel mutation in the UL97 gene of a clinical cytomegalovirus strain conferring resistance to ganciclovir. Antimicrob. Agents Chemother. **39**, 1204–1205.
22. Hirsch, M. S., Schooley, R. T. (1989). Resistance to antiviral drugs: the end of innocence. N. Eng. J. Med. **320**, 313–314.
23. Hirsch, M. S. (1994). Herpes group virus infections in the compromised host. *In* Rubin, R. H., Young L. S. (ed.), Clinical Approach to Infection in the Compromised Host (3rd. ed.), pp. 379–422. Plenum Medical Book Co., New York.
24. Ho, M. (1991). Epidemiology of cytomegalovirus infection in man. *In* Ho, M. (ed.), Cytomegalovirus. Biology and infection (2nd. ed.), pp.155–187. Plenum Medical Book Co., New York.
25. Ho, M. (1991). Human cytomegalovirus infection in immunosupressed patients. *In* Ho, M. (ed.), Cytomegalovirus. Biology and infection (2nd. ed.), pp. 249–300. Plenum Medical Book Co., New York.
26. Knox, K. K., Drobyski, W. R., Carrigan, D. R. (1991). Cytomegalovirus isolate resistant to ganciclovir and foscarnet from a marrow transplant patient. Lancet **337**, 1292–1293.
27. Litter, E., Stuart, A. D., Chee, M. S. (1992). Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. Nature **358**, 160–162.
28. Lurain, N. S., Spafford, L. E., Thompsom, K. D. (1994). Mutation in the UL97 open reading frame of human cytomegalovirus strains resistant to ganciclovir. J. Virol. **68**, 4427–4431.
29. Lurain, N. S., Thompsom, K. D., Holmes, E. W., Read, G. S. (1992). Point mutations in the DNA polymerase gene of human cytomegalovirus that result in resistance to antiviral drugs. J. Virol. **66**, 7146–7152.
30. Mar, E. C., Cheng, Y. C., Huang, E. S. (1983). Effect of 9(1,3-dihydroxy-2-propoxymethyl) guanine on human cytomegalovirus replication in vivo. Antimicrob. Agents Chemother. **24**, 518–521.
31. Mar, E. C., Chiou, J. F., Cheng, Y. C., Huang, E. S. (1985). Inhibition of cellular DNA polymerase α and human cytomegalovirus-induced DNA polymerase by the triphosphates of 9-(2-hydroxyethoxymethyl)guanine and 9-(1,3-dihydroxy-2-propoxymethyl)guanine. J. Virol. **53**, 776–780.
32. Matthews, T., Boehme, R. (1988). Antiviral activity and mechanism of action of ganciclovir. Rev. Infect. Dis. **10**, S490–S494.
33. Metzger, C., Michel, D., Schneider, K., Lüske, A., Schlicht, H. J., Mertens, T. (1994). Human cytomegalovirus UL97 kinase confers ganciclovir susceptibility to recombinant vaccinia virus. J. Virol. **68**, 8423–8427.
34. Plotkin, S. A., Drew, W. L., Felsenstein, D., Hisrch, M. S. (1985). Sensitivity of clinical isolates of human cytomegalovirus to 9-(1,3-dihydroxy-2-propoxymethyl)guanine. J. Infect. Dis. **152**, 833–834.
35. Snoeck, R., Andrei, G., de Clercq, E. (1996). Patterns of resistance and sensitivity to antiviral compounds of drug-resistant strains of human cytomegalovirus selected in vitro. Eur. J. Clin. Microbiol. Infect. Dis. **15**, 574–579.
36. Snoeck, R., Sakuma, T., de Clercq, E., Rosemberg, I., Holy, A. (1988). (S)-1-3-(hydroxy-2-phosphonylmethoxypropyl)cytosine, a potent and selective inhibitor of human cytomegalovirus replication. Antimicrob. Agents Chemother. **32**, 1839–1844.
37. Stanat, S. C., Reardon, J. E., Erice, A., Jordan, M. C., Drew, W. L., Biron, K. K. (1991). Ganciclovir-resistant cytomegalovirus clinical isolates: mode of resistance to ganciclovir. Antimicrob. Agents Chemother. **35**, 2191–2197.
38. Sullivan, V., Biron, K. K., Talarico, C., Stanat, S. C., Davis, M., Pozzi, L. M., Coen, D. M. (1993). A point mutation in the human cytomegalovirus DNA polymerase gene confers resistance to ganciclovir and phosphonylmethoxyalkyl derivatives. Antimicrob. Agents Chemother. **37**, 19–25.

39. Sullivan, V., Coen, D. M. (1991). Isolation of foscarnet-resistant human cytomegalovirus patterns of resistance and sensitivity to other antiviral drugs. J. Infect. Dis. **164**, 781–784.
40. Sullivan, V., Talarico, C. L., Stanat, S. C., Davis, M., Coen, D. M., Biron, K. K. (1992). A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. Nature **358**, 162–164.
41. Tatarowitz, W. A., Lurain, N. S., Thompson, K. D. (1992). A ganciclovir-resistant clinical isolate of human cytomegalovirus exhibiting cross-resistance to other DNA polymerase inhibitors. J. Infect. Dis. **166**, 904–907.
42. Wagstaff, A. J., Bryson, H. M. (1994). Foscarnet. A reappraisal of its antiviral activity, pharmacokinetic properties and therapeutic use in immunocompromised patients with viral infections. Drugs **48**, 199–226.
43. Wolf, D. G., Smith, I. L., Lee, D. J., Freeman, W. R., Flores-Aguilar, M., Spector, S. A. (1995). Mutations in human cytomegalovirus UL97 gene confer clinical resistance to ganciclovir and can be detected directly in patient plasma. J. Clin. Invest. **95**, 257–263.

MICROBIOLOGÍA SEM **13** (1997), 353–356 **PERSPECTIVES**

Sergei I. Kuznetsov (1900–1987). The founder of Russian aquatic microbiology

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This year is the tenth anniversary of the death of Professor Sergei Ivanovich Kuznetsov, outstanding Russian microbiologist and pioneer of microbial aquatic ecology in Russia. Sergei I. Kuznetsov was born in Moscow on November 17, 1900. His father, Ivan S. Kuznetsov, was a talented, well known architect. The buildings he designed still can be seen in Moscow and in cities along the Black Sea shoreline.

In 1919 Kuznetsov entered the Physics and Mathematics Faculty of Moscow University and in 1923 he graduated in the Department of Biology of this faculty. Granted a position as Instructor in the Department of Plant Physiology, he worked under the guidance of Prof. E. E. Uspensky, who was to exert a great influence on his scientific development.

Kuznetsov's interest in microbiological processes in aquatic environments began very early when he was a student, and developed as he worked as a chemist-bacteriologist at the hydrobiological station at Deep Lake (Moscow region). These interests continued throughout his long life. From 1931 to 1941, Kuznetsov headed the microbiological laboratory of the limnological station in Kosino, near Moscow. His scientific production from this period was to be relevant both for him and for the opening and continuation of a line of research in the field of limnology and aquatic ecology.

The major articles he published at that time, all corresponding to his stay in Kosino, were *A comparative study of nitrogen, phosphorus and oxygen regime in lakes Glubokoe and Beloe* (1934), *The effect of reserves of easily-hydrolysed nitrogen in the mud on the overall character of reduction processes in different lakes* (1937), and *Determination of the rate of oxygen uptake from the water mass of a lake at the expense of bacteriological processes* (1939). Despite of the difficulty had the rest of the scientific community understanding Russian, translations (in some cases), communications and exchange allowed his works to be known abroad. Researchers could take advantage of Kuznetsov's contributions to limnology and ecology of aquatic microorganisms and to the knowledge of biogeochemical processes.

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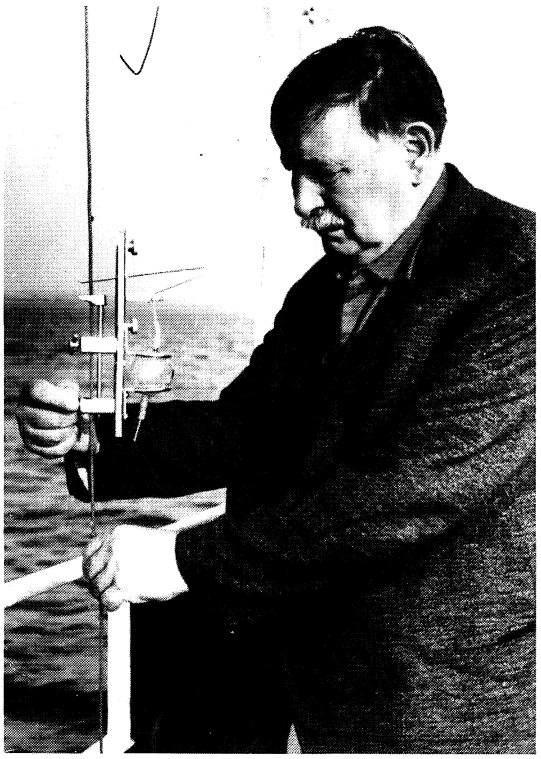

FIG. 1. Sergei I. Kuznetsov (1900–1987).

At the outbreak of Word War II, Kuznetsov became head of the Microbiology Laboratory of wastewater purification plant in Lublino. From 1941 he also chaired the Department of Microbiology of the Department of Biology at Moscow State University.

He worked at the Institute of Microbiology of the Soviet Union Academy of Sciences in the Department of Geological Activities of Microorganisms, which had been previously chaired by the academician B. L. Isachenko—who was then director of the Institute. Kuznetsov worked at the Institute of Microbiology for 41 years. In 1953 he created and chaired a microbiology laboratory at the Institute of Biology of Inland Waters of the Academy of Sciences. Although later this laboratory was headed by his disciple J. Sorokin, and later by V. Romanenko, Kuznetsov continued to serve as a consultant for many years.

In 1934, Kuznetsov married Cleopatra Apushkina. They had four children, but only one daughter is now alive.

The scientific interests of Kuznetsov were wide-ranging. Many of his studies were devoted to the biogeochemical cycles of carbon, sulfur, nitrogen, iron, manganese, and other elements present in fresh water. Numerous expeditions to different lakes of the central parts of the former USSR—in Siberia, Karelia, Middle Asia, and other regions—enabled him to make a great number of observations that were generalized in his monograph: *Role of Microorganisms in Geochemical Cycles in Lakes*, published in 1952. In 1970, he published *Microflora of Lakes and Its Geochemical Activity*, a book without equal in the microbiological literature. His approach to microbial phenomena in fresh waters and the coverage are impressive.

For this book, Kuznetsov was awarded the Sergei Winogradski Prize, without any doubt, a much deserved prize. He had always admired Winogradski, the founder of the ecology of soil microorganisms. Differently from the classical methods to study microorganisms, Winogradski had outlined new approaches and considered that both the study of ecology and the search for new organisms needed to be preceded by the acquaintance with the natural phenomena. Kuznetsov and his collaborators followed the same path by studying the ecology of microorganisms in inland waters. The study of aquatic microbiology is essential for many practical problems—productivity of reservoirs, prognosis of hydrochemical and biological regimes, environmental problems.

Kuznetsov was one of the first scientists in Russia to use modern research techniques, especially radioactive isotopes, to study natural processes. The application of such modern tech-

niques allowed him to quantify and determine characteristics of the activity of sulfate-reducing, photosynthetic, methanogenic and other groups of bacteria, as well as their roles in the cycles of carbon, sulfur and oxygen in lakes. His use of electron microscopy for the study of aquatic microorganisms greatly contributed to our knowledge about their morphology.

Kuznetsov published a handbook of methods for aquatic microbiologists, limnologists and hydrobiologists.

His theoretical investigations in the field of aquatic microbiology established a basis for forecasting the hydrochemical state and productivity of newly created water reservoirs that found an application in wastewater treatments. He had collaborated in the development of methods for the treatment of industrial wastewaters containing perchlorates and chromates

His monograph *The Ecology of Aquatic Microorganisms*, written in 1977 with his disciples V. M. Gorlenko and G. A. Dubinina, deals with the ecology of aquatic microorganisms—their variety, methods of isolation, and description of new genera and species. The book is considered to be a major treatise on aquatic microbiology. All important microbial reactions of the essential inorganic and organic compounds were treated there, and it was established that the major external factors that affect the ecology of microorganisms were: the presence of nutrient elements into the water, light penetration, and the aerobic state of the water mass. The book concludes that "...there exists in nature the finest regulation of all biological processes."

The cycles of organic matter, sulfur, iron, nitrogen, phosphorus and other elements are most closely bound with each other, and before attempting to interfere with the equilibrium existing in nature, before trying to change nature, it is essential to assess what it may lead to". The last monograph that Kuznetsov published in his lifetime was *Microbiological Processes of Carbon and Nitrogen Cycle in Lakes*, coauthored with A. I. Saralov and T. N. Nazina. It is a quantitative description of the biogeochemical cycles of carbon and nitrogen.

In the mid 1950's Kuznetsov began to investigate, together with his collaborators, the role of microorganisms in geochemical processes in sulfur and oil deposits. The study of the role of sulfate-reducing bacteria in the formation of hydrogen sulfide in floated oil deposits helped to develop the methods to prevent metal corrosion of oil equipment. Kuznetsov participated in the development of microbiological techniques for the prospect of oil deposits and oil recovery.

The significant roles played by microorganisms in both the formation and destruction of mineral deposits were reflected in a monograph coauthored with M. V. Ivanov and N. N. Lyalikova: *Introduction to Geological Microbiology*, published in 1962. During his long, fruitful scientific career, Kuznetsov published more than 160 papers and monographs. All Kuznetsov's monographs were translated into foreign languages in Germany, Japan, the United States and other countries.

He was a member of the editorial board of *Hydrobiology* and the *Geomicrobiological Journal*. He was elected an honorary member of the International Society of Limnologists, and was awarded the Naumann Gold Medal in 1971. He received several orders and medals from the Soviet Government. Kuznetsov was very active in teaching and in leading scientific investigations.

His kindness and wide knowledge attracted many students to science, to whom he devoted much time and effort. By directing more than 50 master and doctoral dissertations, he created schools of microbiologists who contributed to studies of petroleum, mineralogy and aquatic microbiology in Russia and other countries. His

students and colleagues held him in great esteem. The memory of Sergei I. Kuznetsov is greatly cherished not only by Russian scientists but also by foreign microbiologists and limnologists.

He died in Moscow on February 27, 1987, at the age of 86.

Outstanding works by Sergei I. Kuznetsov

Kuznetsov. S. I. (1942). Sulphur cycle in lakes. Mikrobiologiya **11**, 218–241.

Kuznetsov. S. I. (1949). The application of microbiological methods to the study of organic matter in water bodies. Mikrobiologiya **18**, 203–214.

Kuznetsov. S. I. (1951). Comparative characterization of the biomass of bacteria and phytoplankton in the surface layer of water of Middle Baikal. Trudy Baikal'skoi Limnol. Stancii Akad. Nauk SSSR **13**, 217–224.

Kuznetsov. S. I. (1952). The role of microorganisms in the cycle of matter in lakes. Moscow. Akad. Nauk SSSR. 300 p.

Kuznetsov. S. I. (1956). Microbiological characteristics of the waters and the sediments of Lake Baikal. Trudy Baikal'skoi Limnol. Stancii Akad. Nauk SSSR **15**, 388–296.

Kuznetsov, S. I. (1967). Application of electron microscopy to the study of water microflora. Mikrobiologiya.

Kuznetsov, S. I. (1970). Microflora of lakes and its geochemical activity. C. H. Oppenheimer (ed.), University of Texas Press.

Kuznetsov, S. I. (1975). The role of microorganisms in the formation of lake bottom deposits and diagenesis. Soil Sci. **119**, 81–88.

Kuznetsov, S. I., Dubinina, G. A. (1989). Methods of investigation of aqueous microorganisms.

Kuznetsov, S. I., Dzyuban, I. N. (1960). Utilization of humic substances in the development of mycobacteria. Byull. Inst. Biol. Vod **27**, 3–5.

Kuznetsov, S. I., Gorlenko, V. M., Dubinina, G. A. (1983). The ecology of aquatic microorganisms. Nägele u Obermiller. 288 p. (ed. original 1977).

Kuznetsov, S. I., Ivanov, M. V., Lyalikova, N. N. (1963). Introduction to geological microbiology. English edition edited by C. H. Oppenheimer. McGraw-Hill. N.Y.

Kuznetsov, S. I., Romanenko, V. I. (1963). Oxidation-reduction potential in surface layers of mud sediments in lakes of different type. Dokl. Akad. Nauk SSSR **151**, 679–682.

Kuznetsov, S. I., Romanenko, V. I. (1974). Ecology of microorganisms in fresh water reservoirs 193 p.

Kuznetsov, S. I., Saralov, A. I., Nazina, T. N. (1985). Microbial processes of carbon and nitrogen cycles in lakes. Nauka. 213 p.

Kuznetsov. S. I., Speranskaya, T. A., Konshin, V. D. (1939). The composition of organic matter of mud sediments in different lakes. Trudy Limnol. Stancii Kosino **22**, 75–104.

MICROBIOLOGÍA SEM **13** (1997), 357–360 **PERSPECTIVES**

Irreversibility of information and its implications for living systems

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The distinguished physicist Erwin Schrödinger, born and died in Vienna (1887–1961), was awarded the 1933 Nobel prize in Physics, shared with the theoretical physicist Paul Dirac. In part due to Nazi wickedness, and also because of his love whims, he enjoyed a most stimulating life. Women probably felt prone to forgive him, for his emphatic statement, that a society without women was hateful and barbaric. He made other statements about academic life in the same, or even more strong, style. He also found comfort in poetry; I think his poems have inspiration and strength.

In 1934 he was invited to visit Spain. He gave six lectures at the International Summer University of Santander, which that year was directed by the Spanish philosopher José Ortega y Gasset. He also lectured in Madrid. The Santander lectures were translated into Spanish by Xavier Zubiri, and were published in 1935 (*Cursos de la Universidad Internacional de Verano en Santander*, Signo, Madrid).

From 1939 to 1956 he stayed in Dublin working at the Institute for Advanced Studies, created for him as a result of the Irish leader de Valera's mathematical interests. There, in 1943, he delivered a series of lectures on the topic "What is life?", that were attended by more than 400 people. President De Valera and other authorities from the Irish administration and Catholic church, as well as representatives of Gaelic intellectuality—who really appreciated that Schrödinger had preferred Dublin to Oxford—were among the audience.

The book produced from the series of lectures and published under the title *What is Life? The physical aspect of the living cell* (Cambridge University Press, 1944) had great success, and has been translated into many languages. There are, at least, two independent translations into Spanish. I wrote an introductory comment for one of them which was translated by Ricard Guerrero (Editorial Avance, Barcelona, 1976). (The other translation, many years its senior, was published already in 1947 in Buenos Aires, by Espasa-Calpe Argentina S. A. Finally, Tusquets Edito-

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res, Barcelona, started in 1983 its well known series "Metatemas" with R. Guerrero's translation.) That comment was signed by a certain Ramon Margall, pen name for which I was not responsible, and which came out from an entropic circumstance. Upon re-reading myself I still find the text quite acceptable, although today I see that youth and hope inspired me to write that "the book contains an optimistic message, that life has retained all its potentialities from its beginning". I also added that man could be more optimistic than ever before, provided he looked ahead and not back. Now I suspect that, since then, almost all of us have tended to look back more than ahead. This is the best formula for us to stumble.

It is true that Erwin Schrödinger, who wrote before the discovery of the DNA double helix (J. D. Watson and F. H. C. Crick, 1953, "Molecular structure of nucleic acids", Nature, 171: 737–738), could only vaguely refer to a kind of periodic crystal as a vehicle for genetic information. Linus Pauling wrote a short chapter, entitled "Schrödinger's contributions to chemistry and biology" (pp. 225–233) for a volume published on the occasion of the centennial of Schrödinger's birth (*Schrödinger, Centenary Celebration of a Polymath*, ed. by C. W. Kilmister, Cambridge University Press, 1987). Pauling honored Schrödinger by strongly critizising him, stating for example—as far as the ideas he expressed in *What is life* is concerned:"When I first read the book, more than forty years ago, I was disappointed. It was, and still is my opinion, that Schrödinger made no contribution to our understanding of life". "Schrödinger's discussion of thermodynamics is vague and superficial to an extent that should not be tolerated even in a popular lecture." And there is much more along the same line.

All this serves as an introduction to the things I would like to say on the irreversibility of information and its implications for living systems. Thermodynamics was born at the beginning of the XIX century, influenced by the brand new industrial age. It developed to deal with the efficiency problems in steam engines, in terms of pressures, volumes and temperatures. Any desired local effect forms part of a cycle that can not be indefinitely repeated, but requests new energy input that will also be degraded, this is to say, energy can not be reutilized in the same way. In fact, the concept of entropy looks as unreal as phlogiston; so, the double negative of negative entropy can be criticized. In the best of the cases, entropies might be added up, as something that will end up in the basket of the Universe wastes.

What thermodynamicists did not think about was that the concept of entropy usually becomes complementary—although with a regrettable waste—to a more or less persistant growth of information. An example of this could be the wearing of cylinders, or other engine elements that, even being undesirable accidents in this case, result in inputs of more persistant information and organization, which will affect the future.

We have all read that Karl Marx sent to Charles Darwin a copy of *Das Kapital*. Darwin never opened the book completely. (At that time, even later, as we, older generation, remember, books arrived to us with their pages folded and tied, uncut, so it was far more difficult to boast about being an enthusiastic reader.) Marx saw in the mass production of objects or tools a program which actually required energy and thus an increase in entropy, but which offered the possibility of improving the production's process. The most successful tools were taken as a prototype to be copied in a completely parallel interpretation to Darwin's natural selection. The first edition of Darwin's *On the Origin of the Species* dates from 1859. Karl Marx, who was nine years

younger than Darwin, to whom he wanted to dedicate his work, published *Das Kapital* in the year 1867.

However, the cost of increasing persistant information, which can be transmitted and improved, and in any case reverts in itself, so it multiplicates, is an increase in a somewhat unreal entity which simply accumulates as a waste of the universe.

If the concept of entropy had not been so generalized, that entity would not even deserve a particular name. However, we can assume that entropies can add up, whereas it is true that information can, and usually do, multiply. This concept might be difficult to understand when the way of expressing and dealing with information was discussed by Kolmogoroff, Shannon, Ashby, and Chaitin, among others. Nowadays, however, this concept is better understood and can be even considered trivial because of the treatment imposed on all kind of information. In fact, the value of the stores of information for the survival of the systems that handle them is never as essential as genetic systems are for organisms.

Actually life's success in handling information depends on the low cost of copy work, more or less as it happens with photocopying machines, and on mechanisms that preserve the probability of keeping some probability of unfaithful reproductions. This is what has become popular talking of monkey typists and the chances of their repeating a given sequence of symbols already known and especially appreciated, such as fragments of the Bible, Don Quixote, or works by Shakespeare.

We must point out that the first version of this idea comes from Jonathan Swift (1667–1745, born in Ireland). In his *Gulliver's Travels*, he tells that, at the Great Academy of Lagado, Gulliver is received by a famous scholar in the care of a device out of the usual. This is a kind of board with axes at right angles, around which about forty youngsters turn the cranks to shake several wooden dice with words written on each side. After a while they stop the shaking and leave the dice alone. As a result of the student's movement, a text is produced at random. This text is copied by quick scribes, in the hope of joining everything that can be possibly written and, thus, all possible sciences, both divine and humane. It was just a matter of patience.

We could say that entropy, if we want to continue using this apparently empty concept —speaking about negative entropy would sound even emptier—in an elementary and rough way, adds up, whereas information multiplies. If we establish a connection between entropy and information, according to the traditional entropy-information binomial association, and accepting that both the copy and the generation of new information must be linked to an increase in entropy, information always has advantages. However, catastrophic destruction of accumulated information happens frequently. This is a satisfactory news about the chances of the universe to persist; a universe whose essence might be its ability to create. As Pascal said in one of his pensées, "L'homme se lassera plûtot d'imaginer que la nature de creer".

We know that a computer program, if it is already complex from the beginning, can be considerably modified in the form of a supplementary, relatively small amount of information. So, it is not uncommon that a difference which in biological terms can be considerable, such as that existing between us and the living primates closest to humans (chimpanzees), could be attributed to small genetic differences which act on relatively large amounts of information.

Age makes me point out—more to comfort myself than to regret it—another fact, which I think is very significant and unavoidable if information, as I have already stated, multiplies. Firstly, it is impossible to obtain all information

accumulated, and exactly in a way that follows backwards the order followed as accumulation has taken place. Obviously, you could find here the reason, regarding the fact of realizing the loss of the easiness that usually we had for recovering "threads" of information, when this was part of a quicker system. I think this could be a model of the dificculty of manipulating in the opposite way the access to information, in an ordered way, comparable to a way opposite to that at which the information was generated. Obviously, as copy systems are so inexpensive and effective, the problem's solution to keep on mantaining the thin skin of life around the world, with a thermodynamics reasonable cost, is entrusted to natural selection. Death must be understood as an absolutely and necessary event inside this scheme.

Darwin's ideas agree with all thermodynamic-information speculation, both in life and in death. It is actually true that organisms as living systems can be considered to have self-organizing power, that is to say, autopoietic in a sense similar, if not identical, to that proposed by Chilean researchers Maturana and Varela in their book *Autopoiesis and Cognition: The Realization of the Living* (Reidel Publ., Dordrecht, Holland, 1980; originally published, in part, as *De máquinas y seres vivos*, Editorial Universitaria, S.A., Chile, 1972).

MICROBIOLOGÍA SEM **13** (1997), 361–362 **OPINION**

Comments on "Two generations of spore research: from father to son"*

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Science is done by people and in a human and historical setting. Therefore, this memoir by a leader for many years of microbial spore research is welcome and valuable. A sad measure of the rapid progress in our science in the last generation is that students and younger workers too often are a bit like popular musicians and lack such perspective.

Harlyn Halvorson's view covers an unusually long time, from childhood in the home of an important microbiology researcher, teacher and science administrator through a long and productive career in these activities himself. I recall the first, as well as the most recent, time I have talked with Harlyn, the two some 40 years apart: In the first exchange he rejected me from registering in his microbiology course at the University of Michigan, as he judged me as being still wet behind the ears. I learned then not to turn away young enthusiastic students. In the last, he had helped to organize and run a scientific symposium on phosphate metabolism (polyphosphates remain a lifelong interest of his) at his favorite institution, the Marine Biological Laboratory (MBL) in Woods Hole, at which he has gone from student to Director. At the end of his time at MBL, he was not treated very well—for having done the required job vigourously, people found faults in his efforts. His enthusiasm for the MBL remains bright. He taught perspective and judgement, as always.

One aspect of his virtuosity as scientist, teacher, organizer, administrator, and educator that fails to emerge adequately from this fascinating discussion of his father and his spores is Harlyn's wry and remarkable sense of humor. He laughs, teases and inspires originality by, in the midst of gravely serious issues, never taking himself or those around him too seriously.

* See Halvorson, H. O., *Microbiología SEM* **13**, 131–148 (1997)

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Throughout his career, Professor Halvorson has been enthusiastic for both science and science administration. As he mentions here, he is proud to be half of (one of only two) father and son pairs to both be presidents of the American Society for Microbiology (ASM), an organization for which he has worked productively for many years. Institutions have always been, and clearly still are, important for Harlyn Halvorson. At the University of Illinois, H. Orin Halvorson, the father, mentored young microbiology faculty (notably Salvador E. Luria, Sol Spiegelman and Irwin C. Gunsalus) in a golden department at a golden era for microbiology. Gunther S. Stent (3), who is also an Urbana product from this period, has written a different view of this wonderful time and place, and especially of Harlyn Halvorson's research mentor, Sol Spiegelman. The first class tradition at Urbana has continued to today with the explosion of science on the third type of life, the Archaea, associated most closely with the evolutionary interests of Carl Woese and the metabolic biochemistry of Ralph Wolfe. Research enthusiasm transferred from parent to child continued, for example, with the sons (both named George) of Z. John Ordal and Sol Spiegelman (both Urbana microbiology faculty) still active in the field.

At the University of Wisconsin, Harlyn Halvorson built and headed the Laboratory of Molecular Biology, and then he moved to Brandeis University, Waltham, Massachusetts, where his administrative responsibilities included building as well as fund raising for the Rosenstiel Center. From there he went on to the directorship of an 100-year old world-class institution, the MBL. He has long been interested in the relationship between science, the institutions in which it happens, and the society that pays the bills. Halvorson passes lightly over the early relationship between spores studies and Fort Dietrich, the military bacteriological warfare laboratories, and does not mention the early cooperative and later strained relationship between ASM and Fort Dietrich. Much more recently, his interest in and encouragement of science in Cuba, especially microbiology, again raises the relationship between public policy and science, a subject that he has developed for years at Woods Hole in conjunction with Alex Keynan (from Israel) and Sir Hans Kornberg (from the U.K.). It is a rich, complex fabric in which experimental science occurs.

Harlyn Halvorson's longest span of research concerned spore germination and yeast development. Yet, although there are now twenty *ger* (germination) genes in the newest genetic map for *Bacillus* (1), germination research seems to have been reduced to a minor topic, compared with the continuing highly active research on *Bacillus* sporulation (2, 4). Sadly for its strong North American roots, the current complete DNA sequencing of the genome of *Bacillus subtilis* (1) is basically listed as a European–Japanese cooperative effort, with Americans mostly missing!

References

1. Biaudet, V., Samson, F., Anagnostopoulos, C., Ehrlich, S. D., Bessiëres, P. (1996). Computerized genetic map of *Bacillus subtilis*. Microbiology **142**, 2669–2729.
2. Sonenshein, A. L., Hoch, J. A., Losick, R. (1993). *Bacillus subtilis* and Other Gram-Positive Bacteria. Biochemistry, Physiology and Molecular Biology. American Society for Microbiology, Washington, D.C.
3. Stent, G. (1994). Sol Spiegelman. Adv. Cancer Res. **65**, 203–212.
4. Straigier, P., Losick, R. (1996). Molecular genetics of sporulation in *Bacillus subtilis*. Annu. Rev. Genet. **30**, 297–341.

MICROBIOLOGÍA SEM **13** (1997), 363–370 **OPINIÓN**

Microbiología en Internet

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Summary

Internet is a vast net of information, services and global communication tool. The main services available are electronic mail (e-mail), connection to a remote computer (telnet), file transfer protocol (ftp), seeking (gopher) and the world wide web (www). In microbiology Internet allows to search for data bases, submitting articles to journals, communication with other scientists, participation in newsgroups, clinical meetings, multilocus training trials and the acquisition of products and services. The benefits of Internet grow quickly together with the improvement of scientific and technical resources in the net and its use is becoming simpler and friendly. It is an important tool at academic and research level.

Key words: Internet, world wide web, data processing nets, applications data processing, electronic addresses

Resumen

Internet es una extensa red de fuentes de información y servicios, y una eficaz herramienta de comunicación global. Los servicios más importantes en la red son: correo electrónico (e-mail), conexión remota a otro ordenador (telnet), transferencia de ficheros (ftp), buscadores (gopher) y world wide web (www). En microbiología, Internet permite consultar bases de datos, enviar artículos a revistas, comunicarse con otros científicos y participar en cursos de formación, foros de discusión, reuniones clínicas y ensayos multicéntricos, así como adquirir productos y servicios. Las ventajas

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de Internet crecen rápidamente a la par que los recursos científicos y técnicos en la red, su manejo se hace más sencillo y amigable, y aumenta su utilidad en docencia y investigación.

Introducción

El desarrollo tecnológico de las comunicaciones ha experimentado un crecimiento exponencial. Con las nuevas tecnologías informáticas, cada día un mayor número de ordenadores se comunican entre sí, formando un entramado de redes que han venido a denominarse autopistas de la información (8). Internet, la mayor de estas redes informáticas, fue creada a finales de la década de 1960 por militares norteamericanos para lograr una comunicación más eficaz en caso de guerra nuclear. Inicialmente estaba formada por la interconexión de tan sólo cuatro ordenadores, pero su desarrollo en años posteriores fue vertiginoso. Aunque es imposible conocer el número de ordenadores actualmente conectados a la red, se estima que es alrededor de 40 millones y su número aumenta anualmente sobre un 10% (8, 10). Se prevé que en el año 2000 más de 150 millones de ordenadores estarán conectados a la red (11).

La conexión a Internet es fácil (8,11): basta un ordenador (PC o Apple Macintosh), un módem (conectado a la línea telefónica) y el software de comunicaciones de la red, que suele proporcionar el proveedor sin coste alguno (TCP/IP: Transmission Control Protocol/Internet Protocol). Una vez contactado el proveedor de acceso a la red, éste proporcionará una cuenta de acceso. Los distintos proveedores y tarifas en nuestro país están disponibles en: hppt://www.ole.es/Páginas/Internet/Proveedores_de_Internet/España.

Dos puntos a tener presente cuando se realiza la conexión son: seleccionar un proveedor próximo al domicilio o lugar de trabajo (así las llamadas telefónicas se facturan como locales), y elegir un módem de alta velocidad para recibir rápidamente la información.

Los ordenadores conectados a Internet se identifican mediante direcciones numéricas y alfabéticas. Cada ordenador tiene asignada una dirección IP, única y específica, de cuatro números, cada uno de ellos comprendido entre 0 y 255, separados entre sí por un punto (5). Su asignación está coordinada por el Centro de Información de Redes (Network Information Center, NIC). Ahora bien, el manejo de las direcciones numéricas IP resulta incómodo, ya que son difíciles de recordar y se cometen frecuentes errores al teclearlas. Por ello, se ha establecido un método de direcciones alternativo basado en nombres. El nombre del ordenador está constituido por varios grupos de caracteres (palabras, abreviaturas), separados entre sí por puntos y ordenados de izquierda a derecha: ordenador.nombre_subred. nombre_red.dominio.

En la práctica, los ordenadores se comunican por su dirección IP, de modo que el nombre del ordenador es transformado automáticamente a su correspondiente dirección numérica por una base de datos, denominada sistema de nombres de dominio (Domain Name System, o DNS), y que también está coordinada por el NIC (5).

Las características más importantes de Internet son: es multinacional, universal y con carácter no lucrativo; es decir, no tiene propietario y su uso es gratuito para el usuario. Internet es utilizado por individuos y organizaciones de todo tipo, privadas y públicas. En un próximo futuro, el conocimiento y manejo de esta tecnología será un factor determinante en todos los ámbitos, particularmente en el campo de la ciencia, debido a la masiva información científica existente en la red y a su rápida actualización (2, 8).

Las posibilidades de Internet se pueden considerar ilimitadas, dependiendo únicamente de la preparación del usuario y de la utilización que éste le quiera dar. El objetivo del presente trabajo es describir el funcionamiento y las aplicaciones de Internet en el ámbito de la microbiología.

TABLA 1. Herramientas accesibles desde WWW y su denominación en URL

Herramienta	Denominación en URL
Correo electrónico	mailto://
File Transfer Protocol	ftp://
Gopher	gopher://
Newsgroups	news://
Telnet	telnet://
World Wide Web	http://

Aplicaciones de Internet

El manejo de Internet se realiza mediante las herramientas de trabajo en la red, siendo cada una de ellas un conjunto de programas informáticos y utilidades para una determinada tarea (11). La Tabla 1 muestra las herramientas accesibles desde la World Wide Web (WWW, o W3). Estos servicios funcionan en un modo de interacción entre ordenadores denominada relación cliente/servidor (3, 11). En Internet, la información y los servicios se obtienen de otros ordenadores o equipos denominados servidores; mientras que los clientes son los ordenadores que solicitan esa información. Para ello, el software del ordenador cliente interpreta las instrucciones del usuario y envía órdenes al ordenador servidor o remoto, donde otro programa informático lleva a cabo las instrucciones enviadas por el usuario (3). Todas las aplicaciones de Internet utilizan uno de los servicios básicos que se describen a continuación.

Correo electrónico (e-mail). Es una de las aplicaciones más utilizadas. Permite enviar y recibir cualquier documento susceptible de ser transformado en bits (textos, imágenes, sonidos) de forma instantánea y fiable (6–8). Todos los usuarios de Internet tienen una dirección de correo electrónico específica. El formato de esta dirección es el siguiente: leyendo de derecha a izquierda, las dos primeras letras corresponden al país o dominio, seguidos de la organización y el nodo al cual está conectado el ordenador. Debe tenerse cuidado en la utilización de las letras mayúsculas y minúsculas, pues no siempre son equivalentes en las direcciones electrónicas. Estados Unidos no utiliza código de país, debido a que allí se inició esta red.

El procedimiento (2, 3) es muy sencillo. Se prepara un mensaje con un procesador de textos ASCII y se incorpora a la red poniendo en la cabecera la dirección del destinatario. Se puede verificar que el mensaje ha sido recibido correctamente; si no es así, será devuelto con una indicación del motivo (fallo de comunicación, usuario y/o destino desconocido, etc.).

El mismo mensaje se puede enviar de manera automática a un grupo de personas distanciadas entre sí mediante las listas de distribución, previa suscripción. Cada lista se mantiene en un ordenador central (Listserv) al cual deben remitirse todos los mensajes que se deseen distribuir. Estas listas constituyen un foro real de opinión de temas diversos. Su principal inconveniente es que, además de sobrecargar el tráfico de datos en la red, el buzón de correos del usuario se satura rápidamente en cuanto éste se suscribe a varias listas. Para evitar este inconveniente se crearon los noticiarios ("newsgroups"), cuyo funcionamiento es similar al de un tablón de anuncios (2, 8). La información es depositada en un ordenador del nodo de la red transmitiéndose una sola vez, entonces el usuario debe acceder al mismo y buscarla, y si le interesa puede importarla

a su ordenador, imprimirla, etc. Existen más de 5500 noticiarios, que abarcan los más diversos temas (2).

Junto con los mensajes del correo electrónico, también se pueden enviar documentos o ficheros elaborados mediante otros programas informáticos, con la aplicación anexo ("attach"). Para que el destinatario del correo pueda utilizar estos ficheros, debe poseer estos programas u otros, llamados visores, capaces de abrir los ficheros recibidos, teniendo entonces la posibilidad de copiarlos y modificarlos o almacenarlos. Para contestar un mensaje de correo electrónico basta con utilizar la función responder ("reply") y escribir el mensaje que se desee.

Conexión remota a otro ordenador. Otra función básica de Internet es la conexión de un usuario a otro ordenador servidor situado a gran distancia. Esta utilidad es más conocida en los protocolos TCP/IP como Telnet y permite utilizar otro ordenador como si se estuviese conectado a un terminal local (2). Cuando se establece una conexión Telnet, el ordenador remoto solicita al usuario su nombre ("username" y/o "login") y una contraseña ("password") para permitirle el acceso. De esta manera, el usuario puede utilizar los recursos disponibles en el otro ordenador según el grado de acceso permitido por su sistema de seguridad. Su principal ventaja, además de la facilidad de uso, es que la velocidad de transmisión es similar a la de un terminal local. Muchas instituciones científicas y gubernamentales tienen carácter abierto, lo que significa que puede accederse a sus bases de datos con sólo una contraseña genérica.

Transferencia de ficheros. O también protocolo de transferencia de ficheros, FTP ("File Transfer Protocol"), es otra de las aplicaciones básicas de Internet. Su funcionamiento es parecido al de Telnet, pero en lugar de utilizar los recursos de otro ordenador, FTP permite navegar entre sus directorios y enviar y/o recibir ficheros de un ordenador a otro. FTP es el único sistema que permite transmitir ficheros binarios como programas, imágenes, sonido o películas en formato digital (3–8). Como en Telnet, para acceder a las áreas de libre utilización en otro ordenador hay que conocer su contraseña, que suele ser la palabra "anonymous", seguida de la dirección de Internet del usuario ("username") que establece la conexión (2, 4).

Buscadores. La gran cantidad de información disponible en Internet ha obligado al diseño de utilidades para navegar en este océano de información, permitiendo búsquedas selectivas. Archie facilita la localización de ficheros en Internet, realizando búsquedas de ordenadores con FTP anónimo. Su resultado es un listado desglosado de los ficheros disponibles en cada sitio y de su contenido, indicando cuáles son los más adecuados en la solicitud de búsqueda (2, 4). Gopher es un servicio de obtención de documentos a través de menús interactivos. Permite conectarse a un servidor general e ir avanzando mediante menús desplegables hasta encontrar la información que interesa (3, 11).

World Wide Web (WWW). Desarrollado en Ginebra por CERN (12), es el método más sencillo y potente para proveer y obtener información y servicios a través de Internet (8, 11, 12). Facilita el acceso a documentos multimedia interactivos, en los que la información se puede presentar en forma de texto, imágenes, sonido y/o vídeo. Está basado en la tecnología hipertexto, es decir, páginas en las que determinadas palabras (o imágenes), subrayadas y resaltadas en un color distinto del resto del texto, constituyen un enlace ("link") con otras páginas a las que están relacionadas, con independencia de su localización. La extensión de una página Web es ilimitada y dependerá del espacio libre que el servidor de Internet deje disponible para tal fin (3).

Las páginas WWW están diseñadas en un nuevo lenguaje de programación denominado

HyperText Markup Language (HTML), el cual permite especificar la información contenida en un documento, el modo de presentarla (textos e imágenes), facilitando también el acceso a otras direcciones electrónicas ("link"). Además, es capaz de convertir toda la información multimedia en simples archivos de texto, a los que se reconoce por su extensión .html (3).

Sin necesidad de conocer las direcciones electrónicas de cada una de las páginas, e incluso sin conocimientos específicos de informática, es posible navegar de una página a otra valiéndose simplemente de la ayuda del ratón, aunque estén localizadas en lugares opuestos del planeta (11). Se accede al WWW por medio de una página propia o por la del servidor y por medio de los navegadores ("browsers") como por ejemplo, Netscape Navegator, Internet Explorer o Mosaic. Es posible trasladarse de una página a otra o ir directamente a una página concreta tecleando su dirección (3–8). La dirección de las páginas siempre va precedida por la abreviatura http:// (hipertext transfer protocol).

También existen numerosos buscadores de información en la WWW, españoles o extranjeros, con características propias, particularmente en lo referente al tipo de información que catalogan, a la sintaxis del texto que debe especificarse para llevar a cabo la búsqueda y a la forma de presentar los resultados de la misma. No obstante, todos ellos funcionan de manera semejante, utilizando el sistema de búsqueda por palabras clave en toda la WWW y proporcionando un listado en forma de hipertexto para localizar la información deseada (11). Los buscadores más utilizados, aunque existen muchos más son los siguientes:

Altavista	http://www.altavista.digital.com/
Lycos	http://www.msn.lycos.com/
Magellan	http://www.mckinley.com/
Yahoo	http://www.yahoo.com/

Otras utilidades de Internet. Internet permite la comunicación entre dos usuarios a tiempo real por escrito, mediante la herramienta IRC (Internet Relay Chat, charla en tiempo real), apareciendo la pantalla del ordenador dividida en dos mitades, una para cada usuario (3, 11). El sistema de videoconferencia permite la comunicación a tiempo real mediante imágenes y sonido, si bien su costo es todavía elevado.

Internet aplicada a la microbiología

La creciente popularidad de Internet y su impacto en los centros universitarios y de investigación españoles, se ha traducido en una importante presencia de investigación y desarrollo (I + D) en la red. En España, este sector representa más de 200.000 páginas WWW (1).

Las utilidades de Internet en microbiología abarcan desde la microbiología básica (taxonomía, bacteriología, virología, micología, parasitología, biología molecular) hasta la microbiología aplicada, integrada por microbiología clínica (quimioterapia), microbiología ambiental y microbiología industrial (microbiología de alimentos, microbiología farmacéutica, ingeniería genética). Así pues, esta información puede variar desde la búsqueda, comparación y análisis de cualquier secuencia de DNA o proteínas mediante bancos de datos genéticos (9), hasta la consulta de un tratamiento farmacéutico para combatir una intoxicación alimentaria, o tratar una enfermedad infecciosa.

Otras aplicaciones son la consulta y petición de cepas microbianas directamente de ciertas colecciones tipo de microorganismos (ATCC, CECT, etc.), así como la posibilidad de enviar y recibir trabajos a revistas científicas para su publicación, reduciendo de esta manera el tiempo requerido en estos casos. Generalmente, tanto las instrucciones para los autores como los

resúmenes de los trabajos publicados (e incluso, a veces, el artículo en su totalidad) pueden consultarse en las páginas WWW de la revista. Sin embargo, todavía son pocas (*Microbiología SEM* es una de ellas) que permiten esta vía de publicación, siendo éste un campo que se desarrollará en los próximos años (10).

La utilización masiva del sistema interactivo WWW ha permitido un acceso más rápido y ordenado a todo tipo de información. En microbiología, como en otros campos científicos, sus aplicaciones son ilimitadas y permite a través del correo electrónico una comunicación rápida y sencilla entre científicos en cualquier parte del mundo. Asímismo, es posible, por medio de la conexión remota a otro ordenador, consultar bases de datos para la búsqueda de bibliografía científica, manuales de protocolos experimentales, líneas de investigación de grupos científicos, resultados de ensayos clínicos, etc. La Tabla 2 proporciona direcciones electrónicas de servicios de búsqueda bibliográfica.

Desde la aparición de la WWW, la utilización de Internet ha aumentado espectacularmente, así como sus distintas aplicaciones. En el ámbito científico, y concretamente en microbiología, es posible utilizar la WWW con diferentes fines (3–11): conocer las líneas investigadoras de una determinada institución con la que se desea estar en relación o visitar; participar en debates, conferencias y foros de discusión sobre temas profesionales de actualidad; colaborar en grupos de trabajo específicos; desarrollar ensayos clínicos multicéntricos; asistir a cursos y programas de formación continuada a distancia de modo interactivo; participar en seminarios con apoyo audiovisual o no ("talking"); adquirir productos comerciales para el laboratorio, libros, revistas. Todo ello sin necesidad de efectuar desplazamientos. En la Tabla 3 se incluyen las direcciones electrónicas de revistas y sociedades científicas de microbiología.

Conclusión

La WWW es el sistema más importante y sencillo de comunicación de Internet y desde su aparición es la razón del gran incremento de usuarios en los últimos años. Se estima que a finales de 1996 existían más de 150 millones de páginas Web (11).

TABLA 2. Direcciones electrónicas de servicios de búsqueda bibliográfica en Internet

Servicio	Dirección electrónica
Grateful Med Medline Journal Article Search	http://igm.nlm.nih.gov/
HealthGate Medline	http://www.healthgate.com/
HealthWorld Medline	http://www.healthy.net/library/search/medline.htm
Infotrieve Medline Service Provider	http://www.infotrieve.com/
Medline Database at Community of Science, Inc.	http://muscat.gdb.org/repos/medl/
Medscape Medline Search	http://www5.medscape.com/home/search/search.mhtml
NlightN Medline Search	http://www.nlightn.com/
OhioLINK	telnet://cat.ohiolink.edu
Ovid On Call Medline and Medical Databases	http://preview.ovid.com/
PaperChase Medical Literature Searching	http://enterprise.bidmc.harvard.edu/paperchase
PHP	http://php2.silverplatter.com/

TABLA 3. Servidores y direcciones de páginas WWW de revistas de microbiología en Internet

Servidor	Dirección electrónica
Annales de l'Institut Pasteur	http://www.elsevier.nl/inca/publications/store/5/0/5/8/0/7/505807.pub.shtml
Antimicrobics and Infectious Diseases Newsletter	http://www.elsevier.nl/inca/publications/store/5/0/5/7/4/8/505748.pub.shtml
British Medical Journal	http://www.tecc.co.uk/bmj/bmjpubs/
Cell	http://207.20.62.102/cell/index.htm
Clinical Microbiology Newsletter	http://www.elsevier.nl/inca/publications/store/5/0/5/7/5/5/505755.pub.shtml
Current Advances in Applied Microbiology & Biotechnol.	http://www.elsevier.nl/inca/publications/store/7/3/7/737.pub.shtml
Diagnostic Microbiology and Infectious Disease	http://www.elsevier.nl/inca/publications/store/5/0/5/7/5/9/505759.pub.shtml
EMBO Journal	http://www.oup.co.uk/embojo/hdb
FEMS Immunology and Medical Microbiology	http://www.elsevier.nl:80/estoc/publications/store/4/09288244/
FEMS Microbiology Letters	http://www.elsevier.nl:80/section/life/fems/docs/contents.htm
FEMS Microbiology Reviews	http://www.elsevier.nl:80/estoc/publications/store/5/01686445/
Gene	http://www.elsevier.nl:80/estoc/publications/store/9/03781119
Genetics	http://www.faseb.org/genetics/gsa/jou-gsa.htm
Infection and Immunity	http://www.at-home.com/ASM/IAI
International Journal of Antimicrobial Agents	http://www.elsevier.nl/inca/publications/store/5/0/5/5/2/1/505521.pub.shtml
International Journal of Food Microbiology	http://www.elsevier.nl/inca/publications/store/5/0/5/5/1/4/505514.pub.shtml
Journal of Bacteriology	http://www.at-home.com/ASM/JB
Journal of Clinical Microbiology	http://www.at-home.com/ASM/JCM
Journal of Medical & Veterinary Mycology	http://www.blackwell-science.com/products/journals/jmvm.htm
Journal of Microbiological Methods	http://www.elsevier.nl/inca/publications/store/5/0/6/0/3/4/506034.pub.shtml
Microbiología SEM	http://morgat.udg.es/microbsem
Microbiological Reviews	http://www.at-home.com/ASM/MR
Microbiology: Bacteriol., Mycol. Parasitol. & Virol.	http://www.elsevier.nl/inca/publications/store/5/0/5/9/7/3/505973.pub.shtml
Molecular and Cellular Biology	http://www.at-home.com/ASM/MCB
Molecular Microbiology	http://www.blackwell-science.com/products/journals/mole.htm
Nature Biotechnology	http://biotech.nature.com/
Nature Genetics	http://genetics.nature.com/
Nature Medicine	http://medicine.nature.com/
New England Journal of Medicine	http://www.nejm.org
Proceedings of the National Academy of Sciences	http://www.pnas.org
Research in Microbiology	http://www.elsevier.nl/inca/publications/store/5/2/2/4/9/3/522493.pub.shtml
Science	http://science-mag.aaas.org/science/home/contents-by-date-alt.htm
The Lancet	http://www.thelancet.com/
Trends in Biotechnology	http://www.elsevier.nl/inca/publications/store/4/0/5/9/1/7/405917.pub.shtml
Trends in Microbiology	http://www.elsevier.nl/inca/publications/store/4/2/4/1/0/0/424100.pub.shtml
Veterinary Microbiology	http://www.elsevier.nl/inca/publications/store/5/0/3/3/2/0/503320.pub.shtml

En general, el científico tiene una avidez innata por conocer los últimos adelantos en su campo. A través de Internet puede acceder a resultados de investigaciones incluso antes de su publicación en revistas sin la pertinente revisión (10). Los peligros de difundir ciertos tipos de información (p.e., médica y terapéutica) no contrastada a una audiencia potencial de millones de personas son obvios (7) ya que no siempre puede el usuario determinar la credibilidad de la fuente de información.

Muchas de las posibles ventajas de la utilización de Internet, como el acceso a bases de datos y a sociedades científicas, pueden volverse contraproducentes. Cualquier persona sin acreditación alguna puede acceder a la información en todos los campos científicos pudiendo utilizarla para fines poco éticos o dañinos (7, 10). A todos estos potenciales inconvenientes hay que añadir el "miedo a la nueva tecnología" por parte de muchos profesionales, debido al analfabetismo informático y a su actitud reacia al conocimiento de esta tecnología (2).

Esperamos que este trabajo sirva para estimular el interés por el uso potencial de Internet, y así ser capaces de incorporar esta revolución tecnológica a nuestro más inmediato futuro, o mejor dicho, al más actual presente.

Los autores de este artículo disponen de las direcciones electrónicas de numerosas instituciones, centros de investigación y sociedades científicas que pueden ser de interés para microbiólogos cualquiera que sea su especialidad, que, por razones de espacio no se incluyen en este trabajo. Los lectores interesados pueden solicitar esa información a la dirección electrónica del autor correspondiente.

Bibliografía

1. Aguillo, I. F. (1996). Ciencia e Internet en España. Mundo Científico **174**, 1032–1062.
2. Belmonte Serrano, M. A. (1995). Internet en la medicina del 2000. Med. Clín. **104**, 744–752.
3. D'Emanuele, A. (1995). The Internet: (1) A guide to the information superhighway. Pharm. J. **254**, 29–31.
4. D'Emanuele, A. (1995). The Internet: (2) Connecting and searching for information. Pharm. J. **254**, 58–59.
5. Jarabo, F., Elortegui, N. (1995a). Internet: red de redes. *In* Jarabo, F., Elortegui, N. (ed.), Internet. pp. 1–23. Ed. Paraninfo, S.A., Madrid.
6. Jarabo, F., Elortegui, N. (1995b). Correo electrónico: e-mail. *In* Jarabo, F., Elortegui, N. (ed.), Internet. pp. 25–43. Ed. Paraninfo, S.A., Madrid.
7. Klein, C. N. (1995). Pharmacy and the Internet. Am. J. Health-Syst. Pharm. **52**, pp. 2095.
8. Millman, A., Lee, N., Kealy, K. (1995). The Internet. Br. Med. J. **311**, 440–444.
9. Nieto, J. M. (1996). Internet para microbiólogos. *Microbiología SEM* **12**, 481–484.
10. Piqueras, M. (1997). Internet, nueva fuente de información. *Microbiología SEM* **13**, 229–236.
11. Ronchera-Oms, C.L., Martí-Bonmatí, L. (1996). Aplicaciones de Internet en farmacia (1): ¡Bienvenido a la red, internauta! Noticias Farmacoterapéuticas **14**, 3–5.
12. Tennant, R., Ober, J., Lipow, A.G. (1993). Crossing the Internet Threshold: An Instructional Handbook. Library Solutions Press, Berkeley.

MICROBIOLOGÍA SEM **13** (1997), 371–374 **OPINION**

Scientific writing: revising basic communication strategies

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Writing is an essential professional skill. Well done, it is an exciting, rewarding experience; done poorly, it can be frustrating and disappointing. Writing is extremely important for moving ahead in any profession. Scientists spend up to one third of their time writing reports. Papers are one of the major products of their research. Nevertheless, all too frequently the time and effort given to writing up the results is minimum. By underestimating the time and effort you put into your writing, you are in a sense underestimating your research.

Writing is not an easy task, as you will remember from your school days. Memories of low marks on writing assignments, together with unsympathetic remarks from demanding teachers, can have a negative effect on your writing even in later years. However, writing for scientific purposes is not the same as writing for academic assessment. Too often junior scientists think they are still writing university essays and tend to emphasize all they know about a topic instead of limiting their contribution to the essentials.

Assigned essays are written by people who are "learning" for people who already "know". It is the student's job to "impress" the teacher by resorting to sophisticated vocabulary, complex sentence structure and other literary devices that will best fulfil that purpose. In professional life, however, both the purpose and audience are different. Readers of scientific papers do not read them to assess them, they read them to learn from them. And because many writers overlook this basic difference, their writing can sometimes get out of focus. What is needed is more simplicity, not more sophistication!

Communicating vs. writing

One basic assumption to bear in mind is that writing—making information available on paper—and communicating are not necessarily synonymous. The main underlying purpose of a scientific paper is to communicate information, not just to put information into written form. Communication implies "sharing". It is a two-way activity involving both the sender of the message and the receiver. There is giving

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and taking; transmitting information and receiving feedback. Feedback refers to the verbal or visual responses the message elicits.

Communication scientists refer to this two-way transaction, or "sharing" of information, as the encoding and the decoding of a given message. Encoding involves the process of putting the information into a code, or means, through which the message is conveyed. When the message is "picked up", the receiver tries to make sense out of it. This process of interpreting the message is known as decoding. The decoder's reaction, or response, to the encoder's message is known as feedback. Feedback tells the sender two things: how well the message was received and how accurately it was interpreted.

In oral communication, this encoding-decoding transaction takes place very rapidly, almost simultaneously, and may involve a number of subtleties. Nods, frowns or other facial gestures rapidly convey agreement, disagreement, comprehension, indifference, doubt, etc. A successful speaker relies heavily on non-verbal feedback to establish rapport with his/her audience.

In written communication, feedback is much more difficult to perceive. How can a writer tell whether a reader has picked up and decoded his/her message the way it was meant? Immediate direct feedback allows individuals to modify their behaviour accordingly. When this type of feedback is lacking, all we can do is assume that the message was decoded correctly. But because breakdowns in communication are so often due to careless encoding, we cannot stress enough the importance of clear, concise writing.

Encoding

Proper encoding is essential for effective results. Business people are very aware of this. The key to being a good salesperson lies in knowing how to stimulate and motivate the customer. Stimulation and motivation are two key concepts in communication. In the introduction of an oral presentation, a good speaker first gets his/her listeners' attention (stimulation to listen) and then shows them how this particular presentation will be of value to them personally (motivation to continue to listen). This, of course, requires a carefully prepared, well-organized presentation. The same is true of good writers and good writing.

Your first "message" to your reader is the title you choose for your paper (stimulation to read). Titles have two jobs: they must inform readers what the research is about, and they must distinguish your research from other research in the field. Titles must not only be precise; they must also be clear. Too many details can confuse the readers. One useful technique for testing the necessary words in a title is to underline just those words that would be used in a keyword index. Those not underlined may be redundant or even distracting.

Next comes the summary; it supplies the details that do not fit in the title and gives the reader just enough information to know whether he or she wants to go on reading (motivation to continue to read). The introduction goes one step further and gives details about the research that could not fit in the title or in the summary. A strong introduction tells the reader why the research is/was important and why it is a real contribution to the field (motivation to continue to read to the end).

Because writing is such an important part of interaction in the scientific community, we must not forget that in scientific writing language is a means, not an end. Hopefully, by thinking of our own irritation as readers, we can learn to be more professional writers. A paragraph represents a chain of thought. A well-written paragraph should contain a topic sentence and several supporting

sentences. Smooth transitions between the sentences is achieved with the use of connectors, or conjunctive adverbs (however, therefore, hence, likewise, furthermore, etc.). Connecting words are like sign posts on a road map. They link one thought with another, giving a sense of continuity and cohesion to the text, greatly facilitating the task of the reader.

Visual devices such as graphs, charts, tables, etc. can be useful in clarifying complex information. Visual presentations have the advantage of representing three dimensions. Prose restricts information to a one-dimensional linear sequence; visual devices enables you to show activities going on in parallel, and movement in varying directions. Your reader can take in all these things "at a glance". It must be remembered, however, that perception and comprehension are not the same thing. Your reader must not only perceive your visual presentations, but also understand them!

Decoding

Efficiency in writing can be measured only in relation to its purpose. As we have seen earlier, the purpose of scientific writing is to inform the scientific community of new developments and research being carried on in the field. The best way to make our writing efficient is by making it readable. Papers that place extra barriers and obstacles between the message and the reader are obviously inefficient and are defeating their purpose.

Although encoding and decoding may seem like fairly simple processes, numerous factors are involved. We all use our own background and experience to encode messages. 'Receivers', on the other hand, use their own background and experience to decode those messages. When this involves the added difficulty of being expressed in a language not one's own, major communication breakdowns are even more likely to occur. The rate at which information is received and processed varies according to the reader. The time of day, stress, attention spans, the language the message is written in first language, second language, third language (L1, L2, L3), interferences, etc. all affect the decoding process. Consider your own experience as a reader. With the huge amounts of written material to be dealt with every day, there is little time to waste on unorganized, wordy messages.

Writers must remain sensitive to their readers and adjust the content, style and format of their writing accordingly. Even the rate at which information is "unloaded" must be taken into consideration. Too fast and you overwhelm our reader, too slow and you bore him/her. Here a reasonable dose of repetition can be helpful. Moreover, repetition of key points can reinforce the message and can make the reader's task less demanding.

Readability is a good barometer for effective writing. Readability research shows that long sentences make texts more difficult for readers to absorb. Nevertheless, many writers feel that long sentences are inevitable if complex interactions have to be expressed. This is a mistaken idea. Any subject can be broken up into longer or shorter items of information. The determining factor is how much the reader can comfortably absorb. As we read a sentence, we store the words in our short-term memory and when we reach the full stop, we decode the sentence as a unit. Our short-term memory is limited. A reader's capacity to absorb information can easily be exceeded. When we reach the end of a long complex sentence the first words have been forgotten. If too many ideas are packed into one sentence a reader can easily get lost. When this happens over and over, reading becomes tedious and frustrations begins to build up, producing negative reactions in your reader.

Feedback

One very clear type of feedback that cannot be ignored occurs when a paper comes back from an editor with a note saying "re-write" with no other explanation. What happened? Where did the message fail? Was it the language? Was it the organization? As we have stated earlier, great pains must be taken in conveying your message with the greatest simplicity and clarity.

Generally, the first person to "read" your paper and form an opinion of it will be a reviewer, or referee. Most writers react defensively to the feedback they receive from a referee. But before taking offence at a referee's remarks, turn back to what we have just said about the many factors involved in decoding written messages. It might be a good idea to keep a record of these comments for future reference.

Paradoxically, people writing in a language not their own are at an advantage as far as feedback is concerned. Before being sent on to the publisher, their paper passes usually through the hands of a translator or a language specialist. Although individual writing styles should be respected, the importance of this early "polishing" stage should not be overlooked. The comments and questions of a language expert can constitute useful feedback. However, because most scientists are more interested in content than form, they tend to ignore the comments of non-specialist translators. Although this is not the time or the place to enter upon the complex topic of translation theory, nevertheless, we would strongly suggest that writers of scientific papers try to find translators who adscribe to the modern reader-centered communicative theories. The semantic theories, in which the idiosincracies of the author are preserved at all costs, may please the writer, but may hinder the reader. Whatever the case, writer and the translator must work in close collaboration.

Because the development of communication strategies is so often related to the business world and the media, we seldom stop to think of its implications in the world of science and technology, and even less in scientific writing and translation. Being reader-sensitive and knowing how to deal with feedback are important skills to master. They will help you to be more aware of the writing process and in the end help you to produce more effective papers. Remember, good writing needs precision and detail, not pompousity and verbosity. Because many writers find it difficult to judge the reactions of a silent and invisible reader, they protect themselves by being over-formal. Pomposity, verbosity and disorganization are barriers to communication.

Further reading

Alley, M. (1988). The Craft of Scientific Writing. Prentice-Hall, Englewood Cliff, NJ.

Blicq, R. S. (1981). Technically–Write! Communicating in a Technological Era. Prentice-Hall, Englewood Cliff, NJ.

Bly, R., Blake, G. (1982). Technical Writing. McGraw-Hill, New York.

Couture, B., Goldstein, J. R. (1985). Cases for Technical and Professional Writing. Scott Foresman, New York.

Hamilton, C., Parker, C. (1987). Communicating for Results. Wadsworth, Belmont, CA.

Kirkman, J. (1975). Readable writing for scientific papers. Bull. Brit. Ecol. Soc. **6**, 5–9.

Lee, M., et al. (1990). The Handbook of Technical Writing: Form and Style. Harcourt Brace Jovanovich, New York.

Luttikhuizen, F. (1996). The ins and outs of scientific writing. *Microbiología SEM* **12**, 477–480.

Newmark, P. (1981). Approaches to Translation. Pergamon, Oxford, UK.

Pauley, S. E., Riordan, D. G. (1990). Technical Report Writing Today. Houghton Mifflin, Boston, MA.

Turk, C., Kirkman, J., (1989). Effective Writing. Chapman & Hall, London.

MICROBIOLOGÍA SEM **13** (1997), 375–384

Revisión de libros

Immunology: The Making of a Modern Science

Richard B. Gallagher, Jean Gilder, G.J.V. Nossal, Gaetano Salvatore (eds.)

Academic Press, London, 1995. 246 pp.
ISBN 0-12-274020-3

Lo que hace importante una obra sobre biología no es la mera descripción de hechos y descubrimientos, o la organización de los conocimientos en una exposición ordenada, aunque ello sea muy necesario. Es el razonamiento, la discusión en profundidad de sus bases fundamentales, de los procesos —biológicos, bioquímicos o de cualquier otra índole— que intervienen. Y ésta es una aportación significativa del libro que comentamos, unida a otras dos que merece la pena destacar: Por un lado, la reflexión filosófica y, por el otro, la comprensión del contexto histórico y social donde se enmarcan los acontecimientos descritos.

En palabras de los coordinadores del libro, éste intenta comunicar las ideas de algunos de los científicos que más han contribuido al avance de la inmunología, y lo hace en forma de narraciones de las circunstancias que condujeron a los principales descubrimientos de esa ciencia. Aunque en los tiempos actuales la filosofía e historia de la ciencia no tengan demasiados seguidores, las grandes figuras de la ciencia, que en uno u otro campo han contribuido a situarla en el lugar que hoy ocupa, han sido también pensadores que han realizado profundas reflexiones sobre el desarrollo científico. Nuevamente, la estéril confrontación entre ciencia y humanidades propicia estereotipos, tópicos que anulan el interés por lo que parece que no conoce una relación directa con el propio tema de estudio. El estudiante de ciencias no conoce, habitualmente, el desarrollo histórico y conceptual de las materias científicas. En pocas ocasiones relaciona ese desarrollo con el estudio y la reflexión profunda sobre la ideología, orientación e interés que subyacen en el fondo de cada actividad humana. En el libro vemos, por ejemplo, que la teoría de la formación de anticuerpos fue modificada en 1940 por Linus Pauling, un químico. Esta modificación, además de los efectos prácticos, suponía una concepción neo-lamarckiana, contrapuesta a las estrictamente darwinianas que habían orientado los presupuestos de Metchnikoff y Ehrlich.

La inmunología arranca de la microbiología para encontrar su lugar como disciplina en los años de la I Guerra Mundial. Muchas de las primeras ideas que la orientaron cayeron en desuso por la falta de tecnología que impedía avanzar y obtener resultados. Ésta sigue siendo una característica común de disciplinas estrechamente dependientes de los avances tecnológi-

cos. En cuanto a los transplantes, el rechazo se había considerado inicialmente un problema de base genética y, por tanto, insuperable. Los fracasos también se habían atribuido a una mala actuación quirúrgica. Pero nunca se había reparado en la extrema precisión del cuerpo para identificar algo como ajeno.

El libro, que consta de 19 capítulos, se articula en torno a cuatro ejes básicos de la investigación y la aplicación: (i) teorías de la inmunidad, (ii) bases celulares, (iii) bases moleculares y (iv) inmunología y medicina. Puede ser de extraordinaria utilidad para todas las personas a quienes interese la inmunología y tengan curiosidad por conocer sus inicios, ya que la sitúa en su contexto histórico y científico entre las demás ciencias. También resultará útil a quienes se interesen por la base epistemológica de las teorías científicas. Subrayaría que, además de instructivo en esos aspectos, resultará ameno y agradable para quien quiera saber cómo se desarrollaba la vida de los investigadores en un laboratorio en los años cincuenta, cómo eran las relaciones interpersonales, cómo se utilizaba un material determinado, y cómo se suplían, mediante inventiva o ingenios caseros, los instrumentos técnicos que tardarían todavía años en inventarse o mejorarse.

Hay que destacar, igualmente, que los aspectos inmunológicos considerados en el libro abarcan hasta su estado actual, ya que también se discuten los resultados y aplicaciones recientes de esta disciplina, así como los problemas que todavía quedan por resolver. La mayoría de inmunosupresores que se utilizan en cirugía de transplantes impiden la división celular y, en consecuencia, la respuesta inmunológica. En el caso de los transplantes, los problemas afectan fundamentalmente a la necesidad de agentes inmunosupresores que depriman la respuesta inmunológica el tiempo suficiente para que el paciente supere el período en el que se produce el rechazo.

El libro procede de uno de los cursos de la Escuela de Ciencias Biológicas celebrado en 1992 en la Stazione Zoologica Anton Dohrn, de Nápoles, que estuvo a cargo de reputados inmunólogos e historiadores de la ciencia. Todos ellos son profesionales en activo de significados centros de Europa y Estados Unidos.

Hay que destacar la labor que a lo largo de los años ha desarrollado la Stazione Zoologica Anton Dohrn, organizando actividades de un alto nivel científico con unos excelentes resultados. En los últimos años esa labor se ha debido, en gran parte, a la actuación dinámica e incansable de su primero director y después presidente, Prof. Gaetano Salvatore, cuyo repentino fallecimiento, en junio de este año, ha dejado en tantos que le conocíamos un triste vacío. La muerte le sorprendó cuando, con su entusiasmo habitual, preparaba la 4th International Marine Biotechnology Conference (22 al 29 de septiembre, 1997), que se ha venido anunciando en las páginas de nuestra revista.

Una característica común a las personas de valía, humana y profesional, es su capacidad para infundir entusiasmo y pasión por el trabajo bien hecho. Esa cualidad del Prof. Salvatore (Nino, como quería que le llamasen los amigos) le llevó a rodearse de un eficaz equipo de personas que pueden seguir adelante con la labor que su súbita desaparición dejó sin terminar. Precisamente durante una reunión preparatoria de esa reunión, fue cuando el Prof. Salvatore me entregó el libro, de indudable interés. Sirva esta reseña también como un respetuoso y amigable homenaje a un médico que tanto ha influido en el desarrollo de la ciencia biológica en Italia.

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Protein Structure Prediction

A Practical Approach

Michael J. E. Sternberg (ed.)

Oxford University Press, Oxford, 1996. 298 pp.
Precio: £ 27,95. ISBN 0-19-963496-3

En el estudio de las proteínas, la predicción de la estructura tridimensional es una cuestión que biólogos y bioquímicos consideran fundamental para el funcionamiento de las células vivas, y a la que dedican notables esfuerzos. Como recuerda el coordinador del libro, autor de uno de los capítulos, la idea que anima el dogma central de la predicción es que lo que determina la estructura tridimensional de una proteína es la secuencia y el medio, sin necesidad de atribuir un papel obligatorio a factores extrínsecos.

El libro se centra en las estrategias para la traducción de las secuencias dentro de las estructuras conformacionales, describiendo sus aproximaciones y validez.En el primer capítulo se resumen los procesos generales de estimación/predicción de la estructura tridimensional, y se centra más adelante en el análisis y comparación de secuencias para identificar homologías. Ofrece dos razones para justificar la comparación y alineación de las secuencias: (i) obtener una alineación precisa, por comparación con proteínas de estructura tridemensional conocida, y (ii) recoger los datos de nuevas secuencias proteicas e identificarlas con posibles funciones por analogía con proteínas bien caracterizadas.

A lo largo de dos capítulos se revisa la estructura secundaria y las predicciones topológicas para proteínas globulares y transmembranales. El principal problema de la predicción de la estructura secundaria es el plegamiento de la proteína general, de modo que pueda predecirse una conexión estructural y funcional entre proteínas cuando hay una débil relación secuencial. La predicción a partir de la secuencia de aminoácidos es más satisfactoria cuando se conoce la estructura de uno o más homólogos.

Esta información estructural puede ser extrapolada para una nueva secuencia y obtener un modelo tridimensional. Las características estructurales de las regiones variables de los anticuerpos son objeto de un capítulo, el cual proporciona la aproximación metodológica al modelo de regiones determinadas complementariamente (CDRs). Se describen nuevos métodos para la predicción de la estructura terciaria —basados en el reconocimiento de pliegues a partir de la secuencia de aminoácidos— y se desarrollan las predicciones de las estructuras secundaria y terciaria. Estos nuevos métodos pueden estudiarse mediante cálculo de energía, definido por una representación de la cadena y una energía asociada con una conformación particular.

En dos capítulos se recoge la aplicación de los cálculos de energía a los modelos de proteínas. Se hace por métodos que forman las bases de los mecanismos moleculares, minimización de energía y dinámica molecular, así como una técnica de acoplamiento para acomodar dos moléculas juntas en configuraciones favorables. Esta obra agrupa hechos y orientaciones en un campo en expansión continua, y cuyos nuevos conocimientos redundan en el avance de otras materias científicas. Resulta muy útil para los no especialistas que requieren una guía de identificación y evaluación de las estrategias de aproximación, y para expertos que requieren información más especializada sobre la aplicación de técnicas avanzadas.

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Cáncer

Genes y nuevas terapias

Alberto Muñoz

Editorial Hélice, Madrid, 1997. 374 pp. Precio: 4400 PTA. ISBN 84-921124-1-7

¿Qué es el cáncer? es la pregunta que encabeza la primera sección de este libro de Alberto Muñoz, con prólogo de Mariano Barbacid y presentación de Rafael Rosell. A la complejidad de la materia se refiere Barbacid al señalar que "abarca más de cien enfermedades distintas con más de mil variedades histopatológicas".

Alberto Muñoz lleva años dedicándose al estudio del cáncer. Con una sólida formación en uno de nuestros mejores centros y afianzada por un período de investigación en Heidelberg, en el European Molecular Biology Laboratory, ha visto reconocida su labor con el Premio Jaime I y el Premio Harington-De Visscher de la European Tyroid Association.

El libro analiza la naturaleza e importancia del cáncer como conjunto de enfermedades, tipos e incidencias en la población, su origen a partir de unos genes críticos y los agentes externos que pueden causarlo. Se discuten los datos más recientes sobre factores de riesgo (edad, tabaco, alcohol, dieta, ambiente, virus y radiaciones) y las posibles medidas de prevención.

En la segunda sección, *La información genética,* se estudia la estructura de la célula, su composición química y la expresión del material hereditario, como base para entender las alteraciones del proceso de división celular, etapa inicial del cáncer. Se revisa la importancia de la adhesión de las células entre sí y al substrato, y el fenómeno de la apoptosis (muerte celular programada), para concluir con los rasgos diferenciadores de las células cancerosas respecto a las normales.

Los genes del cáncer, la tercera y más extensa sección, trata de los proto-oncogenes que intervienen en la transducción de la señal mitogénica hasta el núcleo, y de los proto-oncogenes nucleares, responsables directos del control de los genes que convertirán células normales en cancerosas. Se trata ampliamente la actividad bioquímica y biológica de los genes supresores de tumores (retinoblastoma, p53, MT51), y de los genes de reparación de las lesiones en el DNA, cuya mutación puede conducir a la aparición de un tumor inicialmente benigno, así como de los cánceres de tipo hereditario y de las transformaciones fenotípicas de las células tumorales.

En la última sección, *Terapia actual*, se discuten las causas de la ineficacia de algunos tratamientos y se describen las modernas terapias anticancerosas en experimentación, junto con nuevos enfoques derivados de la biología molecular y celular del cáncer. No faltan las novedosas técnicas de terapia génica basadas en la modificación del genoma de las propias células tumorales.

La obra se completa con un juego de veintiseis diapositivas, que resultan útiles como material docente. Es un libro que puede recomendarse tanto a profesionales como a estudiantes. La claridad del tratamiento la hace también asequible para el público no especializado, pues aporta una visión de conjunto muy comprensible de una materia que para todos tiene interés. Se trata, en definitiva, de un texto científico muy cuidado en la precisión de sus contenidos y en la eficacia didáctica de su desarrollo, al que son aplicables las palabras de Georg Christoph Lichtenberg "El único fallo que tienen los libros verdaderamente buenos es que suelen dar origen a muchísimos malos o mediocres".

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El virus del SIDA
Un desafío pendiente

Luis Carrasco

Editorial Hélice, Madrid, 1996. 252 pp.
ISBN 84-921124-0-9

¿Otro libro sobre SIDA? Ésta es la primera frase que aparece en el libro *El virus del SIDA. Un desafío pendiente*. En realidad, es una pregunta que a buen seguro se plantea más de un profesional relacionado con la investigación, o con el tratamiento de esta enfermedad, ante una obra de este tipo. Quizá por eso es también la pregunta-exclamación inicial que nos ofrece el libro.

Desde que en 1983 se aislara y caracterizara el agente etiológico de una nueva enfermedad infecciosa a la que se denominó "síndrome de la inmunodeficiencia adquirida" (SIDA), han aparecido miles de publicaciones en forma de artículos, libros, tratados, informes y reseñas. Sin contar la cantidad de debates y reportajes en los diversos medios de comunicación de todo el mundo.

Es verdad que, en los quince años desde la aparición de este nuevo agente infeccioso (virus de la inmunodeficiencia humana, VHI), se ha avanzado en todos los campos del conocimiento científico en general y de aquellos aspectos que se relacionan con el origen, mecanismos de actuación del virus y vías de tratamiento. Pero también es cierto que han sido enormes los medios que se han puesto en esta causa, especialmente desde que sus víctimas pertenecen a la llamada sociedad civilizada, pues es a partir de ese momento cuando se repara en ella. Desde entonces ha sido constante el caudal de información aparecida en revistas especializadas y de divulgación, tratando de explicar tanto los últimos hallazgos que van surgiendo sobre el propio virus y su relación con el sistema hospedador, como los aspectos clínicos y sociales de la enfermedad, que transcienden los ámbitos estrictamente científicos y sanitarios.

Como respuesta quizá a esta situación, y a una opinión muy sensibilizada hacia el tema, el libro está pensado para un público amplio; personas con inquietudes y curiosidad sobre los acontecimientos científicos. Está dirigido también a los estudiantes de ciencias de la salud y a quienes se interesen por los aspectos globales de la enfermedad, una perspectiva ésta que cada día va ganando más adeptos. El libro ofrece una visión amplia de la relación hospedador (ser humano)–parásito (VIH), y va todavía más allá cuando examina su repercusión en la sociedad y los cambios que ha producido en muchos comportamientos.

El prólogo del libro está a cargo de Federico Mayor Zaragoza, Director General de la UNESCO. Desde su conocimiento y preocupación por la expansión de la enfermedad, y por sus condiciones y efectos, que son todavía mucho peores en los países en desarrollo, destaca que nadie está libre del ataque del VIH, cualquiera que sea su condición social. Hay que reconocer que, a pesar de los cuantiosos esfuerzos que se realizan y de haberse obtenidos logros significativos, la solución al problema todavía está lejana. Los logros han sido tanto científicos, en el conocimiento y tratamiento de la enfermedad, como sociales, en la adopción de medidas preventivas y en una mentalidad más generosa. Pero el SIDA ha adquirido una dimensión tan compleja que requiere un gran esfuerzo colectivo, no sólo de medios materiales sino también de comprensión por parte de todos los estamentos.

El libro se estructura en cuatro secciones, cada una dividida en capítulos de progresión lógica que permiten una perspectiva general del problema. Se aporta información acerca del impacto global de la enfermedad, de los recur-

sos sanitarios que se le destinan, estructura y caracterización genética del virus, relación con el sistema inmunitario y, finalmente, sobre medidas preventivas. Las ilustraciones que acompañan al texto son de gran calidad, y facilitan la comprensión de los mecanismos del virus del SIDA a un público heterogéneo.

"Ante la evidencia", la primera sección, introduce algunos aspectos históricos claves de la aparición de la enfermedad y las consecuencias epidemiológicas. En el capítulo, "El impacto de una nueva epidemia", se abordan los efectos de la aparición del SIDA y su impacto social y sanitario. Se ofrecen algunos apuntes de la historia y descubrimiento del VIH, una visión global del curso de la enfermedad, los distintos métodos de lucha que se han ido ensayando, resultados y estrategias propuestas. La sección concluye con una perspectiva de la enfermedad en el mundo y con una breve referencia al posible origen de la epidemia.

En la segunda sección, "Tras la pista del VIH", se intenta llegar al conocimiento del VIH a través, primero, de una directrices básicas. "Conocer los virus" es un capítulo introductorio de conceptos básicos relacionados con la biología molecular, ingeniería genética y naturaleza y diversidad de los virus animales. Incluye una explicación conceptual y muy básica de la técnica de la PCR (reacción en cadena de la polimerasa). El capítulo "Sobre los retrovirus" presenta los conceptos generales, su importancia, naturaleza del virión, ciclo replicativo y menciona otros virus relacionados. "El virus de la inmunodeficiencia humana" es un capítulo dedicado exclusivamente al conocimiento del VIH, con la estructura detallada de la partícula vírica, el ciclo de replicación y la síntesis y función a nivel molecular de las proteínas codificadas por el virus. Concluye con la infectividad de las partículas víricas, las salidas de nuevos viriones y su efecto sobre las células infectadas.

La tercera sección, "Tras la pista del SIDA", aborda en tres capítulos los aspectos básicos del sistema inmunitario, para llegar a una posterior comprensión del problema de la relación VIH–célula y de su acción devastadora sobre ese sistema. Se ofrece una revisión básica de la respuesta inmunitaria, con la aparición de anticuerpos, su detección y evolución después de un ataque vírico, tanto de anticuerpos como del RNA vírico o proteína p24. De un modo general se habla de las diferentes fases en que se cataloga la enfermedad, así como de las principales infecciones oportunistas y malignas que afectan a los enfermos que la padecen.

"Hacia una salida" es la última sección, en la que en el capítulo dedicado a "Prevenir el SIDA" se plantea la necesidad del conocimiento de los factores de riesgo, los problemas que supone actualmente la vacunación y los esfuerzos que se realizan para encontrar una vacuna eficaz. El segundo capítulo de este apartado, "Combatir el SIDA", se centra en la búsqueda y selección de agentes antivíricos y se plantea cuáles son las estrategias adecuadas para combatir la infección, así como la problemática del tratamiento con la aparición de resistencias en el VIH.

El libro cumple con los objetivos del autor, que ha conseguido un texto comprensible para un público amplio, con un cierto conocimiento del virus VIH y de la enfermedad que produce, y que ofrece una visión general del problema que se plantea en la sociedad. En realidad, en las actuales circunstancias, el virus del SIDA, es un desafío pendiente y permanente.

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Man and Microbes

Disease and Plagues in History and Modern Times

Arno Karlen

Touchstone, New York, 1995. 266 pp.
Precio: $ 13. ISBN 0-684-82270-9

Arno Karlen, escritor, investigador y psicoterapeuta, tuvo numerosas dificultades para encontrar un editor interesado en publicar un libro sobre enfermedades resurgentes. Recopiló material durante veinte años y, en 1990, empezó a escribir lo que después resultaría este interesante libro, con una idea en mente que sorprendía a muchos. Al explicar Karlen que escribía sobre nuevas enfermedades y su procedencia, le decían "Te refieres al Sida". Alguien mencionaba Lassa, Ebola o las fiebres Marburg. Pero él pensaba en muchas otras enfermedades. Cuando por fin encontró editor, predijo que antes de que su libro apareciese publicado al menos dos o tres enfermedades epidémicas saldrían en titulares. Así fue.

Karlen ofrece en este libro un paseo por las enfermedades que han sacudido a la humanidad, preguntándose el porqué del resurgimiento con más fuerza de muchas de ellas. Trata de concienciarnos de que estamos viviendo una reaparición de enfermedades infecciosas, aportando datos concretos que apoyan sus observaciones y presentando un panorama un tanto dramático a lo largo de la historia de las enfermedades. El libro contiene trece capítulos, a través de los cuales documenta ampliamente sobre la forma, condiciones y morbilidad que estas enfermedades produjeron y están produciendo actualmente, años o décadas después de ser descubiertas, o de haber sido aparentemente erradicadas. Además de documentar históricamente las enfermedades que durante diferentes épocas se han cobrado más víctimas, explora y reflexiona sobre nuevas plagas, y sobre la supervivencia y adaptación mútua de la especie humana con los microorganismos con los que convive.

Según Karlen, los humanos han favorecido la reaparición de estas enfermedades al permitir un elevado número de cambios en el medio ambiente y en su propio comportamiento (respecto a viajes, dietas diferentes, nuevos hábitos y pautas sexuales, etc.). Los países en vías de desarrollo registran el mayor número de personas infectadas, en gran medida debido a las deficiencias de los servicios de sanidad y a su situación. A pesar de que estas enfermedades afectan más al Tercer Mundo, también se extienden por los países ricos, debido principalmente a los siguientes factores, ampliamente analizados a lo largo del último capítulo: la insuficiencia de medidas de los servicios sanitarios, el desarrollo económico y la explotación de los recursos de la tierra, los viajes y comercio internacionales, la industria y tecnología, la demografía y el comportamiento humano, y, finalmente, el cambio y la adaptación microbianos. El libro finaliza subrayando la importancia de la adecuada comprensión de la interacción y coexistencia entre la especie humana y los microorganismos, que necesita una permanente adaptación.

Es una obra amena, de fácil lectura, adecuada para un sector amplio de público, tanto estudiantes como especialistas o cualquier persona interesada en conocer el origen, desarrollo y manera de combatir las enfermedades, así como los motivos de su resurgimiento. Una extensa bibliografía, separada por capítulos y comentada brevemente por el autor, permite ampliar la información. La localización de los temas resulta cómoda gracias a un extenso índice.

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Sixty Years of Biology
Essays on Evolution and Development

John Tyler Bonner

Princeton University Press, Princeton, 1996. 144 pp. ISBN 0-691-02130-9

This collection of five essays by the professor emeritus of biology at Princeton University John Tyler Bonner, a major participant in the development of biology as a experimental science. The book is written for the nonspecialist reader in a language similar to that of an introductory biology text, but with a more personal, reflective style.

The first chapter is a discussion of the influences of genome and the physical properties of the environment on organismic evolution. Through various examples, including D'Arcy Thompson's discussion of the skeleton's of Haeckel's radiolarians (their diversity could not have evolved by natural selection), the author demonstrates the importance of non-genetic, physico-chemical forces of the environment in shaping the evolution of organism's morphology. The author makes some observations and commentaries aside his main argument, such as suggesting that aversion to mathematical modelling as a theoretical approach to science may stem from one's lack of mathematical ability.

Chapter 3 is the most useful chapter in the book. Gene accumulation, the "Baldwin effect," as coined by G. G. Simpson, is "the idea that a repetition of some somatic process over many generations could eventually become hardened into a truly inherited variation". It is the chance appearance of genes which will direct a process that the organism was previously carrying out without any genetic control. In contrast, gene silencing is the removal by gene inactivation of inherited control of a system, making its phenotype more flexible and more subject to active selection. The author argues that gene silencing is behind the evolution of asexuality in plants and animals and that both gene accumulation and gene silencing were involved in the differences between mosaic development, involving within-cell signalling, and regulative development, involving between-cell signalling.

Chapter 4 is less encouraging. The chapter is filled with the examples of the volvocalean green algae, social amoebae (slime molds) and social insects, but none of these examples is used to support his sweeping assertion. *Volvox* has gonidia, specialized colony-forming reproductive cells, and somatic cells that cannot produce colonies while in other volvocaleans any cell in the colony is capable of generating a new colony, but no evidence is presented to show that *Volvox* dominates the waters, driving *Gonium* and *Pandorina* to extinction. Similarly, the solitary soil amoebae coexist with the slime molds. Presumably they occupy different niches, but doesn't that contradict the author's thesis?

These essays provide useful discussions of the evolution of developmental processes. They are light reading. Some will attract your attention to important concepts you might otherwise miss, while others will lead you to organize your challenges because you disagree with them so. In any case, experimental and experienced biologists must discuss biological problems, many times abandoned to philosophers of science, to understand and realize the profound meaning of a science which has revolutionized human thinking in the second part of this century.

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El mundo y sus demonios

La ciencia como una luz en la oscuridad

Carl Sagan

Editorial Planeta, Barcelona, 1997. 493 pp. Precio: 4300 PTA. ISBN 84-08-02043-9

Billions & Billions

Thoughts on Life and Death at the Brink of the Millenium

Carl Sagan

Random House, New York, 1997. 244 pp. Precio: $ 24,00. ISBN 0-679-41160-7

El mundo y sus demonios es el último libro de Carl Sagan (1934–1996) publicado antes de su muerte (la impecable traducción española, de Dolors Udina, es de 1997). *Billions & Billions*, que escribió cuando luchaba ya contra la enfermedad, se publicó póstumamente. Los capítulos de ambos libros, aunque son unidades de información independientes, procedentes en algunos casos de artículos previos o de conferencias impartidas por el autor, mantienen una cierta coherencia en su distribución. Un rasgo común a ambos libros es la frecuente referencia autobiográfica, como una estratagema para acercarse el lector, al mostrarle el lado humano del científico. Ese aspecto se hace más patente, incluso patético, en el último capítulo de *Billions & Billions,* "En el valle de las sombras", donde describe el desarrollo de su enfermedad y sus sentimientos frente a la muerte.

El mundo y sus demonios es un libro de denuncia y alerta, para hacer comprender al lector que la verdad que ofrece la ciencia puede ser tan sorprendente como los falsos prodigios basados en la religión, las supersticiones, los mitos o la ignorancia; pero mucho más fiable. Sagan insiste en la necesidad de poseer unos conocimientos científicos básicos que permitan a cualquier persona no experta distinguir y combatir los "demonios" que nos acechan en muchos frentes, como el de las llamadas paraciencias. Intenta estimular el pensamiento crítico y el escepticismo para iluminar la oscuridad, que es la ignorancia. Buena parte del libro está dedicada a desmontar algunos mitos sobre temas que han tenido engañada a la población, como la presencia de ovnis y extraterrestres que practican "abducciones", seducen a mujeres, y marcan en los sembrados su paso por la Tierra; los falsos poderes de curanderos y chamanes y las supuestas propiedades curativas del magnetismo; los presuntos beneficios de la meditación trascendental y de las doctrinas de la Nueva Era; la aparente base científica de la astrología; la hipotética presencia de endemoniados, brujas y espíritus. Tras una reflexión sobre las reacciones que provocó la publicación resumida de los capítulos anteriores, muestra el camino para aprender a descubrir el complemento pseudocientífico que tiene cada campo de la ciencia. Discurre sobre las dudas que pueden surgir de la aplicación errónea del desarrollo tecnológico; sobre el funcionamiento de la ciencia y el desarrollo del pensamiento científico; y sobre la educación y comunicación científica y la visión que la sociedad tiene del investigador. Advierte de la necesidad de superar el analfabetismo científico y propone algunas soluciones para ello. Indica la conveniencia de adoptar una actitud escéptica también en política y expresa su deseo de que los ciudadanos de cualqueir país se formen siguiendo el método científico y en la democracia.

Billions & Billions es una colección de ensayos cuyo subtítulo, *Pensamientos sobre la vida y la muerte en el límite del milenio*, resume el

significado de la obra, que consta de tres partes. La primera, "El poder y la belleza de la medida", se inicia con un recorrido por el mundo de las matemáticas aplicadas a la vida cotidiana. Pone de manifiesto el analfabetismo numérico imperante en la sociedad actual, evocándonos un librito de gran interés *Innumeracy* (*El hombre anumérico* en su versión castellana), de John A. Paulos, y un ensayo de Stephen J. Gould ("La mediana no es el mensaje", incluido en *Brontosaurus y la nalga del ministro*), que tratan también de la falta de perspectiva numérica, que dificulta el manejo racional de grandes cantidades y de conceptos ligados a la matemática, como las probabilidades o las estadísticas. Eso nos impide alcanzar una visión cuantitativa del mundo y es una de las causas del apogeo de las pseudociencias. Como dice Sagan, la medida de un fenómeno es lo que nos permite adentrarnos en su comprensión. Otros temas tratados son: el auge del deporte-espectáculo como continuación de un ritual ancestral; diferentes aspectos de radiaciones que nos parecen fenómenos muy distintos pero que pueden reducirse, en última instancia, a variaciones de ondas y frecuencias; consideraciones en relación a los mitos sobre los orígenes, comentando las cuestiones en las que ve más posibilidad de avanzar el conocimiento (¿ha habido alguna vez vida en Marte?, ¿es Titán un laboratorio para estudiar el origen de la vida?, ¿hay vida inteligente en algún otro lugar?, ¿cuáles son el origen y el destino del universo? y ¿existen otros sistemas planetarios fuera del nuestro?).

En la segunda parte del libro, "¿Qué conservan los conservacionistas?", trata algunos de los problemas ambientales más acuciantes, como el efecto invernadero, la acumulación de CFC y la consiguiente disminución de la capa de ozono, y destaca la capacidad que tiene el ser humano de cambiar el ambiente. Somos responsables de las alteraciones que se han producido o pueden producirse en nuestro planeta y, por nuestro propio bien, hemos de aprender a mantenerlo en las condicioness adecuadas. Considera posturas encontradas, que recuerdan los mitos de Creso y Casandra. Por una parte, tenemos unos modernos oráculos —los científicos— a quienes acuden los políticos. Éstos no suelen entender de ciencia y pueden interpretar mal las profecías. Por otra parte, como ocurrió a Casandra, condenada a que nadie creyese sus profecías, muchas veces las predicciones de la ciencia tampoco son tomadas en cuenta. Sin embargo, Sagan se siente optimista respecto al futuro, y por primera vez cree advertir un acuerdo entre religión y ciencia en muchos aspectos relacionados con la conservación del planeta.

La última parte del libro, "Donde el corazón y la mente chocan", contiene apreciaciones subjetivas sobre aspectos éticos de diferentes cuestiones: la inutilidad de las guerras y los bloques en un momento de la historia en que los países deben cooperar para combatir el enemigo común que podría surgir de un mal uso de los avances tecnológicos; la intolerancia que suelen demostrar los grupos pro abortistas y los autodenominados "defensores de la vida", aunque a veces defiendan el mantenimiento de la pena de muerte; los códigos morales imperantes en las sociedades actuales; el reto de alcanzar un equilibrio entre los beneficios y las amenazas debidos al avance científico y tecnológico del siglo XX; y finalmente la experiencia de un agnóstico frente a la muerte. Ann Druyan, esposa de Sagan y coautora de algunos capítulos de ambos libros, es también autora de un emotivo epílogo. Estamos, en fin, frente a dos obras que muestran, no sólo la habilidad literaria y comunicativa de Carl Sagan, sino también su profundo amor por la ciencia y por la humanidad.

Mercè Piqueras
Redacción de 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.

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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.

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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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Las referencias a tesis doctorales, originales no aceptados todavía o comunicaciones presentadas a congresos, deben incluirse en el texto del artículo de acuerdo con los siguientes ejemplos: (García, P. et al. 1985, en preparación), (Smith, T. 1985. Tesis doctoral University of Massachusetts, Amherst) o (Suárez, A., González, F. 1975. Res. V Congr. Nac. Microbiol., p. 1845).

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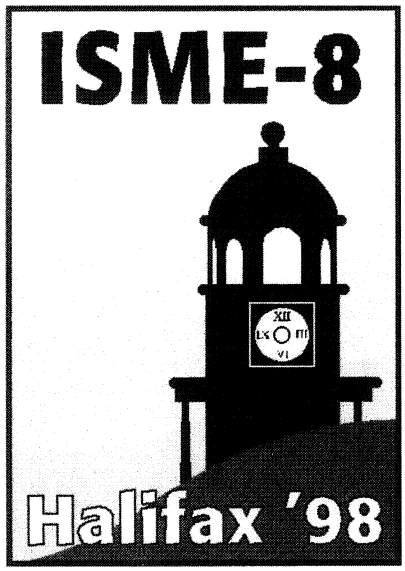

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