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PUBLICACION DE LA SOCIEDAD
ESPAÑOLA DE MICROBIOLOGIA

Microbiología

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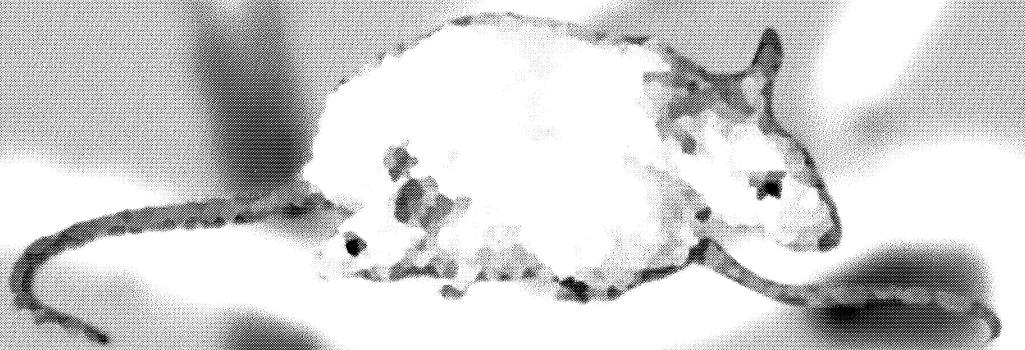

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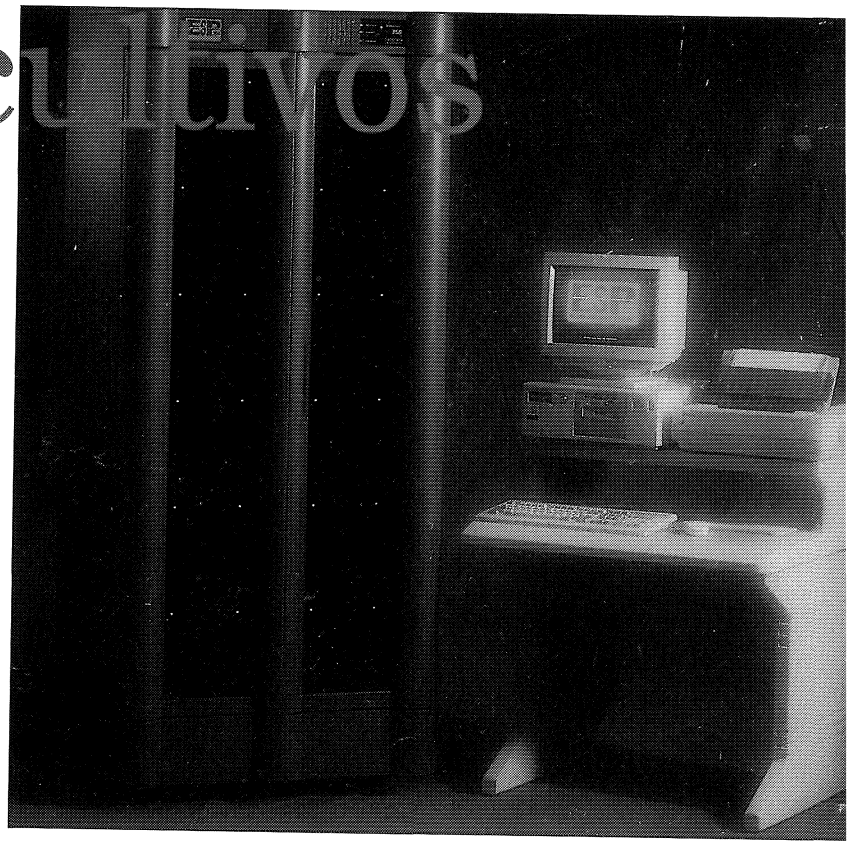

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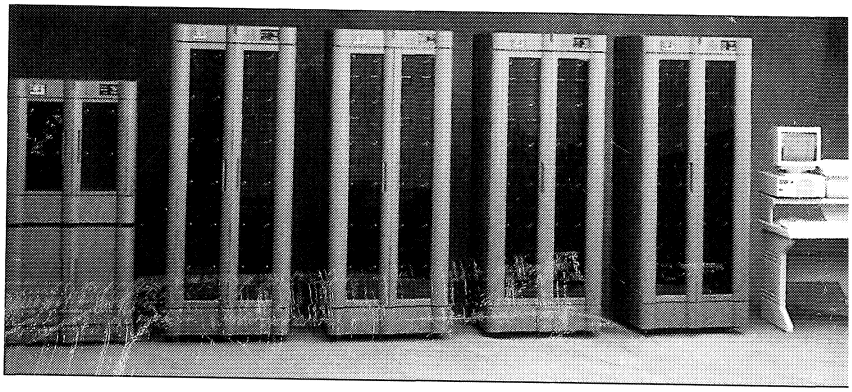

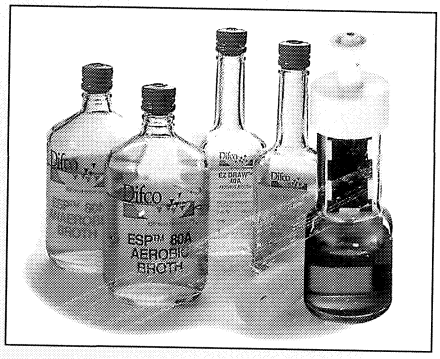

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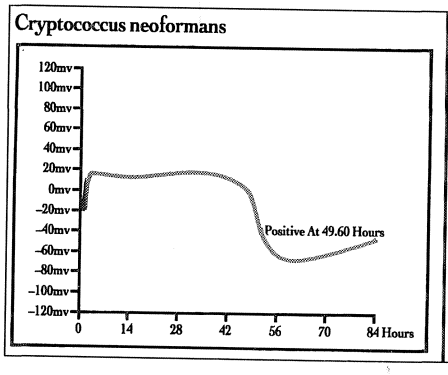

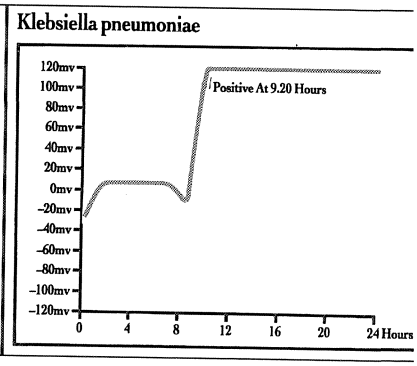

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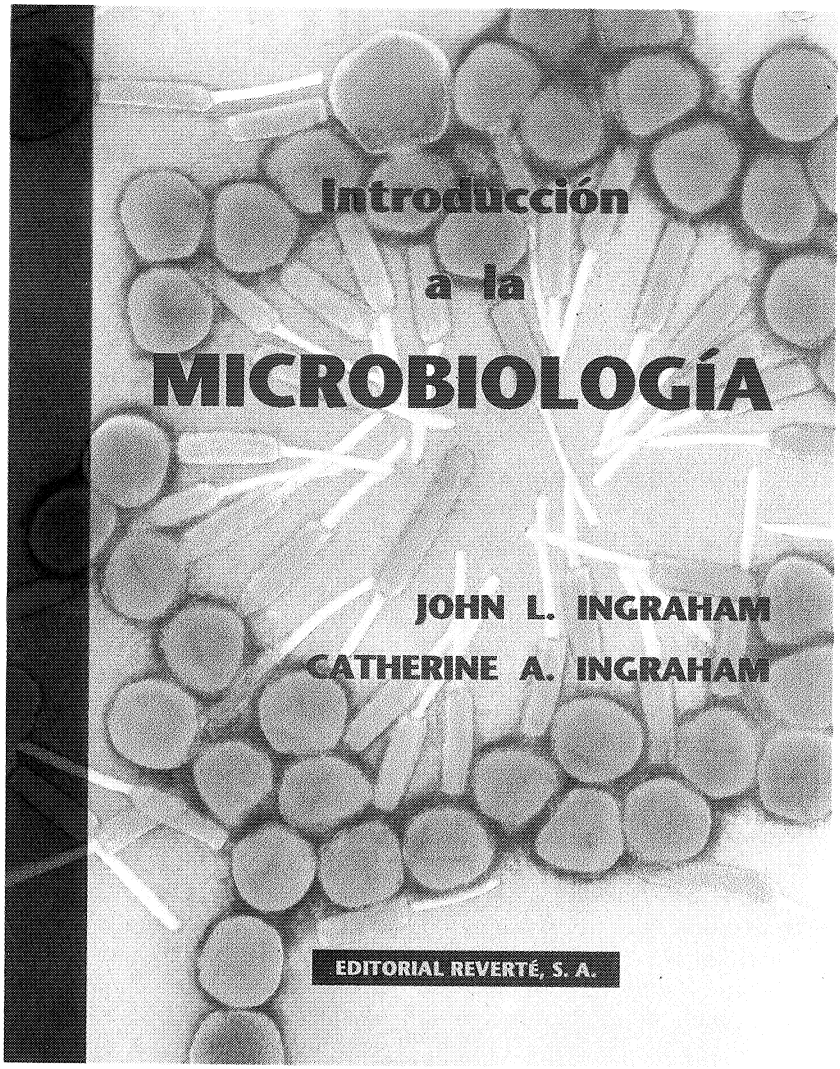

EDITORIAL REVERTÉ, S.A.

INTRODUCCIÓN A LA MICROBIOLOGÍA

JOHN L. INGRAHAM
CATHERINE A. INGRAHAM

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El presente libro de texto ha sido escrito, conjuntamente con su hija, por uno de los anteriores autores de la 4ª edición del clásico libro de Microbiología *The Microbial World*, de R. Y. Stanier y col., y viene a ocupar el hueco dejado por ese libro, cuya última edición data de 1984.

Si bien su objetivo sigue siendo el de introducir al lector en el mundo de los microorganismos, en los diversos métodos y técnicas que se utilizan para su estudio y en el impacto que tienen en nuestra vida, los autores han escogido como motivo central de su obra (lo que supone una diferencia fundamental con la anterior) el estudio de las enfermedades infecciosas que afectan a los seres humanos, los microorganismos que las producen y los procesos que tienen lugar en el cuerpo humano durante el transcurso de la infección y, en su caso, enfermedad. De esta manera, se ha superado una laguna tradicional de la Microbiología de Stanier y col., como era su enfoque excesivamente biológico y que lo limitaba enormemente como libro de texto de Microbiología para los estudiantes de Farmacia, Veterinaria y Medicina.

Para conseguir los fines propuestos y hacer que el texto sea lo más didáctico posible, los autores han incluido en el mismo una serie de casos clínicos basados en hechos reales de la vida cotidiana. Asimismo, al principio de cada capítulo de los dedicados al estudio de las enfermedades en el ser humano producidas por microorganismos se incluye una historia clínica, a modo de viñeta, que sirve como guía para todo el capítulo. Así, a medida que se van estudiando las bases microbiológicas y fisiológicas de la enfermedad se van explicando los fundamentos y causas de los síntomas clínicos observados en el enfermo. Igualmente, a lo largo de estos capítulos, aparecen otra serie de casos clínicos que

ilustran o especifican una materia en particular.

Es especialmente interesante el enfoque didáctico que se ha utilizado para el tratamiento de los temas concernientes al Metabolismo y Genética de los microorganismos. Para ello, se ha utilizado la analogía microorganismo-fábrica celular, siguiendo las directrices de un plan genético, para explicar conjuntamente las reacciones metabólicas con los procesos genéticos asociados.

La obra, que comprende 29 capítulos que se recogen en dos volúmenes, está estructurada en cinco grandes bloques o partes: Principios de Microbiología, Los Microorganismos, Principios de la interacción ser-humano-microorganismo, Enfermedades humanas causadas por los microorganismos y, por último, Beneficiosos y usos de los microorganismos. Asimismo, se acompañan una serie de Apéndices al final del texto y un extenso glosario.

Cada capítulo comienza con unos Objetivos y finaliza con un amplio Resumen. Por otro lado, en todos los capítulos se han incluido una serie de comentarios ilustrativos sobre aquellos aspectos de la Microbiología en donde se están llevando a cabo las investigaciones más recientes, muchos de ellos escritos por los propios investigadores que han alcanzado una mayor notoriedad en dichos campos de investigación. De forma similar, se incluyen también en el libro otra serie de breves comentarios ("Avances decisivos") en los que se describe un hallazgo o técnica que supuso un trascendental avance en diversos campos de la Microbiología, como por ejemplo, el descubrimiento del agar-agar como agente solidificante para el diseño de los medios de cultivo para el aislamiento de los cultivos puros microbianos.

De especial mención es la impresionante calidad de los gráficos y fotografías en color que ilustran el texto, así como la claridad de los esquemas utilizados. En este último aspecto, debemos destacar la enorme utilidad de los mismos en el tratamiento del apartado concerniente a la Inmunología, una de las secciones mejor tratadas a nivel didáctico de toda la obra. Asimismo, hay que comentar que en el tratamiento de las enfermedades humanas producidas por microorganismos se ha seguido el criterio (recogido cada vez más por otros libros de texto de Microbiología) de agrupar a los microorganismos infecciosos en diversos capítulos de acuerdo con las infecciones que causan en los distintos sistemas del cuerpo humano (respiratorio, digestivo, genitourinario, nervioso, cardiovascular y linfático, y las superficies corporales).

Por todo lo dicho, creemos que estamos ante un libro de texto de Introducción a la Microbiología que se adapta muy especialmente a los contenidos que figuran en la mayoría de los nuevos planes de estudio implantados en las universidades españolas en las diversas licenciaturas en las que la Microbiología figura como una disciplina de las mismas (Medicina, Farmacia, Veterinaria, Biológicas). Finalmente, hay que destacar que se ha realizado un gran esfuerzo para que la versión castellana que se presenta no adolezca de los desgraciadamente habituales errores en la terminología científica utilizada, así como para que el vocabulario empleado se ajuste lo más fielmente posible al castellano, evitando términos confusos o incorrectos.

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The Official Journal of the Spanish Society for Microbiology

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MICROBIOLOGÍA SEM **13** (1997), 399–402

Editorial*

La microbiología ambiental en América Latina

En los últimos años se ha experimentado un movimiento hacia el área de la tecnología y las ciencias aplicadas en el mundo entero. Aunque esta dirección parece estar por lo menos parcialmente justificada debido a los cambios político-económicos a escala mundial, es lamentable que los presupuestos asignados para la investigación científica hayan ido reduciéndose o manteniéndose en los niveles de años pasados. La menor cantidad de fondos para la investigación básica ha disparado una consecuente competencia entre científicos por una cantidad de fondos cada vez más escasa. Aunque éste es un problema que aparentemente concierne solamente a los países más industrializados, la misma tendencia se está viendo en los países en vías de desarrollo.

Esta revista ha publicado diversos editoriales realizados por distinguidos científicos de diferentes países hispanos donde se han descrito muy apropiadamente numerosos problemas que aquejan a los científicos de nuestros países. Debido a esto, no merece que se haga un análisis más extenso por el presente autor. Sin embargo, un tema que hasta ahora no se ha tocado en detalle ha sido el de la cada vez más desesperante situación de las generaciones de estudiantes que se alejan de los estudios que requieren la investigación científica, y el consecuente impacto negativo que esto tiene sobre el avance de la ciencia en nuestros países.

Actualmente el ámbito de las ciencias biológicas es visto como el primo pobre de la medicina. La gran mayoría de la población del mundo entero encuentra difícil entender que medicina no es sinónimo de ciencia. Aún entre personas con una educación universitaria existe este problema. El resultado de esto es que las carreras en ciencia no son vistas como una alternativa a la medicina por los futuros profesionales, o simplemente dicha alternativa no existe debido a que el papel de la

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* *Microbiología SEM* continúa en este número la serie de editoriales dedicados a comentar la situación de la ciencia (en general, y de la microbiología en particular) en América Latina. En esta ocasión el artículo ha sido preparado por el prestigioso microbiólogo Gary A. Toranzos, de la Universidad de Puerto Rico.

ciencia en la vida cotidiana no se conoce, o no se entiende. La experiencia personal de este autor indica que son muy pocos los estudiantes que han tomado siquiera en consideración estudios de maestría o doctorales para seguir una carrera en la investigación científica. Estos mismos estudiantes, si tienen la oportunidad de hacer una pasantía en algún laboratorio de investigación, cambian radicalmente el abordaje que tienen hacia la ciencia.

Muchos distinguidos científicos (tales como los microbiólogos de renombre internacional Rita R. Colwell y Ronald M. Atlas, entre otros) y este autor, empezamos nuestras carreras universitarias con un desconocimiento casi total de la ciencia. Muchos de nosotros comenzamos pensando en que la única ruta que nos llevaría a una profesión científica era la medicina. En la mayoría de los casos, alguna persona en particular tuvo un papel crucial que cambió totalmente nuestro enfoque profesional. En el caso de este autor, un curso de microbiología, dictado por un científico que obviamente gozaba de su profesión, y una subsecuente mentoría en su laboratorio, le decidieron a cambiar la medicina por una extremadamente satisfactoria carrera en el campo de la microbiología básica.

Las anécdotas mencionadas no son nada más que una pequeña muestra de la importancia que tienen las mentorías en la formación científica de las futuras generaciones. Esto último cobra un significado muy grande en el mundo entero, pero más especialmente en nuestros países, ya que, de la misma manera que expresaron colegas en anteriores editoriales, si América Latina quiere sobresalir (o tal vez sobrevivir) en términos científicos, se tiene que hacer un esfuerzo en todos los ámbitos para estimular a las futuras generaciones a emprender estudios de investigación y para hacer que esos investigadores puedan desarrollar su trabajo dentro de la sociedad a la que pertenecen.

Es necesaria una política agresiva en los ámbitos universitarios y profesionales para llegar a la meta de tener científicos productivos. Una posible forma de alcanzar estos objetivos es que los científicos seamos más visibles a la población en general, o como dijo Antonio Lazcano (editorial del número 13 [1] de *Microbiología SEM* [pp. 7–10]) "debemos ser eclécticos en el uso de todos los medios disponibles". Es obvio que los científicos no sabemos comunicar nuestra ciencia al público en general. Somos muchos los científicos latinos que asistimos asiduamente a reuniones internacionales, lo cual demuestra el nivel de actividad investigadora. Sin embargo, esta misma ciencia no es descrita apropiadamente a los medios de comunicación y, consecuentemente, la población en general no alcanza a captar su contenido y significado.

Todos estos problemas, sumados a los limitados fondos de investigación, hacen que el número de científicos en los países hispanos sea pequeño; algunas estadísticas de esto ya fueron mencionadas en anteriores editoriales. Es importante hacer ver a los gobernantes que los beneficios aparentemente esotéricos (según creen algunos de ellos) de la ciencia son realmente beneficios directos para el país. Un ejemplo de esto último es el gran interés que ha ido surgiendo en todo lo concerniente a la biodiversidad. Muchos estudios anteriormente llevados a cabo en América Latina han demostrado la gran biodiversidad de especies que presentan nuestros países. Sin embargo, muchos de estos estudios no han sido realizados por científicos latinoamericanos. Un posible problema con esto es que surgen abusos, como el que llevaron a cabo un par de estadounidenses que patentaron una cepa boliviana de quinua (un cereal de alto contenido proteico), que había sido obtenido de una comunidad indígena cercana al lago Titicaca. Es interesante saber que fueron también científicos extranjeros los que denunciaron este abuso.

La diversidad microbiana también está comenzando a ser determinada en nuestros países y es muy probable que se aíslen cepas de mucha importancia industrial. Es necesario informar a los gobernantes en América Latina que personas con una formación científica sólida son absolutamente necesarias para salvaguardar los posibles beneficios de la biodiversidad presentes en nuestros países. Estos mismos científicos son los únicos que podrán llevar a cabo estudios que a su debido tiempo reportarán beneficios para el país.

Si la ciencia en su totalidad está siendo víctima del utilitarismo actualmente existente, la microbiología ambiental sufre aún más. Aunque dicha rama de la microbiología haya sido estudiada desde los tiempos de Louis Pasteur, Martinus Beijerinck, Sergei Winogradsky y otros notables científicos, su importancia ha sido subestimada hasta hace poco. La gran mayoría de la población mundial considera solamente los efectos negativos de los microorganismos, llegando a lo que Ivette García Castro ha llamado "microfobia", sin tener en cuenta el gran papel que éstos desempeñan en todos los ámbitos de la vida cotidiana. Un aspecto de gran repercusión que ha ocupado las noticias de primera plana ha sido el proceso de la biorremediación. Sin embargo, aunque el uso de microorganismos para la degradación de compuestos xenobióticos es ya una realidad, existen relativamente pocos estudios llevados a cabo en América Latina. Lamentablemente contamos con un reducido número de microbiólogos ambientales que puedan realizar estudios genuinamente científicos en el área de la biorremediación.

Aunque la importancia de las investigaciones en todos los aspectos ambientales no puede ser subestimada, los fondos disponibles para este tipo de estudios son casi inexistentes en nuestros países. Microbiólogos ambientales de renombre internacional han tratado por muchos años de estimular la investigación en esta área de la ciencia; lamentablemente, han logrado pocos avances. Maria Therezinha Martins (véase el artículo de Campos, V. y Toranzos, G. A. en *Microbiología SEM* **11**[3], 409–410) fue, antes de su inesperada muerte, una de las más fuertes impulsoras de los estudios ambientales en América Latina. Con esta idea en mente, Therezinha y otros científicos latinoamericanos fundaron el Comité Latino Americano para la Microbiología Ambiental (CLAMA). Por muchos años, el CLAMA ha estado presente de una u otra forma en los diversos congresos y coloquios científicos que se han llevado a cabo en América del Sur.

Entidades como CLAMA desempeñan una función muy significativa en la difusión de la microbiología ambiental en América Latina. Sin embargo, se necesita una acción mucho más fuerte por parte de los científicos ambientales en todos los niveles. Si nuestros países quieren mantenerse competitivos en el campo de las ciencias experimentales, tienen que lograr alcanzar una masa crítica de científicos que estén participando activamente en la investigación. El proceso es largo. Sin embargo, el convencer a los gobernantes de la importancia de los estudios científicos, el atraer nuevo talento a esta área y el formar profesionalmente a estos estudiantes está, por lo menos en parte, en nuestras manos.

Teniendo presente esto último, ya se han iniciado proyectos diseñados para aumentar el nivel de conocimiento microbiológico en profesores de biología en la enseñanza primaria y secundaria. Un proyecto diseñado por Douglas Zook y Lynn Margulis en los Estados Unidos está tomando impulso en esta dirección. El proyecto, llamado "Microcosmos", además de proporcionar conocimientos de microbiología a los maestros y profesores, les enseña técnicas de observación y les estimula el

desarrollo del pensamiento crítico. En Puerto Rico, la ya mencionada Ivette García Castro ha organizado talleres que han despertado el interés de los maestros. Este interés muy seguramente va a ser transmitido a los estudiantes preuniversitarios. La microbiología ambiental puede desempeñar un papel muy destacado para despertar la curiosidad de los estudiantes por medio de experimentos sencillos que están al alcance de cualquier escuela, independientemente de la existencia de instalaciones de laboratorio sofisticadas.

Es obvio entonces que los microbiólogos debemos tener una participación muy activa en despertar el interés de los estudiantes por las ciencias biológicas y ambientales. El cómo llevar esto a cabo depende de nuestra entrega a esta meta, como científicos, educadores y, sobre todo, como mentores de las próximas generaciones de científicos latinoamericanos.

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Comentarios del año

En el aspecto científico, 1997 ha sido un año fértil tanto in vivo como in vitro, y de muchas otras maneras que el diccionario todavía no ha incorporado. Sin duda, la noticia más destacada fue el nacimiento de Dolly, la ovejita de aspecto angelical, que aparecía en la portada de *Nature* del 27 de febrero, y en el artículo discretamente colocado en el interior (pp. 810–813) con la descripción del primer mamífero clonado a partir de una célula adulta. Dolly es el resultado de una técnica de clonación en la que el núcleo de una célula mamaria de una oveja adulta se implantó en un oocito, de una segunda oveja, desprovisto de su propio núcleo. Este óvulo se implantó en una tercera oveja, que fue la que, finalmente, alumbró a Dolly. La alarma ante una gesta que los investigadores de otros campos no podemos más que adivinar, por cuanto supone de avance tecnológico, no se hizo esperar. Máxime cuando tras el éxito obtenido parecía que otros grupos estaban en disposición de conseguir resultados similares. No es la primera vez, sin embargo, que los avances científicos suscitan temor ante las consecuencias de unas aplicaciones indeseadas. Aunque no hay que desestimar esas posibles consecuencias, sí hay que ofrecer la información más clara posible para evitar sustos innecesarios. De lo que no hay duda es que se ha disparado una supuesta consciencia ética en ámbitos más políticos que científicos que, como casi todo lo que atañe a la esfera política, corre el peligro de convertirse en una traba burocrática que no soluciona ninguna de las preocupaciones reales del ciudadano. Eso sí, a tenor de los últimos rumores alrededor de los proyectos a los programas de la Unión Europea, puede que se cree una oficina de control ético, a cargo de "personal especializado", que garantice que ningún proyecto presentado a las convocatorias atentará contra los principios inalienables e incontrovertibles de la vida en sus múltiples manifestaciones reales o potenciales. Los necesarios controles y salvaguarda que la sociedad debe poner ante cualquier nueva tecnología no deben impedir el desarrollo de la investigación, que, como se ha demostrado repetidamente, ayuda tanto al conocimiento básico de los procesos biológicos como a la mejora de las condiciones de vida de nuestra especie.

La carrera que se iniciara en julio de 1995 con la publicación de la secuencia completa del DNA de *Haemophilus influenza* ha continuado imparable y en este año se ha podido conseguir la secuenciación de *Escherichia coli*, el organismo estrella de la bacteriología. El conocimiento de su genoma y de los procesos biológicos en que interviene, ayudarán a desentrañar la función de cada uno de sus genes. Pero no hay que olvidar las otras secuencias completadas durante 1997. En el mes de julio aparecía en Internet el genoma de *Borrelia burgdorferi*, espiroqueta causante de la enfermedad de Lyme, que transmiten las garrapatas. *Nature* la ha publicado en su número del 11 de diciembre. En agosto era el genoma de *Helicobacter pylori* el que aparecía secuenciado y en octubre los de *Bacillus subtilis* y *Archaeoglobus fulgidus*, un miembro del dominio Archaea y primer microorganismo metabolizador del azufre secuenciado. Con éstas, son trece las secuencias completas de microorganismos publicadas (doce procariotas y *Saccharomyces cerevisiae*). Han aparecido, principalmente, en las revistas *Nature*, *Science*, *DNA Research* y *Journal of Bacteriology*.

El año 1997 ha visto desaparecer figuras muy destacadas en el desarrollo de la biología y la medicina. El 8 de enero moría Melvin Calvin, Premio Nobel de Química en 1961 por sus estudios sobre los mecanismos de la fotosíntesis. El 22 de mayo moría Alfred D. Hershey, que aportó grandes conocimientos sobre los mecanismos de reproducción de los virus y estableció la función del DNA como molécula de la herencia. Compartió el Nobel de Fisiología o Medicina con Max Delbrück y Salvador E. Luria en 1969. Sir John C. Kendrew falleció el 23 de agosto; había compartido con Max Perutz el Premio Nobel de Química, en 1962, por sus estudios sobre las hemoproteínas.

Entre los premios concedidos en 1997 y que se relacionan con algún aspecto de la microbiología, podemos destacar el importante Premio de la Fundación Asashi Glass, de Tokyo, para el químico atmosférico James E. Lovelock, por su contribución a la solución de los problemas ambientales a escala global. El Premio Nobel de Fisiología o Medicina ha recaído este año en Stanley Prusiner, por su contribución a la identificación del agente causante de la encefalopatía contagiosa espongiforme. Prusiner describió los priones en 1982 y mantiene la hipótesis de que estas partículas proteicas son los agentes causantes de esa enfermedad. Es preciso destacar, sin embargo, que se ha concedido el Premio a una hipótesis que todavía no se ha podido demostrar por completo.

Sobre la revista de la SEM, se sigue manteniendo el optimismo en cuanto a la recepción de material. Van llegando artículos de investigación y contribuciones en cantidad y calidad ascendente, un total de 102 este año. No sucede lo mismo con el esperado aumento de recursos por publicidad, de lo cual podemos hablar de fracaso sin paliativos. Es éste un factor limitante a la expansión de la revista. En el año que acabamos, los cuatro números del volumen 13 comprenden un total de 560 páginas, con 42 artículos de investigación y revisiones, 20 artículos de contribuciones u opinión, y 32 reseñas de libros. Ha habido muchas citas a nuestra revista y una petición por parte de la Israel Society of Microbiology para reproducir en su *ISM News* el artículo de Harlyn O. Halvorson "Two generations of spore research: from father to son" (*Microbiología SEM* **13**, 131–148), y también dos contribuciones sobre Thomas S. Kuhn y Ludwick Fleck publicadas en nuestra revista.

Hay que destacar el interés que han despertado los ocho editoriales dedicados a América Latina. Han sido colaboraciones muy apreciadas, de americanos del norte y del sur, que se han prestado gustosos a analizar el proceso que ha seguido el desarrollo de la ciencia en sus distintos campos. Ofrecen una visión crítica y realista, que a nuestro entender es la mejor forma de encarar los problemas a los que el avance científico tiene que enfrenterse ante realidades complejas. El análisis es esperanzador por: (1) el potencial humano, joven y seguro de la necesidad de una formación científica competente; (2) los inmensos recursos naturales, cuyo estudio puede ser enormemente beneficioso para el conocimiento científico y, por ende, para la implantación de una política higiénica, sanitaria y agrícola que beneficie a toda la población; (3) el deseo de mostrar al resto de la comunidad científica motivación, capacidad y rigor en los planteamientos y logros alcanzados en los distintos campos de la ciencia. Una consecuencia directa del tratamiento de este tema y de los contactos iniciados en relación con ellos, ha sido la invitación a participar en el II Taller sobre Publicaciones Científicas en América Latina (Guadalajara, México 27–29 noviembre 1997). La participación de nuestra revista en esta reunión, para la cual no se consiguió ningún tipo de ayuda oficial, sirvió para estrechar los contactos con diversas publicaciones científicas y con microbiólogos de esas tierras, tan cercanas a nosotros en el corazón.

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MICROBIOLOGÍA SEM **13** (1997), 405–412 **MINIREVIEW**

Trends in wine microbiology*†

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Summary

During the last few years many winemakers have started to use pure *Saccharomyces cerevisiae* strains, frequently isolated from their own geographical regions, to produce wines of more reproducible quality. This microbiological simplification has opened the way for the genetic modification of wine yeast strains. This review concerns the application of molecular techniques in oenology, not only from the point of view of the construction of recombinant strains but also for the study of the population dynamics of wine fermentations.

Key words: *Saccharomyces cerevisiae*, recombinant yeast strains, lactic/alcoholic fermentations, population dynamics, wine production

Resumen

Durante los últimos años muchos bodegueros usan cultivos puros de *Saccharomyces cerevisiae*, frecuentemente aislados de la propia región enológica, para producir vinos de calidad más reproducible. Esta simplificación microbiológica ha permitido aplicar técnicas de ingeniería genética a las levaduras vínicas. En este artículo se presenta una revisión de la aplicación de las técnicas de biología molecular en enología, tanto para la construcción de levaduras recombinantes como para el seguimiento de las dinámicas poblacionales durante la fermentación vínica.

*This article is dedicated to the memory of my friend and colleague José Antonio Pérez-González, who died on August 9, 1997.

†Lecture given in Barcelona in the 16th SEM National Congress (Barcelona, July 14–17, 1997), on the occasion of receiving the 6th Biennial Award of the Spanish Society for Microbiology (see p. 412).

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Selection of wine yeasts

Wine is one of the few fermented foods made from non-sterile raw materials. Indeed, the transformation of grape juice into wine is a complex microbiological reaction involving the sequential development of various yeast strains and lactic acid bacteria. Traditionally, wines have been produced by natural fermentations carried out by yeasts originating from both the grapes and the cellar. Members of the genera *Kloeckera* and *Hanseniaspora* are the predominant species on the surface of the grape, but it is also possible to detect species of *Candida*, *Cryptococcus*, *Hansenula*, *Kluyveromyces*, *Pichia* and *Rhodotorula* in the initial must (8). Following three to four days of fermentation, *Saccharomyces cerevisiae* begins to predominate and is responsible for the alcoholic fermentation in the vinification process.

Several studies have revealed the occurrence of great variations in the quantity and distribution of microbial species on grapes. These variations are due to influences such as climatological conditions (19, 32), grape variety, the degree of grape maturity at harvest (36), whether fungicides have been used on the crop (40), and physical damage to the grapes (12, 19). Dissimilarities between most microbiota components are apparent when comparing not only different regions but also different vintages within the same region (25). For this reason, many winemakers started to use pure yeast cultures isolated within their own wine region (41). These cultures, in the form of active dry yeasts, are supplied to wineries, where they are inoculated into the fresh must to perform controlled fermentations. As a result of this practice successive vintages present organoleptical properties typical of the wines produced in that region (17). The selection of wine yeasts is based on different criteria (see Ref. 3, for an excellent review). For example, we selected a wine yeast strain from Alicante musts using a selection scheme based on the following: (i) the production of killer factor, (ii) the nature of the fermentation profile, and (iii) the sensorial properties of the wine produced (31). In many Spanish "Denominación Origen" sites (D.O.; equivalent to the French "Appelation Controlé") similar schemes of selection have been carried out. As a result, a collection of hundreds of wine yeast strains of biotechnological significance isolated from various Spanish D.O. has been constructed. This collection is currently kept at the Instituto de Fermentaciones Industriales (IFI, Madrid), a center depending on the Spanish Consejo Superior de Investigaciones Científicas (Council for Scientific Research, CSIC). CSIC is currently co-ordinating this collection with the Spanish Type Culture Collection to maintain and increase their capabilities.

Use of molecular biology techniques to understand the population dynamics of wine fermentations

There are two main reasons of interest for characterizing wine yeast strains: firstly to facilitate quality control during the production of the dry yeast; secondly, to provide a means to ensure that the fermentation process is actually conducted by the inoculated yeast. Most wine yeast strains belong to the *S. cerevisiae* group and cannot be readily distinguished by classical microbiological methods. Several molecular techniques have recently been employed to try to identify different strains: DNA–DNA hybridization (42), analysis of chromosomal DNA profiles (13, 22, 43), mitochondrial DNA (mtDNA) restriction analysis (21, 31, 44), and analysis of random amplified polymorphic DNA by the Polymerase Chain Reaction (PCR) (11, 34). A com-

parative study of these methods has shown that mtDNA restriction analysis is an excellent option as it is relatively easy to perform, inexpensive and does not require highly specialized equipment. As a consequence, a simplified method for mtDNA restriction analysis has been developed (29) which is rapid, reproducible and able to differentiate yeast strains isolated from the same must (Fig. 1).

Using the mtDNA restriction method, the population dynamics of natural and inoculated wine fermentations have been studied (28, 30). Considerable diversity of wild *S. cerevisiae* strains was detected in initial musts (from 40 to 60 strains, depending on the fermenter) but only some strains were present in all samples at significant numbers (28). So, must is a natural reservoir of *S. cerevisae* strains, but making wine is done by only a few of them. In natural fermentations, when mtDNA restriction pattern similarities between the initial *S. cerevisiae* strains (measured as the proportion of shared mtDNA restriction fragments) were low, a clear sequential substitution of predominant strains during each fermentation phase was observed. When the similarity was high, although a sequential substitution could also be observed between secondary strains, a clearly predominant strain was present during the whole fermentation process (30). In inoculated fermentations, an imposition of the inoculated strain at frequencies varying from 60 to 90% (depending on the fermenter) was detected (28). Note that the imposition of the inoculated strain does not suppress significant development of the natural strains over the first stages of the fermentation process, when they can exert beneficial effects on wine flavour (28).

The use of mtDNA analysis has also been applied to the study of other special vinification processes, such as the biological ageing of sherry wines. The occurrence of a single and stable strain in the film of individual barrels was identified, but this strain varied between barrels (15). The mtDNA technique has been used also to examine the correlation between the genetic relatedness of different *S. cerevisiae* strains and ecological, geographical factors (14), and also to study the evolution of *S. cerevisiae* strains isolated from the cellars of various vineyards, over a period of six years (45).

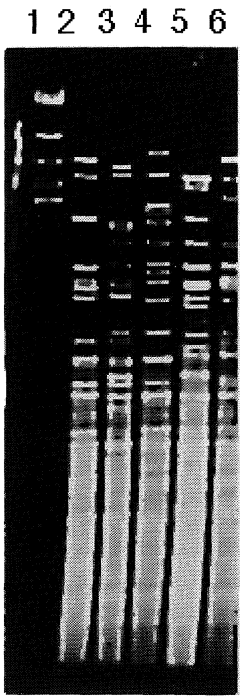

FIG. 1. mtDNA restriction analysis of different winer yeast strains (lanes 2–6). Lane 1: molecular weight markers.

Our knowledge of the microbiology of vinification processes has increased dramatically during the last few years, due to the use of molecular techniques.

Genetic engineering of wine yeasts

The use of active dry yeasts and the formal demonstration of their imposition during the

TABLE 1. Examples of recombinant wine yeast strains

Expressed gene	Reference
Aspergillus niger α-L-arabinofuranosidase	37
Candida molischiana β-glucosidase	38
Fusarium solani pectate lyase	10
Lactobacillus casei L(+)-lactate dehydrogenase	6
Lactococcus lactis malic enzyme	46
Schizosaccharomyces pombe malate permease	46
Saccharomyces cerevisiae K1 toxin	2
Trichoderma longibrachiatum endoglucanase	23

vinification process, together with advances in yeast biotechnology, open the way for the genetic modification of active dry yeasts. The construction of strains that express metabolic activities which exert beneficial effects on the organoleptical characteristics of wines is also possible. Genetic modification of wine yeast strains requires knowledge of the gene promoters that are specifically activated under fermentation conditions. For this reason, several laboratories have started projects aimed at understanding the regulation of gene expression in *S. cerevisiae* during wine production. Besides, different promoters that are specifically induced during exponential or stationary-phase growth are now available (24, 26, 35). In addition, a transformation protocol that yields GRAS (generally recognized as safe) recombinant wine yeasts has been developed recently (27).

Using various strategies, several different recombinant wine yeast strains have been generated (Table 1, for a review, see Ref. 33). Wine yeast strains that offer environmental advantages because they contain both genes encoding the K1 and K2 killer toxins was reported some years ago (2); a laboratory *S. cerevisiae* strain that expresses the L(+)-lactate dehydrogenase gene from *Lactobacillus casei* has also been constructed (6). The latter strain is able to perform a mixed lactic acid/alcoholic fermentations and can be used to increase the acidity of wines of naturally low acidity, a frequent problem in hot oenological regions.

Apart from the alcoholic fermentation, malolactic fermentation is also a major step in the production of most red wines and some white wines in cold regions (20). This fermentation is normally conducted by lactic acid bacteria, mainly *Oenococcus oenos*, and decreases the acidity of wine by virtue of the decarboxylation of L-malic acid to L-lactic acid. The reaction is catalyzed by the so-called "malolactic enzyme", which has been purified from several lactic acid bacteria. The gene coding for this enzyme has been cloned from *Lactobacillus delbrueckii* (48), *Lactococcus lactis* (1, 4) and *O. oenos* (18). *S. cerevisiae* strains cannot metabolize malate in grape must efficiently; genetic engineering of this microorganism to perform alcoholic and malolactic fermentations simultaneously has been explored for several years. Expression of different genes coding for the malic enzyme in yeast strains produces an inefficient malic fermentation due to the absence of an active transport system for malate (5). Recently, however, an efficient pathway for malate degradation has been introduced into a laboratory strain of *S. cerevisiae* (46, 47). The recombinant strain contains both a *Schizosaccharomyces pombe* gene coding for malate permease and the *L. lactis* gene encoding the malic enzyme, and is able to ferment 4.5 g/l of malate in synthetic grape must

in only four days. As one of the most prestigious scientists in the field recognized in a recent editorial: "it is now a great pleasure to behold the final product of this gleam in the eye of the oenologists" (16).

Finally, another interesting example of the use of recombinant DNA technology in oenology is the construction of wine yeast strains that express genes coding for cellulases and hemicellulases. The addition of these enzymes to solve filtration problems or to increase aroma is a frequent practice in wineries, but the heterogeneity of the available commercial preparations (a mixture of enzymes that varies batch to batch) has made their use unpredictable. Recently we constructed several recombinant wine yeast strains containing yeast or filamentous fungal genes coding for α-L-arabinofuranosidases, β-glucosidases, endo-β-(1,4)-glucanases, or endo-β-(1,4)-xylanases (23, 37, 38, unpublished results). All such strains are capable of secreting the corresponding enzymes to the must in sufficient amounts to carry out the technological process, and then produce wine with adequate oenological properties. In addition, a recombinant wine yeast strain containing a fungal gene coding for a pectate lyase has been constructed to address filtration problems (10). With regard to possible future developments, these various strains can be used as tools to investigate the role of each enzyme with a view to constructing recombinant fungal strains that produce combinations of enzymes for application in controlled vinifications (7, 9, 39).

Conclusions

It is obvious that a new era in microbial oenology is emerging. The recent approval of the EU regulation for novel foods opens the way to commercialize recombinant wine yeast strains. As a preliminary step, the Office International de la Vigne et du Vin (OIV) organized a meeting in Paris to discuss the industrial use of these strains. Another meeting of that kind will be held at the end of this year in Buenos Aires. The results obtained so far using molecular biology techniques can be summarized as follows: (i) an increase in our knowledge of the wine fermentation process, and (ii) the construction of new yeast strains that have consistent effects on the organoleptic characteristics of the resulting wines. Unexpected five years ago, can oenology close its eyes to this revolution?

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SEM Biennal Prize

The Spanish Society for Microbiology (SEM) Biennal Prize comes back to 1983, when our Society decided that a lecture was given by a young researcher at each SEM National Congress. The nominates would be elected among the SEM membership; they should be under 40, and should have carried out research of excellence in some microbiology field.

The researchers awarded with the SEM Biennal Prize have been the following (the centers indicated are those where the scientist worked when they received the Prize):

- First: **Juan Ortín Montón**, Center for Molecular Biology (CBM), Autonomous University of Madrid (10th SEM National Congress, Valencia,1985)
- Second: **Enrique Herrero Perpiñán**, Department of Microbiology, University of Valencia (11th SEM National Congress, Gijón, 1987).
- Third: **Ernesto García López**, Center for Biological Research (CIB), CSIC (12th SEM National Congress, Pamplona, 1989)
- Fourth: **Antonio Ventosa Ucero**, Department of Microbiology, University of Sevilla (13th SEM National Congress, Salamanca, 1991)
- Fifth: **Alicia Estévez Toranzo**, Department of Microbiology, University of Santiago de Compostela (14th SEM National Congress, Zaragoza, 1993)
- Sixth: **Sergio Moreno Pérez**, Department of Microbiology, University of Salamanca (15th SEM National Congress, Madrid, 1995)
- Seventh: **Daniel Ramón Vidal**, Department of Biotechnology, Institute for Agrochemistry and Food Technology (IATA), CSIC (16th SEM National Congress, Barcelona, 1997). The text of his lecture is published in this issue of *Microbiología SEM* (pp. 405–411)

MICROBIOLOGÍA SEM **13** (1997), 413–426 **MINIREVIEW**

Microbiological quality of natural waters

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Summary

Several aspects of the microbiological quality of natural waters, especially recreational waters, have been reviewed. The importance of the water as a vehicle and/or a reservoir of human pathogenic microorganisms is also discussed. In addition, the concepts, types and techniques of microbial indicator and index microorganisms are established. The most important differences between faecal streptococci and enterococci have been discussed, definying the concept and species included. In addition, we have revised the main alternative indicators used to measure the water quality.

Key words: index and indicator organisms, faecal bacteria, most probable number, coliphages, water quality

Resumen

En este artículo se revisan diferentes aspectos de la calidad microbiológica de las aguas naturales, con especial referencia a aquellas que se utilizan con fines recreativos. La importancia del agua como vehículo y/o reservorio de patógenos humanos, los diferentes indicadores de la calidad del agua, así como las técnicas de análisis también han sido discutidas. Se han establecido las diferencias más sobresalientes entre estreptococos fecales y enterococos, definiéndose el concepto y las especies incluidas. Se revisan los principales indicadores alternativos utilizados para determinar la calidad del agua.

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Introduction

A major aspect of aquatic microbiology is the acquisition of several human diseases transmitted by the water route. The significance of human morbidity and mortality associated with waterborne infectious disease has led to the design and development of epidemiological surveillance studies (33).

Most human diseases associated with microbiologically polluted waters are produced by pathogenic microorganisms that include bacteria, viruses and protozoa, which are discharged into the aquatic environments through faecal contamination from infected persons or animals. The most relevant waterborne infectious diseases are summarized in Table 1.

The main reservoirs for pathogenic microorganisms present in natural waters can be either humans, animals, or the environment itself (23). Sewage seems to be a major source of pathogenic microorganisms found in aquatic environments. Wastewater treatment processes significantly reduce the incidence of illness among the human population, especially in the case of bacterial diseases; however, protozoa cysts and many pathogenic viruses are very resistant to the chemical, physical treatment processes (29).

Numerous infectious agents have been transmitted by ingestion, contact, or inhalation of water, and the main diseases associated with waterborne infections include gastroenteritis, hepatitis, skin lesions, wound infections, conjunctivitis, otitis, respiratory infections, and generalized infections (40). Several factors are critical in the transmission of pathogenic microorganisms through the water route, such as: (i) source of the infectious agents; (ii) specific water-related modes of transmission; (iii) survival and multiplication capabilities of the pathogens into the aquatic environment; (iv) infectious dose and virulence factors of the microorganisms; and (v) host susceptibility factors.

A major factor determining microbial health-hazard associated with the water route is the infectious dose of the pathogenic microorganism. The infectious dose (ID) is defined as the minimum number of organisms required to cause infection and it varies considerably according to the type of organism. In general, viruses and protozoa have low ID, typically from 1 to 50 PFU or cysts (39), whilst bacterial pathogens possess ID_{50} (number of organisms resulting in a 50% infection rate) ranging from 10^2 to 10^8.

Some agents of waterborne diseases, such as *Legionella* spp., *Vibrio* spp., *Aeromonas hydrophila* and *Pseudomonas aeruginosa* are indigenous of aquatic environments; therefore, infections caused by these microorganisms will depend on the exposure to natural waters. However, most microbial waterborne pathogens of concern originate in the enteric tracts of humans and animals, and enter the aquatic environment via faecal contamination. The concentration of these pathogens in natural waters will depend on the number of infected persons and/or asymptomatic carriers in the community and the effectiveness of the wastewater treatment before the discharge. In addition, the dilution and the natural self-depuring capability of the receiving waters will also reduce the concentration of contaminant microorganisms.

Epidemiological and microbiological studies indicate that *Staphylococcus aureus* skin and ear infections are often associated with recreational use of water, and the source of these organisms may be other bathers or the water itself (39). *Vibrio vulnificus* and *Aeromonas* spp. can cause serious wound infections when an injury to skin occurs in marine and freshwater, respectively (32, 34). Cyanobacterial toxins have been associated with contact irritation after bathing in marine or freshwater environments (11). An

TABLE 1. Pathogenic microorganisms associated with water health-hazards acquired by ingestion and by contact with recreational water

Causative agent	Disease/syndrome	Source
BACTERIA		
Vibrio cholerae O1	Cholera	Human faeces
Non-O1 *V. cholerae*	Diarrhoea	Human faeces
V. vulnificus	Gastroenteritis/wound infections	Marine water
V. parahaemolyticus	Gastroenteritis/wound infections	Marine water
Salmonella spp.	Gastroenteritis	Human and animal faeces
Salmonella typhi	Typhoid fever	Human faeces and urine
Shigella dysenteriae	Bacillary dysentery	Human faeces
Yersinia enterocolitica	Bloody diarrhoea	Animal faeces and urine
Enterohaemorrhagic *E. coli*	Bloody diarrhoea	Human and animal faeces
Enteroinvasive *E. coli*	Dysentery	Human faeces
Enteropathogenic *E. coli*	Diarrhoea	Human faeces
Enterotoxigenic *E. coli*	Diarrhoea	Human faeces
Campylobacter jejuni	Acute gastroenteritis	Human and animal faeces
Aeromonas hydrophila	Diarrhoea/wound infections	Freshwater
Plesiomonas shigelloides	Bloody diarrhoea	Freshwater, fish
Legionella spp.	Legionnaires' disease	Freshwater, aerosols
Mycobacterium spp.	Skin and subcutaneous lesions	Marine and freshwaters
Pseudomonas aeruginosa	Dermatitis/ear and eye infections	Faeces, water, humans
Staphylococcus aureus	Dermatitis/ear and eye infections	Humans
Leptospira icterohaemorrhagica	Leptospirosis	Animal urine
Cyanobacteria	Dermatitis/toxin poisoning	Cyanobacterial blooms
VIRUSES		
Norwalk and Norwalk-related	Acute gastroenteritis	Human faeces
Rotavirus (groups A and B)	Acute gastroenteritis	Human faeces
Hepatitis A virus	Hepatitis	Human faeces
Hepatitis E virus	Hepatitis	Human faeces
Astrovirus	Acute gastroenteritis	Human faeces
Calicivirus	Acute gastroenteritis	Human faeces
Enteroviruses (polio, echo, coxsackie)	Respiratory illness/encephalitis/ paralytic disease/meningitis/ diarrhoea	Human faeces, shellfish
Adenovirus (serotypes 1,3,4,7,14)	Conjuctivitis/pharyngitis	Humans
PROTOZOA		
Naegleria spp.	Meningoencephalitis	Freshwater
Balantadium coli	Bloody diarrhoea	Human and animal faeces
Cryptosporidium spp.	Diarrhoea/dysentery	Human and animal faeces
Entamoeba histolytica	Dysentery	Human faeces
Giardia lamblia	Giardiasis	Human and animal faeces
Acanthamoeba spp.	Conjunctivitis/skin lesions	Water

additional cause of recreational water infections are *Leptospira* species and Adenoviruses, which are neither enteric nor aquatic organisms (40).

In addition, other water-related infections involve the transmission of infectious agents by inhalation of water aerosols and by the consumption of raw or undercooked contaminated shellfish. The major pathogens associated with aerosol transmission are *Legionella pneumophila* and nontuberculous mycobacteria. Bivalve shellfish have served as vehicles of enteric disease transmission because of their ability to concentrate enteric organisms from faecally contaminated water in their tissue. Numerous outbreaks have been attributed to the consumption of raw bivalves. These infections can be caused by hepatitis A and E viruses, Norwalk-related viruses, pathogenic *Escherichia coli*, *Salmonella typhi*, and species of *Vibrio*, *Shigella*, *Plesiomonas* and *Aeromonas* (25).

Index and indicator organisms

Natural waters are subject to important changes in their microbial quality due to occasional discharges of sewage or wastewater as a result of human activity or storm-water run off. Sewage effluents contain a wide range of pathogenic microorganisms, that may pose a health-hazard to the human population when they are discharged into recreational waters. The density and variety of these pathogens are related to the size of the population, the seasonal incidence of the illness, and the dissemination of pathogens.

Many waterborne pathogens are difficult to detect and/or quantify in water, and the optimal specific methodology to detect them in environmental water samples has still to be developed. The specific detection procedures currently available are complex, and they involve concentration processes and subsequent selective enrichment or amplification by molecular biology methods. In addition, the recovery efficiency of the procedures is low and these techniques are limited to specialized research laboratories. A recent revision of the methodologies developed for the detection and/or enumeration of pathogenic microorganisms from waters has been carried out by different authors (12).

Indicator organisms (5) must fulfill the following requirements: (i) Indicators should be present in faeces, sewage and sewage-polluted waters whenever the pathogens are also present, and absent in waters without faecal pollution. (ii) The levels of indicators should have some direct relationship to the density of pathogens in aquatic environments, and be proportional to the extent of the pollution. (iii) Indicators survival rate in water must be at least similar to that of pathogens. In addition, the resistance of indicators to depuration factors and disinfectants should be at least similar to that of the pathogens. (iv) Indicators must be detectable by a simple, inexpensive, accurate, rapid laboratory methodology. (v) Indicators should be non-pathogenic and applicable to all types of water samples that require a monitoring programme. (vi) Their characteristics must be constant. And (vii) indicators must not grow and multiplicate in water.

Mossel (42) defined the term "model organism" which refers to two different functions, index and indicator. "Index organisms" are related, directly or indirectly, either to the health-hazards or to the pathogen presence, and they must fulfill the criteria given in Table 2. On the other hand, "indicator organisms" are related only to the effects of treatment processes or control of water quality. 'Indicator organisms' requirements are less restrictive than those for an index organism: their resistance to the environment must be only similar to that of pathogens and they require more simple laboratory methodologies.

TABLE 2. Criteria for "index" and "indicator" microorganisms

Criteria	Index	Indicator
I. Similar ecology	Yes	No
Presence in human faeces		
Absence in animal faeces		
Detection in polluted waters with human faeces		
Absence in polluted waters with animal faeces		
No multiplication in water environment		
Constant characteristics		
II. Similar resistance and persistence	Yes	Yes
To wastewater treatments		
To disinfection processes		
Survival in marine and freshwaters		
Resistance to sun light		
Relationship with human enteric viruses		
Related to human health-hazards		
III. Simple methodology	Yes	Yes
No specialized installations are required		
Inexpensive		
Rapid		
No pathogen		
Processing of high volumes of water is needed		

The use of "index" or "indicator" organisms to assess the microbiological and sanitary quality of waters is well established and has been practiced for almost a century. Classically, the microbiological quality of waters has been measured by the analysis of indicator microorganisms. The most widely used groups are total coliforms, faecal coliforms, and faecal streptococci.

Total coliforms. They are aerobic and facultatively anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid production in 24–48 h at 36 ± 1°C. Although coliform bacteria belong to the family Enterobacteriaceae, and usually include *E. coli* as well as various members of the genera *Enterobacter, Klebsiella* and *Citrobacter*, the "total coliform" definition is not based on strictly taxonomic criteria, but on specific biochemical reactions or on the appearance of characteristic colonies on selective and/or differential culture media. However, the recent advent of enzyme-specific media and tests has allowed the application of cytochrome oxidase (negative) and β-galactosidase (positive) as additional criteria for the coliform group.

These bacteria have been classically used as indicators of water pollution from sewage and thus they are of sanitary significance. However, several authors have pointed out that some members of the coliform group can originate from non-enteric environments, such as wastes from the wood industry and surfaces of redwood water tanks, biofilms within drinking water distribution systems, and epilithic algal mat communities in pristine streams. The persistence of these bacteria in supply water systems can be compared to that of some waterborne bacterial pathogens, although they are much less persistent than viruses and protozoa. In the case of natural aquatic environments, particularly ma-

rine waters, total coliforms present a survival rate lower than that of pathogenic microorganisms (41). In addition, there are serious shortcomings for the general use of these microorganisms as indicators, such as the ability of some coliforms to grow in natural waters; the low relation between the numbers of coliforms and those of pathogenic microorganisms in natural waters; and the detection of atypical strains of coliforms in water (4). Recent studies have also suggested the low capability of these microorganisms as indicators for bathing waters (20).

Faecal coliforms and *E. coli*. Faecal coliforms are the subset of total coliforms that are more closely related with homeothermic faecal pollution. These bacteria conform to all criteria used to define total coliforms, but in addition they grow on lactose and ferment it with production of gas and acid at 44.5 ± 0.2°C within the first 48 h of incubation. For this reason, the term "thermotolerant coliforms" may be more appropriate to name this group.

Thermotolerant coliforms comprise strains of the genera *Klebsiella* and *Escherichia*, that are shown to correlate with faecal contamination from warm-blooded animals (43). The physiological basis of the high temperature phenotype in faecal coliforms has been described as a thermotolerant adaptation to the temperatures found in the enteric tracts of animals, which lies on a higher stability of proteins to heat. Dufour (18) suggested the redefinition of the faecal coliform group with the addition of several biochemical characteristics, such as their impossibility of using citrate as the only carbon source, the presence of tryptophanase activity and the lack of urease activity, the negative Voges–Proskauer test, and the positive Methyl Red test (positive). These characteristics are found almost exclusively in *E. coli*, which is a most useful microbial indicator of water quality because it is more specific for the presence of faecal contamination that the faecal coliform group. It can be easily distinguished enzymatically by the lack of urease and the presence of β-glucuronidase, enzymes that form the basis of recently developed differential methods (22).

However, several studies indicate the limitation of faecal coliforms as ideal indicators or index organisms. Several thermotolerant *Klebsiella* strains have been isolated from environmental samples with high levels of carbohydrates in the apparent absence of faecal pollution. Similarly, other members of the faecal coliform group, including *E. coli* , were detected in some pristine areas (45) and associated with regrowth in potable water distribution systems (37). Therefore, the disadvantages of using *E. coli* as an indicator in water are: (i) its detection from other environments without faecal contamination, and (ii) its low survival capability in aquatic environments (41).

Faecal streptococci and enterococci. Faecal streptococci have received widespread acceptance as useful indicators of faecal pollution in natural aquatic ecosystems, since (i) they show a high and close relationship with health-hazards associated with bathing in marine and freshwater environments, mainly for gastrointestinal symptoms; (ii) they are not as ubiquitous as coliforms are; (iii) they are always present in faeces of warm-blooded animals; (iv) they are unable to multiply in sewage-contaminated waters; and (v) their die-off is less rapid than coliforms decline in seawater, and their persistence patterns are similar to those of potential waterborne pathogenic bacteria (9, 33, 46, 47).

The main problem derived from the universal use of these microorganisms is the recognized lack of a standard methodology for their selective enumeration from natural waters. More than 70 media have been proposed for this purpose until now. However, a standard methodology has not been yet adopted, since few studies have been conducted on their comparative recovery efficiency from water samples (17).

An additional problem related to the selective enumeration of this group of microorganisms is their taxonomic, ecological heterogeneity. The group called "faecal streptococci" comprises species of different sanitary significance and survival characteristics; in addition, the proportion of the species of this group are not the same in animal and human faeces (43). Therefore, a clearer definition of faecal streptococci is necessary to establish a specific standard methodology of enumeration. The taxonomy of this group has been subject to extensive revision recently (36); in our opinion, the following species of the *Enterococcus* and *Streptococcus*, on the basis of their taxonomical inclusion and the exclusively faecal origin of the species: *Enterococcus faecalis*, *E. faecium*, *E. durans*, *E. hirae*, *E. avium*, *E. gallinarum*, *E. cecorum*, *Streptococcus bovis*, *S. equinus*, *S. alactolyticus*, *S. intestinalis*, *S. hyointestinalis* and *S. acidominimus* (3, 19).

Enterococci include all species described as members of the genus *Enterococcus*, which also fulfill Sherman's criteria: growth at 10°C and 45°C, resistance to 60°C for 30 min, growth at pH 9.6 and at 6.5% NaCl, and the ability to reduce 0.1% methylene blue.

Alternative indicator organisms. None of the above mentioned bacterial indicators used to establish recreational standards fulfill all the criteria desirable for an ideal indicator, and most are more susceptible to environmental conditions than are pathogenic microorganisms (13), and viruses. As a result, many other microorganisms are being evaluated as alternative indicators of water quality.

– Sulphite-reducing clostridia: The use of the sulphite-reducing members of the genus *Clostridium* (*C. perfringens*/*C. welchii*) as indicators of faecal pollution was originally proposed in 1890 by Houston, but until 1930 they were not used as indicators of water quality in certain specific situations, for example in the control of drinking-water treatment and in the microbial quality of groundwater (21, 50).

C. perfringens has been proposed as a water quality indicator because (21): (i) it is consistently present in the faeces of all warm-blooded animals as well as in sewage; (ii) it is more stable in environmental waters than most pathogens; and (iii) it has been successfully used to monitor sewage-contaminated waters. Nevertheless, sulphite-reducing clostridia are considered ubiquitous in aquatic sediments and several problems with their specific detection and recovery methodology limit their potential value as indicator organisms. Due to the great resistance and longevity of their spores, these bacteria can be useful indicators of remote faecal pollution (50).

– *Staphylococcus aureus*: The results of several epidemiological studies show higher incidence of non-diarrhoeal diseases, such as eye, ear, mucous and skin infections. In this regard, many swimmers acquire staphylococcal skin infections after swimming in recreational waters (39), and thus, there is a need for an indicator of water quality which addresses skin infections rather than gastrointestinal infections.

Evidence to support the use of *S. aureus* as indicator of water quality includes the following: (i) this microorganism is stable and shows a great resistance to environmental conditions, especially in marine waters; (ii) its concentration in water has been shown to represent the load of microorganisms being shed by swimmers; and (iii) methods for its specific recovery from marine waters are now available (7, 10).

– *Pseudomonas aeruginosa*: Members of the genus *Pseudomonas* are frequently isolated from aquatic environments. Therefore, their presence does not necessarily imply a possible risk to public health. However, *P. aeruginosa* has been linked to infections associated to exposure to recreational waters and thus it has been

proposed as an indicator of recreational water quality (2, 39).

P. aeruginosa was found to be more resistant than other indicators and pathogenic microorganisms to treatment processes of water, and to environmental factors. These findings demonstrate the usefulness of *P. aeruginosa* as an indicator in analysis of recreational waters such as swimming-pools and whirlpools.

– Bacteriophages: The use of bacteriophages as indicators is not a new concept, since they were proposed as faecal and viral indicators by Kott (35).

The term bacteriophages is a wide concept; the bacteriophage groups proposed as indicators are somatic coliphages, F-specific RNA bacteriophages, and phages of *Bacteroides fragilis* (15, 31, 38, 51).

Somatic coliphages are specific bacteriophages of *E. coli* , and have been the phage group most studied. They are commonly used as indicators of faecal and/or sewage pollution, on the basis of their direct correlation with the presence of enteric viruses in marine waters, freshwater ecosystems and sewage effluents. Recently, Borrego (6) reviewed the indicator potential of somatic coliphages. He concluded that considering the differences in origin and ecology between enteric viruses and somatic coliphages, it is doubtful that this phage group could be used successfully in all situations as viral indicators; however, they are good indicators of faecal pollution and of the microbiological water quality.

Several authors have proposed other bacteriophage groups as alternative indicators. Primrose et al. (44) proposed the use of F(male)-specific RNA bacteriophages (FRNA phages) as faecal pollution indicators, on the basis of their inability to replicate in the water ecosystem, since fimbria are not synthesized below 30°C. In 1984, Havelaar and Hogeboom (27) developed a method for the detection of this bacteriophage group, and proposed them as model viruses in water hygiene, because: (i) the group comprises viruses similar in size, shape, and genetic makeup to human enteric viruses, which are responsible for most of waterborne diseases; (ii) it represents viruses which are more stable than human enteroviruses in environmental waters and more resistant to disinfection; and (iii) the viruses concentrations in environmental waters have been reported to correlate with sewage contamination.

The low incidence of the FRNA phages in faeces, and human faeces in particular (28), and their high concentration in sewage, suggests that FRNA phages must be able to multiply in the sewage system (30). Hence, the presence of FRNA phages in water is primarily an index of sewage pollution rather than just of faecal pollution (30). FRNA phages may be more appropriate to be used as simulators for the environmental behaviour of enteric viruses (e.g. Norwalk) in water and wastewater treatment than as general indicators of faecal pollution.

Analytical methods

Most Probable Number (MPN). MPN analysis is a statistical method based on the random dispersion (Poisson) of microorganisms per volume in a given sample. Classically, this assay has been performed as a multiple-tube fermentation test. Although the technique is rather time-consuming (5–7 days), there are laboratories thet prefer it to other methods of water analysis, because it is applicable to all sample types.

The MPN technique is generally conducted in three sequential phases (presumptive, confirmatory, and complete), each phase requiring 1 to 2 days of incubation. In the presumptive phase, three volumes of samples (usually 10, 1, and 0.1 ml) are inoculated into 3, 5, or 10 tubes

containing the appropriate medium to allow the target bacteria to grow. In this test, it is assumed that any simple viable target organism will result in growth or a positive reaction in the medium.

After the incubation period, all the inoculated presumptive positive tubes must be inoculated into a more selective medium to confirm the presence of the target bacteria (confirmatory phase). The confirmed test is reliable evidence but not proof that the target bacteria have been detected. Therefore, subsamples of the confirmed positive reactions should be inoculated onto a selective agar medium, and several verification tests (Gram stain, and biochemical, serological or enzymatic tests) should be carried out. This completed test is generally conducted on 10% of the positive confirmed samples as a quality control measure. For practical purposes, the number of positive and negative tubes in the confirmed phase of the technique is generally used to determine the MPN of the target bacteria by using the tables of positive and negative tube reactions given in Standard Methods (2).

The major advantages of the MPN technique are the following: (i) it will accept both clear and turbid samples; (ii) it inherently allows the resuscitation and growth of injured bacteria; (iii) the results may be recorded by personnel with minimal skill; and (iv) minimal preparation time and effort are required to start the test, and therefore processing of samples can be initiated at any time of the day. On the contrary, the MPN technique may have several disadvantages, such as: (i) the total time, labour, material, and costs required to analyse one sample; (ii) the substantial increase in reagents, tubes, incubation space and cleanup requirements when multiple samples need to be analysed or when the sample volume must be increased to 100 ml; (iii) the multiphase nature of the technique, each phase requiring 24 or 48 h incubation period; and (iv) the fact that MPN is a simple estimated number, while the true number (95% confidence limit) may show extreme variation from the MPN.

Membrane filtration (MF). MF technique is the most widely used method in microbiological water analyses. The technique is based on the entrapment of the bacterial cells by a membrane filter (pore size of 0.45 μm). After the water is filtered, the membrane is placed on an appropriate medium and incubated. Discrete colonies with typical appearance are counted after 24 to 48 h, and population density of the target bacteria, usually described as colony forming units (CFU) per 100 ml, in the original sample, can be calculated from the filtered volumes and dilutions used. This technique is more precise than MPN, but MF test presents the limitation of its exclusive use for low-turbidity waters with low concentrations of background microorganisms.

P–A test. This test was designed for the microbiological monitoring of drinking-water, in which the presence or absence (P–A) of indicator bacteria in a standardized volume (i.e., 100 ml) of water is determined, using a liquid medium. One of the major advantages of this approach is that it is easy to perform and permits to analyze greater numbers of samples.

Although the P–A test can yield valuable information on the prevalence of microbiological problems, it has a drawback, for this approach fails to provide data on the magnitude of such occurrences. In addition, the common connotation of 'absence' may be misleading in the case of injured bacteria, which are frequent in treated drinking water systems or marine environments and that fail to grow on established media.

Laboratory procedures

Total coliforms. Classically liquid media containing lactose, such as lauryl tryptose broth, lactose broth, or MacConkey broth, have been

used with the MPN technique for the detection of total coliforms in water samples. Positive reaction of the presumptive phase is the gas and acid production at 36 ± 1°C incubated for 48 h. Confirmation of presumptive reactions is done by inoculating the positive aliquots into brilliant green lactose bile broth tubes; gas production in these tubes after 48 h at 36 ± 1°C is interpreted as representing confirmed test. Bacterial density and the 95% confidence limits can be estimated with the use of MPN tables for the volumes and number of aliquots used (2).

The MF analysis of water for total coliforms can involve the use of the following media: LES-Endo agar, m-Endo agar, Tergitol agar, and Teepol agar, Endo media being the most commonly used. Characteristic colonies appear in Endo media as pink to dark red colonies with a metallic green sheen. Only plates containing between 20 and 80 typical colonies should be counted (2).

New technological innovations have been introduced in the analysis of total coliforms from waters, including the application of m-T7 medium that allows the detection of injured coliforms; the use of the enzymatic activity β-galactosidase in the Colilert and Coliquick tests; and the employment of molecular methods, such as PCR technique and gene probes for lacZ.

Faecal coliforms. EC and A-1 media are the most widely recommended for the presumptive test with MPN technique of faecal coliforms (2). The differences between them are based on the incubation periods, 44.5 ± 0.2°C for 24 h in the case of EC medium, and 36 ± 1°C for 3 h and transfer to 44.5 ± 0.2°C for 21 h in the case of the A-1 medium. The tubes containing gas and acid in EC medium are confirmed in the same medium by subsequent incubation at 44.5 ± 0.2°C for 24 h. A-1 medium does not require a confirmation test. Faecal coliform density and the 95% confidence limits can be estimated by using the MPN tables as described for total coliforms (2).

The most frequently medium used to quantify faecal coliforms in water samples when the MF technique is used is mFC agar. Petri dishes containing filters are incubated at 44.5 ± 0.2°C for 24 h. Typical faecal coliform colonies will appear with various lines of blue, although atypical *E. coli* may be pale yellow, whereas non-faecal coliform colonies are grey to cream. Only plates containing from 20 to 60 typical colonies should be counted (2).

As *E. coli* has been demonstrated to be a more specific indicator for the presence of faecal contamination than the faecal coliform group, improvements in both MPN and MF techniques have been carried out for the rapid and selective enumeration of *E. coli*, including a rapid 7-h membrane filter test for quantification of *E. coli* from water samples. Fluorogenic and chromogenic assays using 4-methylumbellipheryl-β-D-glucuronide (MUG) have been applied, in MPN and MF techniques, for the detection of β-glucuronidase that is specific of *E. coli* (22). Different media based on this principle have been developed for the use in MF, MPN, and P–A techniques. Commercially available media include Colisure (Millipore), Colilert (Idexx), m-ColiBlue (Hach), ColiComplete (BioControl), Chromocult (Merck), and MicroSure (Gelman). Similar media to detect *E. coli* in water have also been described. Molecular methods have also been designed to the specific detection of *E. coli* from water samples, such as PCR-gene probes for *uid*, enzymatic activities, enzyme capture, and radioisotopes.

Faecal streptococci and enterococci. Early attempts to quantify faecal streptococci relied on enrichment tube procedures associated with the use of the MPN technique, Rothe Azide Dextrose broth followed by a confirmation in Ethyl Violet Azide (Litsky) broth being the procedure most widely accepted by researchers. The enumeration of faecal streptococci by a MF proce-

dure using a selective medium was first reported by Slanetz and Bartley (48). Since then, several media have been proposed, including Thallous Acetate agar, KF agar, PSE agar, Kanamycin Aesculin Azide (KEA) agar, mSD agar, and mE agar. Other media formulations and incubation procedures for faecal streptococci have been proposed for specific situations, increasing the membrane incubation period from 48 to 72 h to recover stressed faecal streptococci. A new medium, designed as M2, has been designed to distinguish between human and animal pollution sources, and recently a rapid system for enumeration of faecal streptococci and enterococci from water samples using a miniaturized fluorogenic assay has been proposed (43).

The methodologies for enumeration of faecal streptococci from natural waters have been compared by different authors. Dionisio and Borrego (17) compared eight methods for the specific recovery of faecal streptococci from natural freshwater and marine waters on the basis of their following characteristics: accuracy, specificity, selectivity, precision and relative recovery efficiency. The results obtained indicated that none of the tested methods showed an optimal selectivity. The methods that showed the best performance characteristics were the MPN technique (with Rothe and Litsky media) and the m-*Enterococcus* agar in conjunction with the MF technique. The latter is the only method recommended in the *Standards Methods* for faecal streptococci in conjunction with membrane filtration (2). A rapid confirmation technique based on the transplantation of the membrane from m-*Enterococcus* agar to Bilis-Esculin-Azide (BEA) agar improves the low specificity of the m-*Enterococcus* agar, enabling the confirmation of 100% of the colonies (19).

Recently, Audicana et al. (3) designed and tested a modification of the KEA agar, named Oxolinic acid-Aesculin-Azide (OAA) agar, to improve the selectivity in the enumeration of faecal streptococci from water samples by MF. OAA agar showed specificity, selectivity and recovery efficiency higher than those obtained by using m-*Enterococcus* and KF agars. In addition, no confirmation of typical colonies was needed when OAA agar was used. This shortens significantly the time of sample processing (only 24 h) and increases the accuracy of the method. A standardization trial performed at European scale and funded by the EC, demonstrated that the m-*Enterococcus* agar with total confirmation of the colonies (19), the OAA medium (3), and the miniaturized MPN method produced the best results (43).

Somatic coliphages. Several methods have been advocated for the recovery of phages from aquatic habitats (1, 8). Depending on the level of pollution of the water sample, these methods may be comprised in two groups: (i) direct plaque assay from the sample, and (ii) concentration techniques. However, other authors have used different detection procedures based on physicochemical or structural properties of the phages, such as precipitation by polyethylene glycol, magnetic-organic flocculation, adsorption to hydroxylapatite, electron microscopy, and ultracentrifugation. (i) Direct plaque assays: The double-agar-layer (DAL) method is widely used for phage detection and enumeration. Havelaar and Hogeboom (26) described a single-agar-layer (SAL) method that yielded phage recovery comparable to that of the DAL method. In both procedures the sample (raw or decontaminated) is mixed with a log-growth adequate host culture, and then the mixture is poured onto Petri dishes with bottom agar. If phage is present in the sample, it will lyse its specific bacterial cells and will produce a clear spot (lysis plaque) on the bacterial lawn; then, the number of plaques on lawns will be a reflection of the phage concentration (expressed as plaque forming units,

PFU) in the sample. The amount of sample assayed by these procedures is necessarily small. Therefore, direct plaque assay methods are suitable when phages are present in high numbers. However, Kott (35) designed a MPN assay with a theoretical detection limit of 2 PFU per 100 ml of water sample, using 65 ml of sample. The most commonly used *E. coli* strains as host for coliphage recovery are *E. coli* C and *E. coli* K12. A rapid coliphage enumeration technique named ACART has two advantages regarding DAL and MPN techniques, its lower detection limit and faster analysis (6 h). Grabow and Coubrough (24) described a direct SAL plaque assay for the direct detection of coliphages in 100 ml of water samples. The theoretical minimum detection limit for this method is 1 PFU per 100 ml.

F-RNA specific bacteriophages. The specific methodology of this bacteriophage group consisted in the application of direct plaque assay using bacterial hosts that have the specific phage receptor (sex pili). Primrose et al. (44) used a mixture of *E. coli* K12 Hfr and *Salmonella typhimurium* LT2 as host system to avoid the recovery of somatic coliphages. Later, Havelaar and Hogeboom (27) developed a method for the detection of F-specific coliphages using a genetically manipulated *Salmonella* strain (*S. typhimurium* WG49) as bacterial host. This strain was constructed by the introduction of F'42 lac::tn5 plasmid into the *S. typhimurium* WG45 strain. Therefore the bacterium is able to produce sex pili but does not contain the receptor sites for coliphages. By using *S. typhimurium* WG49 with the DAL technique it is possible to isolate F-specific coliphages from water samples, and by differentiation on the basis of sensitivity to RNase in the assay medium, more than 90% were found to be RNA phages. DeBartolomeis and Cabelli (16) used a mutant *E. coli* strain (*E. coli* C3000, ATCC 15597) to recover F male specific bacteriophages. Finally, Sobsey et al. (49) developed and evaluated a simple membrane filtration method for concentrating and enumerating F-specific coliphages in natural and treated waters.

Phages of *Bacteroides fragilis*. Tartera and Jofre (51) compared the DAL technique and a modification of the MPN method using modified blood agar base and *B. fragilis* HSP 40 as host for the specific enumeration of these phages. Prior to the assay, the samples were decontaminated by filtration or by chloroform treatment. In addition, to prevent germination of spores, the samples were supplemented with potassium sorbate. In those conditions, these authors found that the DAL technique had a lower efficiency in the enumeration of *B. fragilis* phages.

Finally, Cornax et al. (14) developed a direct assay for *B. fragilis* phages which consisted in the culture of host bacteria in modified Brucella broth vials with an atmosphere of $CO_2 \pm N_2$, and in the use of modified blood agar as bottom agar layer. In the case of samples with a high degree of pollution, these authors suggested supplementing the medium with kanamycin and vancomyxin mixture.

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MICROBIOLOGÍA SEM **13** (1997), 413–426 **MINIREVIEW**

Microbiological quality of natural waters

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Summary

Several aspects of the microbiological quality of natural waters, especially recreational waters, have been reviewed. The importance of the water as a vehicle and/or a reservoir of human pathogenic microorganisms is also discussed. In addition, the concepts, types and techniques of microbial indicator and index microorganisms are established. The most important differences between faecal streptococci and enterococci have been discussed, definying the concept and species included. In addition, we have revised the main alternative indicators used to measure the water quality.

Key words: index and indicator organisms, faecal bacteria, most probable number, coliphages, water quality

Resumen

En este artículo se revisan diferentes aspectos de la calidad microbiológica de las aguas naturales, con especial referencia a aquellas que se utilizan con fines recreativos. La importancia del agua como vehículo y/o reservorio de patógenos humanos, los diferentes indicadores de la calidad del agua, así como las técnicas de análisis también han sido discutidas. Se han establecido las diferencias más sobresalientes entre estreptococos fecales y enterococos, definiéndose el concepto y las especies incluidas. Se revisan los principales indicadores alternativos utilizados para determinar la calidad del agua.

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Introduction

A major aspect of aquatic microbiology is the acquisition of several human diseases transmitted by the water route. The significance of human morbidity and mortality associated with waterborne infectious disease has led to the design and development of epidemiological surveillance studies (33).

Most human diseases associated with microbiologically polluted waters are produced by pathogenic microorganisms that include bacteria, viruses and protozoa, which are discharged into the aquatic environments through faecal contamination from infected persons or animals. The most relevant waterborne infectious diseases are summarized in Table 1.

The main reservoirs for pathogenic microorganisms present in natural waters can be either humans, animals, or the environment itself (23). Sewage seems to be a major source of pathogenic microorganisms found in aquatic environments. Wastewater treatment processes significantly reduce the incidence of illness among the human population, especially in the case of bacterial diseases; however, protozoa cysts and many pathogenic viruses are very resistant to the chemical, physical treatment processes (29).

Numerous infectious agents have been transmitted by ingestion, contact, or inhalation of water, and the main diseases associated with waterborne infections include gastroenteritis, hepatitis, skin lesions, wound infections, conjunctivitis, otitis, respiratory infections, and generalized infections (40). Several factors are critical in the transmission of pathogenic microorganisms through the water route, such as: (i) source of the infectious agents; (ii) specific water-related modes of transmission; (iii) survival and multiplication capabilities of the pathogens into the aquatic environment; (iv) infectious dose and virulence factors of the microorganisms; and (v) host susceptibility factors.

A major factor determining microbial health-hazard associated with the water route is the infectious dose of the pathogenic microorganism. The infectious dose (ID) is defined as the minimum number of organisms required to cause infection and it varies considerably according to the type of organism. In general, viruses and protozoa have low ID, typically from 1 to 50 PFU or cysts (39), whilst bacterial pathogens possess ID_{50} (number of organisms resulting in a 50% infection rate) ranging from 10^2 to 10^8.

Some agents of waterborne diseases, such as *Legionella* spp., *Vibrio* spp., *Aeromonas hydrophila* and *Pseudomonas aeruginosa* are indigenous of aquatic environments; therefore, infections caused by these microorganisms will depend on the exposure to natural waters. However, most microbial waterborne pathogens of concern originate in the enteric tracts of humans and animals, and enter the aquatic environment via faecal contamination. The concentration of these pathogens in natural waters will depend on the number of infected persons and/or asymptomatic carriers in the community and the effectiveness of the wastewater treatment before the discharge. In addition, the dilution and the natural self-depuring capability of the receiving waters will also reduce the concentration of contaminant microorganisms.

Epidemiological and microbiological studies indicate that *Staphylococcus aureus* skin and ear infections are often associated with recreational use of water, and the source of these organisms may be other bathers or the water itself (39). *Vibrio vulnificus* and *Aeromonas* spp. can cause serious wound infections when an injury to skin occurs in marine and freshwater, respectively (32, 34). Cyanobacterial toxins have been associated with contact irritation after bathing in marine or freshwater environments (11). An

TABLE 1. Pathogenic microorganisms associated with water health-hazards acquired by ingestion and by contact with recreational water

Causative agent	Disease/syndrome	Source
BACTERIA		
Vibrio cholerae O1	Cholera	Human faeces
Non-O1 *V. cholerae*	Diarrhoea	Human faeces
V. vulnificus	Gastroenteritis/wound infections	Marine water
V. parahaemolyticus	Gastroenteritis/wound infections	Marine water
Salmonella spp.	Gastroenteritis	Human and animal faeces
Salmonella typhi	Typhoid fever	Human faeces and urine
Shigella dysenteriae	Bacillary dysentery	Human faeces
Yersinia enterocolitica	Bloody diarrhoea	Animal faeces and urine
Enterohaemorrhagic *E. coli*	Bloody diarrhoea	Human and animal faeces
Enteroinvasive *E. coli*	Dysentery	Human faeces
Enteropathogenic *E. coli*	Diarrhoea	Human faeces
Enterotoxigenic *E. coli*	Diarrhoea	Human faeces
Campylobacter jejuni	Acute gastroenteritis	Human and animal faeces
Aeromonas hydrophila	Diarrhoea/wound infections	Freshwater
Plesiomonas shigelloides	Bloody diarrhoea	Freshwater, fish
Legionella spp.	Legionnaires' disease	Freshwater, aerosols
Mycobacterium spp.	Skin and subcutaneous lesions	Marine and freshwaters
Pseudomonas aeruginosa	Dermatitis/ear and eye infections	Faeces, water, humans
Staphylococcus aureus	Dermatitis/ear and eye infections	Humans
Leptospira icterohaemorrhagica	Leptospirosis	Animal urine
Cyanobacteria	Dermatitis/toxin poisoning	Cyanobacterial blooms
VIRUSES		
Norwalk and Norwalk-related	Acute gastroenteritis	Human faeces
Rotavirus (groups A and B)	Acute gastroenteritis	Human faeces
Hepatitis A virus	Hepatitis	Human faeces
Hepatitis E virus	Hepatitis	Human faeces
Astrovirus	Acute gastroenteritis	Human faeces
Calicivirus	Acute gastroenteritis	Human faeces
Enteroviruses (polio, echo, coxsackie)	Respiratory illness/encephalitis/ paralytic disease/meningitis/ diarrhoea	Human faeces, shellfish
Adenovirus (serotypes 1,3,4,7,14)	Conjuctivitis/pharyngitis	Humans
PROTOZOA		
Naegleria spp.	Meningoencephalitis	Freshwater
Balantadium coli	Bloody diarrhoea	Human and animal faeces
Cryptosporidium spp.	Diarrhoea/dysentery	Human and animal faeces
Entamoeba histolytica	Dysentery	Human faeces
Giardia lamblia	Giardiasis	Human and animal faeces
Acanthamoeba spp.	Conjunctivitis/skin lesions	Water

additional cause of recreational water infections are *Leptospira* species and Adenoviruses, which are neither enteric nor aquatic organisms (40).

In addition, other water-related infections involve the transmission of infectious agents by inhalation of water aerosols and by the consumption of raw or undercooked contaminated shellfish. The major pathogens associated with aerosol transmission are *Legionella pneumophila* and nontuberculous mycobacteria. Bivalve shellfish have served as vehicles of enteric disease transmission because of their ability to concentrate enteric organisms from faecally contaminated water in their tissue. Numerous outbreaks have been attributed to the consumption of raw bivalves. These infections can be caused by hepatitis A and E viruses, Norwalk-related viruses, pathogenic *Escherichia coli*, *Salmonella typhi*, and species of *Vibrio*, *Shigella*, *Plesiomonas* and *Aeromonas* (25).

Index and indicator organisms

Natural waters are subject to important changes in their microbial quality due to occasional discharges of sewage or wastewater as a result of human activity or storm-water run off. Sewage effluents contain a wide range of pathogenic microorganisms, that may pose a health-hazard to the human population when they are discharged into recreational waters. The density and variety of these pathogens are related to the size of the population, the seasonal incidence of the illness, and the dissemination of pathogens.

Many waterborne pathogens are difficult to detect and/or quantify in water, and the optimal specific methodology to detect them in environmental water samples has still to be developed. The specific detection procedures currently available are complex, and they involve concentration processes and subsequent selective enrichment or amplification by molecular biology methods. In addition, the recovery efficiency of the procedures is low and these techniques are limited to specialized research laboratories. A recent revision of the methodologies developed for the detection and/or enumeration of pathogenic microorganisms from waters has been carried out by different authors (12).

Indicator organisms (5) must fulfill the following requirements: (i) Indicators should be present in faeces, sewage and sewage-polluted waters whenever the pathogens are also present, and absent in waters without faecal pollution. (ii) The levels of indicators should have some direct relationship to the density of pathogens in aquatic environments, and be proportional to the extent of the pollution. (iii) Indicators survival rate in water must be at least similar to that of pathogens. In addition, the resistance of indicators to depuration factors and disinfectants should be at least similar to that of the pathogens. (iv) Indicators must be detectable by a simple, inexpensive, accurate, rapid laboratory methodology. (v) Indicators should be non-pathogenic and applicable to all types of water samples that require a monitoring programme. (vi) Their characteristics must be constant. And (vii) indicators must not grow and multiplicate in water.

Mossel (42) defined the term "model organism" which refers to two different functions, index and indicator. "Index organisms" are related, directly or indirectly, either to the health-hazards or to the pathogen presence, and they must fulfill the criteria given in Table 2. On the other hand, "indicator organisms" are related only to the effects of treatment processes or control of water quality. 'Indicator organisms' requirements are less restrictive than those for an index organism: their resistance to the environment must be only similar to that of pathogens and they require more simple laboratory methodologies.

TABLE 2. Criteria for "index" and "indicator" microorganisms

Criteria	Index	Indicator
I. Similar ecology	Yes	No
Presence in human faeces		
Absence in animal faeces		
Detection in polluted waters with human faeces		
Absence in polluted waters with animal faeces		
No multiplication in water environment		
Constant characteristics		
II. Similar resistance and persistence	Yes	Yes
To wastewater treatments		
To disinfection processes		
Survival in marine and freshwaters		
Resistance to sun light		
Relationship with human enteric viruses		
Related to human health-hazards		
III. Simple methodology	Yes	Yes
No specialized installations are required		
Inexpensive		
Rapid		
No pathogen		
Processing of high volumes of water is needed		

The use of "index" or "indicator" organisms to assess the microbiological and sanitary quality of waters is well established and has been practiced for almost a century. Classically, the microbiological quality of waters has been measured by the analysis of indicator microorganisms. The most widely used groups are total coliforms, faecal coliforms, and faecal streptococci.

Total coliforms. They are aerobic and facultatively anaerobic, Gram-negative, non-spore-forming, rod-shaped bacteria that ferment lactose with gas and acid production in 24–48 h at 36 ± 1°C. Although coliform bacteria belong to the family Enterobacteriaceae, and usually include *E. coli* as well as various members of the genera *Enterobacter, Klebsiella* and *Citrobacter*, the "total coliform" definition is not based on strictly taxonomic criteria, but on specific biochemical reactions or on the appearance of characteristic colonies on selective and/or differential culture media. However, the recent advent of enzyme-specific media and tests has allowed the application of cytochrome oxidase (negative) and β-galactosidase (positive) as additional criteria for the coliform group.

These bacteria have been classically used as indicators of water pollution from sewage and thus they are of sanitary significance. However, several authors have pointed out that some members of the coliform group can originate from non-enteric environments, such as wastes from the wood industry and surfaces of redwood water tanks, biofilms within drinking water distribution systems, and epilithic algal mat communities in pristine streams. The persistence of these bacteria in supply water systems can be compared to that of some waterborne bacterial pathogens, although they are much less persistent than viruses and protozoa. In the case of natural aquatic environments, particularly ma-

rine waters, total coliforms present a survival rate lower than that of pathogenic microorganisms (41). In addition, there are serious shortcomings for the general use of these microorganisms as indicators, such as the ability of some coliforms to grow in natural waters; the low relation between the numbers of coliforms and those of pathogenic microorganisms in natural waters; and the detection of atypical strains of coliforms in water (4). Recent studies have also suggested the low capability of these microorganisms as indicators for bathing waters (20).

Faecal coliforms and *E. coli*. Faecal coliforms are the subset of total coliforms that are more closely related with homeothermic faecal pollution. These bacteria conform to all criteria used to define total coliforms, but in addition they grow on lactose and ferment it with production of gas and acid at 44.5 ± 0.2°C within the first 48 h of incubation. For this reason, the term "thermotolerant coliforms" may be more appropriate to name this group.

Thermotolerant coliforms comprise strains of the genera *Klebsiella* and *Escherichia*, that are shown to correlate with faecal contamination from warm-blooded animals (43). The physiological basis of the high temperature phenotype in faecal coliforms has been described as a thermotolerant adaptation to the temperatures found in the enteric tracts of animals, which lies on a higher stability of proteins to heat. Dufour (18) suggested the redefinition of the faecal coliform group with the addition of several biochemical characteristics, such as their impossibility of using citrate as the only carbon source, the presence of tryptophanase activity and the lack of urease activity, the negative Voges–Proskauer test, and the positive Methyl Red test (positive). These characteristics are found almost exclusively in *E. coli*, which is a most useful microbial indicator of water quality because it is more specific for the presence of faecal contamination that the faecal coliform group. It can be easily distinguished enzymatically by the lack of urease and the presence of β-glucuronidase, enzymes that form the basis of recently developed differential methods (22).

However, several studies indicate the limitation of faecal coliforms as ideal indicators or index organisms. Several thermotolerant *Klebsiella* strains have been isolated from environmental samples with high levels of carbohydrates in the apparent absence of faecal pollution. Similarly, other members of the faecal coliform group, including *E. coli* , were detected in some pristine areas (45) and associated with regrowth in potable water distribution systems (37). Therefore, the disadvantages of using *E. coli* as an indicator in water are: (i) its detection from other environments without faecal contamination, and (ii) its low survival capability in aquatic environments (41).

Faecal streptococci and enterococci. Faecal streptococci have received widespread acceptance as useful indicators of faecal pollution in natural aquatic ecosystems, since (i) they show a high and close relationship with health-hazards associated with bathing in marine and freshwater environments, mainly for gastrointestinal symptoms; (ii) they are not as ubiquitous as coliforms are; (iii) they are always present in faeces of warm-blooded animals; (iv) they are unable to multiply in sewage-contaminated waters; and (v) their die-off is less rapid than coliforms decline in seawater, and their persistence patterns are similar to those of potential waterborne pathogenic bacteria (9, 33, 46, 47).

The main problem derived from the universal use of these microorganisms is the recognized lack of a standard methodology for their selective enumeration from natural waters. More than 70 media have been proposed for this purpose until now. However, a standard methodology has not been yet adopted, since few studies have been conducted on their comparative recovery efficiency from water samples (17).

An additional problem related to the selective enumeration of this group of microorganisms is their taxonomic, ecological heterogeneity. The group called "faecal streptococci" comprises species of different sanitary significance and survival characteristics; in addition, the proportion of the species of this group are not the same in animal and human faeces (43). Therefore, a clearer definition of faecal streptococci is necessary to establish a specific standard methodology of enumeration. The taxonomy of this group has been subject to extensive revision recently (36); in our opinion, the following species of the *Enterococcus* and *Streptococcus*, on the basis of their taxonomical inclusion and the exclusively faecal origin of the species: *Enterococcus faecalis*, *E. faecium*, *E. durans*, *E. hirae*, *E. avium*, *E. gallinarum*, *E. cecorum*, *Streptococcus bovis*, *S. equinus*, *S. alactolyticus*, *S. intestinalis*, *S. hyointestinalis* and *S. acidominimus* (3, 19).

Enterococci include all species described as members of the genus *Enterococcus*, which also fulfill Sherman's criteria: growth at 10°C and 45°C, resistance to 60°C for 30 min, growth at pH 9.6 and at 6.5% NaCl, and the ability to reduce 0.1% methylene blue.

Alternative indicator organisms. None of the above mentioned bacterial indicators used to establish recreational standards fulfill all the criteria desirable for an ideal indicator, and most are more susceptible to environmental conditions than are pathogenic microorganisms (13), and viruses. As a result, many other microorganisms are being evaluated as alternative indicators of water quality.

– Sulphite-reducing clostridia: The use of the sulphite-reducing members of the genus *Clostridium* (*C. perfringens*/*C. welchii*) as indicators of faecal pollution was originally proposed in 1890 by Houston, but until 1930 they were not used as indicators of water quality in certain specific situations, for example in the control of drinking-water treatment and in the microbial quality of groundwater (21, 50).

C. perfringens has been proposed as a water quality indicator because (21): (i) it is consistently present in the faeces of all warm-blooded animals as well as in sewage; (ii) it is more stable in environmental waters than most pathogens; and (iii) it has been successfully used to monitor sewage-contaminated waters. Nevertheless, sulphite-reducing clostridia are considered ubiquitous in aquatic sediments and several problems with their specific detection and recovery methodology limit their potential value as indicator organisms. Due to the great resistance and longevity of their spores, these bacteria can be useful indicators of remote faecal pollution (50).

– *Staphylococcus aureus*: The results of several epidemiological studies show higher incidence of non-diarrhoeal diseases, such as eye, ear, mucous and skin infections. In this regard, many swimmers acquire staphylococcal skin infections after swimming in recreational waters (39), and thus, there is a need for an indicator of water quality which addresses skin infections rather than gastrointestinal infections.

Evidence to support the use of *S. aureus* as indicator of water quality includes the following: (i) this microorganism is stable and shows a great resistance to environmental conditions, especially in marine waters; (ii) its concentration in water has been shown to represent the load of microorganisms being shed by swimmers; and (iii) methods for its specific recovery from marine waters are now available (7, 10).

– *Pseudomonas aeruginosa*: Members of the genus *Pseudomonas* are frequently isolated from aquatic environments. Therefore, their presence does not necessarily imply a possible risk to public health. However, *P. aeruginosa* has been linked to infections associated to exposure to recreational waters and thus it has been

proposed as an indicator of recreational water quality (2, 39).

P. aeruginosa was found to be more resistant than other indicators and pathogenic microorganisms to treatment processes of water, and to environmental factors. These findings demonstrate the usefulness of *P. aeruginosa* as an indicator in analysis of recreational waters such as swimming-pools and whirlpools.

– Bacteriophages: The use of bacteriophages as indicators is not a new concept, since they were proposed as faecal and viral indicators by Kott (35).

The term bacteriophages is a wide concept; the bacteriophage groups proposed as indicators are somatic coliphages, F-specific RNA bacteriophages, and phages of *Bacteroides fragilis* (15, 31, 38, 51).

Somatic coliphages are specific bacteriophages of *E. coli* , and have been the phage group most studied. They are commonly used as indicators of faecal and/or sewage pollution, on the basis of their direct correlation with the presence of enteric viruses in marine waters, freshwater ecosystems and sewage effluents. Recently, Borrego (6) reviewed the indicator potential of somatic coliphages. He concluded that considering the differences in origin and ecology between enteric viruses and somatic coliphages, it is doubtful that this phage group could be used successfully in all situations as viral indicators; however, they are good indicators of faecal pollution and of the microbiological water quality.

Several authors have proposed other bacteriophage groups as alternative indicators. Primrose et al. (44) proposed the use of F(male)-specific RNA bacteriophages (FRNA phages) as faecal pollution indicators, on the basis of their inability to replicate in the water ecosystem, since fimbria are not synthesized below 30°C. In 1984, Havelaar and Hogeboom (27) developed a method for the detection of this bacteriophage group, and proposed them as model viruses in water hygiene, because: (i) the group comprises viruses similar in size, shape, and genetic makeup to human enteric viruses, which are responsible for most of waterborne diseases; (ii) it represents viruses which are more stable than human enteroviruses in environmental waters and more resistant to disinfection; and (iii) the viruses concentrations in environmental waters have been reported to correlate with sewage contamination.

The low incidence of the FRNA phages in faeces, and human faeces in particular (28), and their high concentration in sewage, suggests that FRNA phages must be able to multiply in the sewage system (30). Hence, the presence of FRNA phages in water is primarily an index of sewage pollution rather than just of faecal pollution (30). FRNA phages may be more appropriate to be used as simulators for the environmental behaviour of enteric viruses (e.g. Norwalk) in water and wastewater treatment than as general indicators of faecal pollution.

Analytical methods

Most Probable Number (MPN). MPN analysis is a statistical method based on the random dispersion (Poisson) of microorganisms per volume in a given sample. Classically, this assay has been performed as a multiple-tube fermentation test. Although the technique is rather time-consuming (5–7 days), there are laboratories thet prefer it to other methods of water analysis, because it is applicable to all sample types.

The MPN technique is generally conducted in three sequential phases (presumptive, confirmatory, and complete), each phase requiring 1 to 2 days of incubation. In the presumptive phase, three volumes of samples (usually 10, 1, and 0.1 ml) are inoculated into 3, 5, or 10 tubes

containing the appropriate medium to allow the target bacteria to grow. In this test, it is assumed that any simple viable target organism will result in growth or a positive reaction in the medium.

After the incubation period, all the inoculated presumptive positive tubes must be inoculated into a more selective medium to confirm the presence of the target bacteria (confirmatory phase). The confirmed test is reliable evidence but not proof that the target bacteria have been detected. Therefore, subsamples of the confirmed positive reactions should be inoculated onto a selective agar medium, and several verification tests (Gram stain, and biochemical, serological or enzymatic tests) should be carried out. This completed test is generally conducted on 10% of the positive confirmed samples as a quality control measure. For practical purposes, the number of positive and negative tubes in the confirmed phase of the technique is generally used to determine the MPN of the target bacteria by using the tables of positive and negative tube reactions given in Standard Methods (2).

The major advantages of the MPN technique are the following: (i) it will accept both clear and turbid samples; (ii) it inherently allows the resuscitation and growth of injured bacteria; (iii) the results may be recorded by personnel with minimal skill; and (iv) minimal preparation time and effort are required to start the test, and therefore processing of samples can be initiated at any time of the day. On the contrary, the MPN technique may have several disadvantages, such as: (i) the total time, labour, material, and costs required to analyse one sample; (ii) the substantial increase in reagents, tubes, incubation space and cleanup requirements when multiple samples need to be analysed or when the sample volume must be increased to 100 ml; (iii) the multiphase nature of the technique, each phase requiring 24 or 48 h incubation period; and (iv) the fact that MPN is a simple estimated number, while the true number (95% confidence limit) may show extreme variation from the MPN.

Membrane filtration (MF). MF technique is the most widely used method in microbiological water analyses. The technique is based on the entrapment of the bacterial cells by a membrane filter (pore size of 0.45 μm). After the water is filtered, the membrane is placed on an appropriate medium and incubated. Discrete colonies with typical appearance are counted after 24 to 48 h, and population density of the target bacteria, usually described as colony forming units (CFU) per 100 ml, in the original sample, can be calculated from the filtered volumes and dilutions used. This technique is more precise than MPN, but MF test presents the limitation of its exclusive use for low-turbidity waters with low concentrations of background microorganisms.

P–A test. This test was designed for the microbiological monitoring of drinking-water, in which the presence or absence (P–A) of indicator bacteria in a standardized volume (i.e., 100 ml) of water is determined, using a liquid medium. One of the major advantages of this approach is that it is easy to perform and permits to analyze greater numbers of samples.

Although the P–A test can yield valuable information on the prevalence of microbiological problems, it has a drawback, for this approach fails to provide data on the magnitude of such occurrences. In addition, the common connotation of 'absence' may be misleading in the case of injured bacteria, which are frequent in treated drinking water systems or marine environments and that fail to grow on established media.

Laboratory procedures

Total coliforms. Classically liquid media containing lactose, such as lauryl tryptose broth, lactose broth, or MacConkey broth, have been

used with the MPN technique for the detection of total coliforms in water samples. Positive reaction of the presumptive phase is the gas and acid production at 36 ± 1°C incubated for 48 h. Confirmation of presumptive reactions is done by inoculating the positive aliquots into brilliant green lactose bile broth tubes; gas production in these tubes after 48 h at 36 ± 1°C is interpreted as representing confirmed test. Bacterial density and the 95% confidence limits can be estimated with the use of MPN tables for the volumes and number of aliquots used (2).

The MF analysis of water for total coliforms can involve the use of the following media: LES-Endo agar, m-Endo agar, Tergitol agar, and Teepol agar, Endo media being the most commonly used. Characteristic colonies appear in Endo media as pink to dark red colonies with a metallic green sheen. Only plates containing between 20 and 80 typical colonies should be counted (2).

New technological innovations have been introduced in the analysis of total coliforms from waters, including the application of m-T7 medium that allows the detection of injured coliforms; the use of the enzymatic activity β-galactosidase in the Colilert and Coliquick tests; and the employment of molecular methods, such as PCR technique and gene probes for lacZ.

Faecal coliforms. EC and A-1 media are the most widely recommended for the presumptive test with MPN technique of faecal coliforms (2). The differences between them are based on the incubation periods, 44.5 ± 0.2°C for 24 h in the case of EC medium, and 36 ± 1°C for 3 h and transfer to 44.5 ± 0.2°C for 21 h in the case of the A-1 medium. The tubes containing gas and acid in EC medium are confirmed in the same medium by subsequent incubation at 44.5 ± 0.2°C for 24 h. A-1 medium does not require a confirmation test. Faecal coliform density and the 95% confidence limits can be estimated by using the MPN tables as described for total coliforms (2).

The most frequently medium used to quantify faecal coliforms in water samples when the MF technique is used is mFC agar. Petri dishes containing filters are incubated at 44.5 ± 0.2°C for 24 h. Typical faecal coliform colonies will appear with various lines of blue, although atypical *E. coli* may be pale yellow, whereas non-faecal coliform colonies are grey to cream. Only plates containing from 20 to 60 typical colonies should be counted (2).

As *E. coli* has been demonstrated to be a more specific indicator for the presence of faecal contamination than the faecal coliform group, improvements in both MPN and MF techniques have been carried out for the rapid and selective enumeration of *E. coli*, including a rapid 7-h membrane filter test for quantification of *E. coli* from water samples. Fluorogenic and chromogenic assays using 4-methylumbellipheryl-β-D-glucuronide (MUG) have been applied, in MPN and MF techniques, for the detection of β-glucuronidase that is specific of *E. coli* (22). Different media based on this principle have been developed for the use in MF, MPN, and P–A techniques. Commercially available media include Colisure (Millipore), Colilert (Idexx), m-ColiBlue (Hach), ColiComplete (BioControl), Chromocult (Merck), and MicroSure (Gelman). Similar media to detect *E. coli* in water have also been described. Molecular methods have also been designed to the specific detection of *E. coli* from water samples, such as PCR-gene probes for *uid*, enzymatic activities, enzyme capture, and radioisotopes.

Faecal streptococci and enterococci. Early attempts to quantify faecal streptococci relied on enrichment tube procedures associated with the use of the MPN technique, Rothe Azide Dextrose broth followed by a confirmation in Ethyl Violet Azide (Litsky) broth being the procedure most widely accepted by researchers. The enumeration of faecal streptococci by a MF proce-

dure using a selective medium was first reported by Slanetz and Bartley (48). Since then, several media have been proposed, including Thallous Acetate agar, KF agar, PSE agar, Kanamycin Aesculin Azide (KEA) agar, mSD agar, and mE agar. Other media formulations and incubation procedures for faecal streptococci have been proposed for specific situations, increasing the membrane incubation period from 48 to 72 h to recover stressed faecal streptococci. A new medium, designed as M2, has been designed to distinguish between human and animal pollution sources, and recently a rapid system for enumeration of faecal streptococci and enterococci from water samples using a miniaturized fluorogenic assay has been proposed (43).

The methodologies for enumeration of faecal streptococci from natural waters have been compared by different authors. Dionisio and Borrego (17) compared eight methods for the specific recovery of faecal streptococci from natural freshwater and marine waters on the basis of their following characteristics: accuracy, specificity, selectivity, precision and relative recovery efficiency. The results obtained indicated that none of the tested methods showed an optimal selectivity. The methods that showed the best performance characteristics were the MPN technique (with Rothe and Litsky media) and the m-*Enterococcus* agar in conjunction with the MF technique. The latter is the only method recommended in the *Standards Methods* for faecal streptococci in conjunction with membrane filtration (2). A rapid confirmation technique based on the transplantation of the membrane from m-*Enterococcus* agar to Bilis-Esculin-Azide (BEA) agar improves the low specificity of the m-*Enterococcus* agar, enabling the confirmation of 100% of the colonies (19).

Recently, Audicana et al. (3) designed and tested a modification of the KEA agar, named Oxolinic acid-Aesculin-Azide (OAA) agar, to improve the selectivity in the enumeration of faecal streptococci from water samples by MF. OAA agar showed specificity, selectivity and recovery efficiency higher than those obtained by using m-*Enterococcus* and KF agars. In addition, no confirmation of typical colonies was needed when OAA agar was used. This shortens significantly the time of sample processing (only 24 h) and increases the accuracy of the method. A standardization trial performed at European scale and funded by the EC, demonstrated that the m-*Enterococcus* agar with total confirmation of the colonies (19), the OAA medium (3), and the miniaturized MPN method produced the best results (43).

Somatic coliphages. Several methods have been advocated for the recovery of phages from aquatic habitats (1, 8). Depending on the level of pollution of the water sample, these methods may be comprised in two groups: (i) direct plaque assay from the sample, and (ii) concentration techniques. However, other authors have used different detection procedures based on physicochemical or structural properties of the phages, such as precipitation by polyethylene glycol, magnetic-organic flocculation, adsorption to hydroxylapatite, electron microscopy, and ultracentrifugation. (i) Direct plaque assays: The double-agar-layer (DAL) method is widely used for phage detection and enumeration. Havelaar and Hogeboom (26) described a single-agar-layer (SAL) method that yielded phage recovery comparable to that of the DAL method. In both procedures the sample (raw or decontaminated) is mixed with a log-growth adequate host culture, and then the mixture is poured onto Petri dishes with bottom agar. If phage is present in the sample, it will lyse its specific bacterial cells and will produce a clear spot (lysis plaque) on the bacterial lawn; then, the number of plaques on lawns will be a reflection of the phage concentration (expressed as plaque forming units,

PFU) in the sample. The amount of sample assayed by these procedures is necessarily small. Therefore, direct plaque assay methods are suitable when phages are present in high numbers. However, Kott (35) designed a MPN assay with a theoretical detection limit of 2 PFU per 100 ml of water sample, using 65 ml of sample. The most commonly used *E. coli* strains as host for coliphage recovery are *E. coli* C and *E. coli* K12. A rapid coliphage enumeration technique named ACART has two advantages regarding DAL and MPN techniques, its lower detection limit and faster analysis (6 h). Grabow and Coubrough (24) described a direct SAL plaque assay for the direct detection of coliphages in 100 ml of water samples. The theoretical minimum detection limit for this method is 1 PFU per 100 ml.

F-RNA specific bacteriophages. The specific methodology of this bacteriophage group consisted in the application of direct plaque assay using bacterial hosts that have the specific phage receptor (sex pili). Primrose et al. (44) used a mixture of *E. coli* K12 Hfr and *Salmonella typhimurium* LT2 as host system to avoid the recovery of somatic coliphages. Later, Havelaar and Hogeboom (27) developed a method for the detection of F-specific coliphages using a genetically manipulated *Salmonella* strain (*S. typhimurium* WG49) as bacterial host. This strain was constructed by the introduction of F'42 lac::tn5 plasmid into the *S. typhimurium* WG45 strain. Therefore the bacterium is able to produce sex pili but does not contain the receptor sites for coliphages. By using *S. typhimurium* WG49 with the DAL technique it is possible to isolate F-specific coliphages from water samples, and by differentiation on the basis of sensitivity to RNase in the assay medium, more than 90% were found to be RNA phages. DeBartolomeis and Cabelli (16) used a mutant *E. coli* strain (*E. coli* C3000, ATCC 15597) to recover F male specific bacteriophages. Finally, Sobsey et al. (49) developed and evaluated a simple membrane filtration method for concentrating and enumerating F-specific coliphages in natural and treated waters.

Phages of *Bacteroides fragilis*. Tartera and Jofre (51) compared the DAL technique and a modification of the MPN method using modified blood agar base and *B. fragilis* HSP 40 as host for the specific enumeration of these phages. Prior to the assay, the samples were decontaminated by filtration or by chloroform treatment. In addition, to prevent germination of spores, the samples were supplemented with potassium sorbate. In those conditions, these authors found that the DAL technique had a lower efficiency in the enumeration of *B. fragilis* phages.

Finally, Cornax et al. (14) developed a direct assay for *B. fragilis* phages which consisted in the culture of host bacteria in modified Brucella broth vials with an atmosphere of $CO_2 \pm N_2$, and in the use of modified blood agar as bottom agar layer. In the case of samples with a high degree of pollution, these authors suggested supplementing the medium with kanamycin and vancomyxin mixture.

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MICROBIOLOGÍA SEM **13** (1997), 427–436 **MINIREVIEW**

Biology and applications of mycorrhizal fungi

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Summary

Mycorrhizae have been shown to increase growth and yield of plants. They have been identified with both nutrient mobilization and nutrient cycling. Arbuscular (or endo-) mycorrhizae play a significant role in agriculture and most natural ecosystems, whereas ectomycorrhizae have a great potential in forestry and wasteland regeneration. The use of mycorrhizal fungi would reduce dependence on chemical fertilizers besides minimizing environmental pollution. The present review addresses the progress that there has been in the area of the ecto- and endomycorrhizae. It also examines the potential of field applications of mycorrhizal biotechnology in agriculture and forestry.

Key words: arbuscular mycorrhizae, ectomycorrhizae, fungal biomass, biofertilizers, nutrient and water uptake

Resumen

Las micorrizas aumentan el crecimiento y producción de las plantas y son responsables de la movilización y reciclado de nutrientes. Las micorrizas arborescentes (o endomicorrizas) desempeñan un papel esencial en la agricultura y en la mayoría de los ecosistemas naturales, mientras que las ectomicorrizas tienen un gran potencial en los sistemas forestales y en la regeneración de tierras baldías. La utilización de los hongos de las micorrizas disminuye la dependencia de los abonos químicos, además de reducir fuertemente la contaminación ambiental. Esta revisión explica el progreso que se ha producido en el área de las ecto- y endomicorrizas. Examina también el potencial de la biotecnología de las micorrizas en agricultura y sistemas forestales.

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Introduction

The use of biomass, a non-polluting, renewable source of energy contribute to mitigate global problems such as the lack of food and energy sources, and environmental pollution. The large area of wasteland (i.e. 93.6 m ha) available in India could be utilized for biomass production if suitable technologies were available. Biofertilizers are a promising alternative. Among these, mycorrhizae have a great potential in biomass production on wastelands.

Studies on ectomycorrhizae have attracted considerable interest in tropical countries that face acute shortage of fuels and raw materials to satisfy the industrial and domestic demands. Nearly all terrestrial plants have mycorrhizae of one type or another. For plants roots to have mycorrhizae is as normal and essential to the plants as for plants leaves to have chlorophyll.

Mycorrhizae are symbiotic associations between the roots of plants and fungi, in which the growth of the plant is enhanced by the absorption of water and nutrients from the soil (by mechanical and enzymatic reactions). In return, the fungi get their food from plants in the form of carbohydrates. Most vascular plants have evolved with root-inhabiting symbiotic fungi; the resulting structures, mycorrhizae, are a consequence of close relationships and subsequent modifications of both symbionts (52). Phosphorus concentration in plant and soil affects mycorrhizal development (72).

Types of mycorrhizae and culture techniques

The main morphological types are : (i) Ectomycorrhizae, (ii) Endomycorrhizae or arbuscular mycorrhizae (AM). They differ widely both in their structure and in the systematic position of the fungi, but they have essentially the same functions. A variety of techniques of identification have been developed (1). AM fungi are obligately biotrophic, and can grow only in dual culture, with a suitable host plant. Many ectomycorrhizal (EM) fungi can grow in axenic culture, though a variety of vitamins and other growth substances must be added.

Ectomycorrhizae

(i) Morphology, anatomy and taxonomic distribution of ectotrophic fungi. Ectomycorrhizae are most characteristic of forest trees (mainly boreal forests) and they characterized by modification of roots and loss of absorbant hairs. Typically, ectomycorrhizae are formed after fungal spores or hyphae come into contact with the actively growing absorbing roots of trees and subsequently invade them. The fungal propagules are stimulated by root exudates. They grow vegetatively over the feeder root forming the external fungal mantle. The external hyphae that originate from the mantle explore the soil and help in the absorption of nutrients and water by enhancing root surface area, while the internal hyphae establish a close contact with the roots that penetrate into the intercellular spaces of the cortical cells, apparently replacing the middle lamella and forming an interconnecting network known as Hartig net. Although these fungi are usually found only in association with tree roots and between their cortical cells, they never penetrate them. Most of them can grow in pure culture and almost all produce sexual fructifications in their natural habitats.

The ectomycorrhizal fungi belong to Basidiomycetes, viz. species *Amanita*, *Boletus*, *Cortinarius*, *Suillus*, *Laccaria*, *Inocybe*, *Tricholoma*, *Lactarius* and *Thelephora*; Gasteromycetes viz. genera *Astraeus*, *Rhizopogon*, *Scleroderma*, *Pisolithus*, and certain orders in Ascomycetes, such as Eurotales (*Cenococcum graniforme*), Tuberales (truffles) and Pezizales. Many of these species have received wide application in for-

estry because of their favourable support for forest trees to growth and develop (23).

(ii) Host plants. Ectomycorrhizal hosts have received considerably attention due to their economic significance. They include members of Gymnosperm family Pinaceae such as *Picea* (spruce), *Abies* (fir), *Larix* (larch), *Tsuga* (hemlock), *Pseudotsuga* (Douglas fir), *Pinus* (pine)(42) and Angiosperms such as *Salix* (willow), *Populus* (aspen), *Quercus* (oak), *Fagus* (beach), *Eucalyptus*, legumes and Rosaceae. Thousands of species of mycorrhizal fungi have been recorded as mycorrhizae formers on woody trees in North America. Many of these fungi are saprophytes that play a major role in the decomposition of organic matter and nutrient recycling in forest soils. Some fungi are host specific, others have broad host ranges.

Ectomycorrhizal roots are generally short, swollen, dichotomously branched and with distinctive colours of either white, black, orange, yellow or olive green.

(iii) Culturing of ectomycorrhizal fungi. Most of the work on the culturing of ectomycorrhizal fungi has been made with sporocarps instead of making it directly with short roots because cultures from short roots are slow growing. Pure ectomycorrhizal fungi can be derived from fruit body tissue, surface sterilized mycorrhizal roots and by germinating spores in sterilized media. Isolation from rhizomorphs or mycelial strands is also possible. Molina (45) listed 18 genera for the isolation of mycorrhizal fungi from their fruit bodies. A commercial preparation of *Pisolithus tinctorius* with trade name "Mycorrhiz" is available in the market (42).

(iv) Ectomycorrhizae inoculation techniques. Soil, spore, nursery seedlings and vegetative mycelium are the four primary sources of ectomycorrhizae inocula for forest tree nurseries. Detailed procedures for inoculum production and nursery inoculation have been discussed by many researchers (13, 40, 41, 45). Current inoculation procedures use mycorrhizal soil (soil inoculum), basidiospores and pure cultures of mycorrhizal fungi. Soil inocula are cost effective if labour is plentiful and inexpensive. Soil is bulky, and large amounts are needed on a continual basis. Basidiospores of mycorrhizal fungi have been used occasionally as inocula, usually in small experiments (39). Spore inoculation bears many problems such as determination of spore viability, insufficient quantity and the presence of associated contaminants which may affect seedlings health and spore viability. Furthermore, it takes a much longer time for spores to form ectomycorrhizae since the infection process is much slower than with pure culture or soil inocula.

Planting mycorrhizal nurse seedlings into nursery beds as a source of the fungi for neighbouring young seedlings has been successful in Indonesia to inoculate *P. merkusii* seedlings where soil inoculation did apparently not succeed (77). Preparation of vegetative inocula is expensive and time consuming. However, vegetative inocula of some fungi can be purchased from commercial sources. *P. tinctorius*, *Laccaria bicolor* (Marie) Orton, other *Laccaria* spp., some *Hebeloma* spp. and some *Scleroderma* spp. have been grown and used effectively.

Endomycorrhizae

(i) Morphology, anatomy and taxonomic distribution of endotrophic fungi. There is no mantle sheath and no macroscopic fruit bodies of fungi. But the technique developed by Phillips and Hayman (54) gives the morphology and anatomy of fungi.

AM spores occur in physiologically inactive stages in the soil. Spores germinate and grow without plant influence but they multiply only in the presence of actively growing roots. After a root encounters an AM fungus the process of infection will be influenced by both soil factors and by the host root physiology.

The thick walled hyphae penetrate (mechanically and enzymatically) the roots and cause an internal infection. Hyphae spread inter- or intracellularly in the root cortex. These hyphae branched repeatedly form a cluster of dichotomously branched filaments, called arbuscules which may occupy the whole cell. Their function is nutrient transfer between symbionts. They remain active only for very short periods, i.e. for 4–15 days. The cause of their destruction is the digestion of the fungal wall by the host chitinase activity (20). Vesicles are produced intercellulary. The main function of vesicles is regarded to be of storage and vegetative reproduction. Generally, the fungi grow throughout the cortex but do not penetrate the vasculature (22, 76). Gerdmann and Nicolson (22) established procedures for collecting spores from soil, and they described several new species.

AM fungi belong to Zygomycetes, family of Endogonaceae. Members of six genera of Endogonaceae, namely *Glomus*, *Gigaspora*, *Acaulospora*, *Scutellospora*, *Entrophospora* and *Sclerocystis*, have been reported to form endomycorrhizae. The members of the Endogonaceae are placed under three main types, i.e. Zygosporic, Chlamydosporic and Sporangiosporic species. The spores of *Acaulospora*, *Entrophospora* and *Gigaspora* have been termed as azygospores. The spores of *Glomus* and *Sclerocystis* are regarded as chlamydospores, i.e. specialized asexual resting cells. The spores are arranged in sporocarps which can be seen with the naked eye when they are separated from the soil (49). AM fungi cannot be cultured, therefore their identification is based mostly on visual characteristics of soil born resting spores (57).

AM fungi have been found in cultivated soils, non-cultivated soils, moist forests, open woodlands, grassland, savannah, sand dunes, semideserts, anthracite and bituminous coal waste, eroded sites, sodic soils, and others (15, 16, 62, 68, 73). In most mesic ecosystems, high root density and the ubiquity of long lived mycorrhizal plants leads to conditions where inoculum (both spores and hyphal) is probably added to newly germinated seedlings. After disturbance of the vegetation, however, the soils of such infectiveness (58), especially for fungi that colonize new roots predominantly from hyphal rather than spores. *Glomus* spp. have the widest distribution (68). *Gigaspora* and *Sclerocystis* sp. are more common in tropical soils. AM spore populations are also found to have seasonal variations (34). In temperate climates they tend to increase towards autumn and to decline over winter. Further studies on the distribution of mycorrhizal fungi under various agroclimatic conditions have been conducted (9).

(ii) Host plants. AM fungi form symbiotic associations with members of Bryophytes, Pteridophytes, Gymnosperms and Angiosperms throughout the world. Some of the families of Angiosperms which had been reported to be non-mycorrhizal have turned out to harbor mycorrhizae (47, 48). Most agricultural (30) and horticultural crops form AM. Many plantation crops such as onion (51), coffee, tea, rubber, citrus, cocoa, oil palm, timber trees, fruit trees and medicinal plants form AM (18, 75). A limited number of plant species especially belonging to hydrophytes, halophytes and xerophytes are regarded as non-mycorrhizal. So far the reports show that, in the few families viz. Cruciferae, Chenopodiaceae, Caryophyllaceae and Cyperaceae, AM infections are rare or absent (26).

(iii) Culturing of endomycorrhizal fungi. AM fungi being obligate endosymbionts, they are cultured on host plants such as *Trigonella*, *Sorghum*, *Allium cepa* and *Solanum lycopersicon* (21). There are no methods to cultivate these fungi on artificially composed nutrient media. Some of the methods used to culture AM fungi (74) are: isolation and concentration of spores

from soil (22), pot culture methods to produce AM from a single spore, hydroponic nutrient flow culture (46), and hydroponic and aeroponic methods to grow mycorrhizal plants as a source for pure fungal spores (29).

(iv) AM fungi inoculation techniques. Root based bulk inocula is the only means of using AM fungi in agriculture or horticulture. They are mass produced in "Pot Culture" on suitable host plants.

Factors affecting mycorrhizal establishment

(i) Mycorrhizal dependency-plant species. In general, plants having rootlets diameters of more than 0.5 mm and lacking root hairs are highly dependent on mycorrhizae. Legumes, for example, are more mycotrophic than grasses. So, there is a critical effect of AM on legume grasses for phosphorus. Ectomycorrhizal hosts usually require fungal association to thrive. For a given fungus, mycorrhizal dependency values varies depending on host species (3).

(ii) Host specificity. Although most ectomycorrhizal fungi are relatively restricted in their hosts, some have a broad host range. No host specificity seems to be present in AM fungi. These fungi have wide host range. Roots of the same plant are known to be colonized by different mycorrhizal fungi at the same time and hyphal links between roots of two different hosts can also occur (43).

(iii) Photosynthesis. Mycorrhizal fungi are supplied with carbohydrates from the photosynthesis of their autotrophic hosts. Photosynthesis depending on light, high light intensities enhance arbuscule formation.

(iv) Soil conditions. Although there is no correlation between AM and soil pH, soil pH can influence the predominance of a given type of spore. Some species are better adapted to acid soils, whereas others are for neutral and alkaline soils. Soil temperatures from 12°C to 25°C increase mycorrhizal development. Other soil factors, such as temperature, moisture, phosphorus concentration and pH, affecting efficacy of mycorrhizal fungi have been discussed by Sujan Singh (71).

(v) Fertilizers. Excess of fertilizers applied to nursery soil tends to suppress mycorrhizal development (4, 31, 69). Besides, organic matter added to soil seems to lead to better mycorrhizal development (27), thus reducing disease incidence (5). However, Kruckelmann obtained opposite results (38).

Phosphorus levels in the plant, rather than in soil, control the establishment of mycorrhizae. Apparently, the higher phosphorus content in the plant, the lower the soluble carbohydrate content in the roots and exudates, and the lower the frequency of entry points.

(vi) Pesticides/fungicides. Pesticides, especially fungicides, are detrimental to mycorrhizal development (38, 44).

(vii) Soil microorganisms. Soil microorganisms can stimulate the mycelial growth of mycorrhizal fungi and the infection process. The mechanisms involved include production of compounds, such as plant hormones or vitamins by soil microbiota. Interactions related to nutrient cycling have been described for nitrogen-fixing bacteria (*Rhizobium* spp., root nodulating Actinomycetes, *Azospirillum* spp., *Azotobacter* spp.), nitrifying bacteria, phosphate solubilizing microorganisms and others. These beneficial microorganisms can be, in turn, stimulated in the mycorrhizosphere (78).

Functions and applications of mycorrhizae

Nutrient uptake and water absorption. The major role of mycorrhizal fungi is to absorb nutrients and water from the soil and to transfer

them into their hosts. Mycorrhizae can improve P uptake in low-fertility soils by extending their external hyphae beyond the root hairs and the phosphorus depletion zone. Mycorrhizae also stimulate plant uptake of N, Zn, Cu, S, K and Ca (7, 11, 14, 28, 32, 65). In the arbuscules of endomycorrhizae VA, the granules containing phosphorus are reported to soluble and mobile form. Various enzymes appear to play a role in this process.

Some species of certain plant families are highly mycorrhizae dependent. This is the case of garlic, onion, some grasses and fruit plants such as grapes, citrus and strawberries (50). Others are less dependent, viz. wheat, oat and potato. However, recently AM have been reported to increase the yield of potato tuber significantly over control (36).

Role of mycorrhizae in wasteland regeneration. Abbot and Robson (2) stated that "the relationship between the level of mycorrhizal colonization and soil physical and chemical properties are markedly variable." Mycorrhizal fungi, specifically AM fungi, have been adopted to a wide range of environmental conditions (67). AM fungi are aerobic and soil aeration has considerable impact on their distribution. Nonetheless, VA mycorrhizae have been found on aquatic plants. Pfeiffer and Bloss (53) succeeded in increasing the growth of *Parthenium argenatum* by inoculating it with *Glomus intraradices* in a moderate and highly saline-sodic soil. Rozema et al. (61) reported that the sodium content of shoots of mycorrhizal *Aster tripolium* plants was lower than that of non-mycorrhizal plants. AM inoculation was found to promote the establishment of peanut plants under acid stress conditions (25). AM association with root samples in deserts have been found (10, 37, 56). Hayman (26) conducted the experiments on acidic soil with mycorrhizae. AM are major contributors to soil stability. The AM symbionts produce extra radical hyphae that may extend several centimetres out into the soil and exude organic materials that are substrate for other soil microbes (70). These hypha-associated microbes frequently produce sticky materials that cause soil particles to adhere and improve soil structure. Further, by increasing nutrient uptake, they improve plant cover and root proliferation. Plants such as *Alnus*, *Myrica*, *Casuarina* and *Ceanothus* generally have a tripartite association of plant, nitrogen fixing Actinomycetes *Frankia* and mycorrhizae (60).

Nutrient recycling. The mycorrhizal plants produce more dry matter than non-mycorrhizal ones (59). Besides, mycorrhizal associations promote the height of the plant hosts. Mycorrhizae might be responsible for closed nutrient cycles with minimal loss by decomposing litter directly and transporting the nutrients to the hosts. Mycorrhizae are efficient at uptaking from soil solutions to which minimal nutrients are added (33). Root turnover can be rapid in tropical forests. Although the annual production of above ground biomass exceeds by far root production, root death may contribute a significant portion of the annual organic matter input to the soil. Mycorrhizal fungi associated with dying roots could be effective in scavenging nutrients from them in competition with other soil microorganisms for mineralization.

Role of mycorrhizal fungi in biocontrol. Mycorrhizae can help plants withstand root diseases either by protecting the root system against the pathogen attack or by compensating for root damage (63). The role played by AM in the biological control of plant diseases has been the subject of several reviews (8, 12, 18, 64). Biocontrol effects are better understood for ectomycorrhizae in which the fungal sheath provides a mechanical barrier to infection by soil-borne root pathogens, and in which the fungi produce antibiotics. Mycorrhizae induce chan-

ges in root exudation; they especially increase the arginine content and thicken cortical root cell walls. This can account for the deterrence of bacteria, fungi and nematodes (66). The altered root exudation induces changes in the composition of microorganisms in the rhizosphere soil. Both the improved mineral uptake from the soil and altered root exudation make plants healthy and resistant to plant diseases.

Role of mycorrhizae other than improving host nutrition. Mycorrhizal fungi synthesize compounds such as auxins, giberellins, and cytokinins. When a mycorrhizal fungus sets up a symbiotic association with a plant root, the morphology and physiology of the plant is altered. As a consequence, microbial communities in the mycorrhizosphere differ from those in non-mycorrhizal rhizosphere soils. The alteration of the microbiota in mycorrhizosphere is mediated through root exudates rather outcome of improved P nutrition (6). Increased cytokinin via mycorrhizae may increase root nodulation for *Rhizobium*. Cytokinin also facilitates phosphorus utilization, and phosphorus level influence not only the frequency of mycorrhizal infections but also the process of nodulation in leguminous species, since legumes are poor competitors for soil phosphates (55). Mycorrhizal nodulated plants exhibited higher levels of nitrogenase and nitrate reductase than to non-mycorrhizal plants. This increase in the nitrate reductase system may contribute to increase the symbiotic effectiveness (17). Moreover, enhanced cytokinin levels can also promote plant growth and elevate photosynthetic rate.

Conclusions

Mycorrhizae are distributed world wide. Nearly all terrestrial plants as well as some aquatic plants have mycorrhizae. Plant growth and health depend mainly on the physical, chemical, biological balances in the soil. Mycorrhizae play a crucial role in facilitating both microbial and plant functions as mediators of exchanges between both kinds of organisms. They improve the health of both plant and soil by enhancing nutrition, modifying the plants physiology, reducing plant response to environmental stresses and suppressing the activity of root pathogens. The extraradical mycorrhizal hyphae extend out in soil and generate significant changes in soil aggregation, organic matter accumulation and microbial activity in soil, which results in healthier soils. Mycorrhizae can play a major role in wasteland regeneration and afforestation programmes. The extensive exploitation of these mycosymbionts would help economically, besides minimizing environmental pollution.

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Selenite bioremediation potential of indigenous microorganisms from industrial activated sludge

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Summary

Ten bacterial strains were isolated from the activated sludge waste treatment system (BIOX) at the Exxon refinery in Benicia, California. Half of these isolates could be grown in minimal medium. When tested for selenite detoxification capability, these five isolates (members of the genera *Bacillus*, *Pseudomonas*, *Enterobacter* and *Aeromonas*), were capable of detoxifying selenite with kinetics similar to those of a well characterized *Bacillus subtilis* strain (168 Trp^+) studied previously. The selenite detoxification phenotype of the Exxon isolates was stable to repeated transfer on culture media which did not contain selenium. Microorganisms isolated from the Exxon BIOX reactor were capable of detoxifying selenite. Treatability studies using the whole BIOX microbial community were also carried out to evaluate substrates for their ability to support growth and selenite bioremediation. Under the appropriate conditions, indigenous microbial communities are capable of remediating selenite in situ.

Key words: *Bacillus subtilis*, bioremediation, selenite, oil refinery, activated sludge

Resumen

Se aislaron diez cepas bacterianas del sistema de tratamiento de fangos activados (BIOX) perteneciente a la refinería Exxon en Benicia, California. Cinco de dichas cepas (las que se pudo

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cultivar en el medio mínimo empleado en este estudio y que pertenecían a los géneros *Bacillus*, *Pseudomonas*, *Enterobacter* y *Aeromonas*), presentaron una capacidad de destoxificación del ion selenito muy similar a la observada en una cepa bien caracterizada (*Bacillus subtilis* 168 Trp^+). El fenotipo de destoxificación de selenito presentado por estas cepas aisladas del sistema de fangos activados se mantuvo estable, incluso después de haberlas subcultivado repetidas veces en un medio carente de selenio. Así mismo, se llevaron a cabo estudios para determinar el efecto del substrato en la capacidad de destoxificación del selenito. Se concluye que, en condiciones fisiológicas adecuadas, la comunidad bacteriana del sistema de fangos activados es capaz de eliminar el ion selenito del efluente.

Introduction

Selenium pollution is one of the most widely encountered and extensively studied examples of group V and VI metal hazards (2). Although required in trace amounts as an essential nutrient for humans and animals, selenium is also potentially toxic to living organisms even at very low concentrations. Environmentally toxic compounds of selenium are found as pollutants in groundwater, smelting effluents, agricultural and municipal wastewater, waste disposal sites, power plant cooling reservoirs and oil refinery waste streams.

The problem of selenium accumulation were highlighted by the ecological disaster of Kesterson National Wildlife Refuge, in California's San Joaquin Valley (11). Intense agricultural activity led to the accumulation of toxic concentrations of selenium in the agricultural drainage released in the Kesterson Reservoir. Selenium concentration resulted in deformities and deaths primarily in developing and newborn birds.

Selenium is a subsurface contaminant and, among other locations, is widely distributed in the western United States. It is found naturally in the San Joaquin Valley, California, generally in association with Cretaceous shales which, in turn, are associated with crude oil deposits. A major source of selenium in the San Francisco Bay is wastewater from the numerous oil refineries along the Carquinez Strait. In this fact, selenite is the oxidized form of selenium that predominates in oil refinery wastestreams. Although oil refineries often have treatment facilities, such as activated sludge bioreactors that use microorganisms to degrade hydrocarbons and nitrogen compounds, these facilities do not effectively remove or convert toxic metal contaminants such as selenium compounds.

Although a reduction in the selenium loading of the environment is desirable, there is currently no cost-effective selenium remediation technology available. Physical and chemical treatment technologies have been extensively evaluated over the last ten years without a successful outcome. On the contrary, biological treatment technologies are a promising solution to the problem.

The cycling of selenium in the environment is largely dependent on oxidation-reduction reactions performed by microorganisms, such as the conversion of soluble toxic forms, namely selenite and selenate, into a relatively nontoxic form (i.e., elemental selenium) (3). Selenite has been found to be an electron acceptor in an anaerobic form of respiration (8) and, also, to be reduced independently of dissimilatory electron transport (7) in an aerobic mechanism of selenite detoxification.

To understand the ecophysiology of bacterial reduction of selenite further, several strains were isolated from the activated sludge bioreactor,

"BIOX", of the Benicia Exxon Refinery in California. The purpose of this investigation was to study the microbial ecology of the BIOX system to determine (i) whether selenite detoxifying microorganisms were present that could account for the observed selenium removal, and (ii) whether selenite could be more effectively removed by altering culture conditions. Treatability studies of the complete BIOX microbial community were also carried out using sludge from the reactor to test the effect of carbon, nitrogen and phosphorus sources on growth and on selenite reduction.

Materials and methods

A series of samples was collected from the Exxon Benicia BIOX activated sludge bioreactor over a two-year period. The samples were immediately plated onto a complex rich medium (Difco Tryptose Blood Agar Base [TBAB]) or a chemically defined minimal medium (i.e., minimal glucose medium [MG]) containing per liter: K_2HPO_4, 14 g; KH_2PO_4, 6 g; $(NH_4)_2SO_4$, 2 g; $Na_3Citrate{\cdot}2H_2O$, 1.9 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; $Ca(NO_3)_2{\cdot}4H_2O$, 0.024 g; $MnCl_2{\cdot}4H_2O$, 0.020 g; $FeSO_4{\cdot}7H_2O$, 0.278 mg and 1% glucose. The MG did not contain selenite. The streak or spread plates were incubated at 37°C. This temperature value was chosen for comparison purposes with some previous studies carried out in our laboratory with a well characterized *Bacillus subtilis* strain grown at that temperature (4). In addition, although it fluctuates slightly depending on the refinery processes, the temperature of the bioreactor is usually around that value. Representative colony types were tested for growth on both MG and TBAB plates containing 1 mM sodium selenite (79 ppm selenium).

Immediately following isolation, individual strains were grown to mid-log in TBAB liquid medium, amended with 10% glycerol, and stored at –80°C. Subsequently, the Biolog Identification System was used to identify the isolates from the BIOX activated sludge.

Isolates of interest were grown in chemically defined MOPS (3-[N-Morpholino] propanesulfonic acid) medium (10) to evaluate their selenite detoxification capability. The MOPS medium contained: 10% (v/v) of a MOPS 10× mixture [400 mM MOPS, 40 mM tricine, 0.1 mM $FeSO_4$, 2.76 mM K_2SO_4, 1.4 mM $CaCl_2$, 40 mM $MgCl_2$, 1.0 mM $MnCl_2$, 3×10^{-5} mM $(NH_4)_6Mo_7O_{24}$, 4×10^{-3} mM H_3BO_3, 3×10^{-4} mM $CoCl_2$, 10^{-4} mM $CuSO_4$, and 10^{-4} mM $ZnSO_4$], 3 mM K_2HPO_4, 10 mM NH_4Cl and 70 mM glucose. Cultures were grown in 250 ml Erlenmeyer flasks with continuous shaking (275 rpm). Minimal medium plates were made by combining the above specified ingredients with 1.5% (w/v) agar (Difco). The growth temperature was 37°C.

Growth was initiated in shake flasks by using inocula from minimal medium plates. Before initiating liquid medium experiments, cultures were transferred twice into fresh medium, over a period of approximately 24 h. Reinoculations were timed to ensure that cultures were under excess nutrient conditions, and thus in an environment allowing balanced exponential growth. By using this pre-inoculation procedure, no lag phase was observed in the control treatments described below. For growth experiments with selenite, 35 ml of MOPS medium containing the indicated concentration of selenite was inoculated to an initial density of 5 Klett units. Growth experiments were repeated a minimum of three times with consistent results. Data from representative experiments are presented.

Growth was routinely monitored with a Klett colorimeter fitted with a red filter. Viability was assessed by serial dilutions of culture samples in growth medium lacking selenite, which were

immediately plated on the same medium to determine the number of viable cells.

In view of the importance of carbon substrates to remediation capability (4), it was of interest to study the response of the whole BIOX microbial community to different substrates—namely, maltose, acetate and propionate. To do so, and in order to increase growth rates, the components of the MOPS medium (i.e., MOPS 10× mixture, 3 mM K_2HPO_4 and 10 mM NH_4Cl) were added to the BIOX activated sludge before supplementation with the appropriate substrate and 150 ppb selenium as sodium selenite. Since the selenium concentration in the BIOX activated sludge samples varied significantly, this additional 150 ppb selenium (as sodium selenite) was added to the BIOX samples to assure a selenium concentration high enough to perform the experiments. The concentrations of the substrates were chosen so as to equalize the number of carbon atoms provided in the culture medium (70 mM glucose, 35 mM maltose, 210 mM sodium acetate and 140 mM sodium propionate). As expected, in this set of experiments, the initial concentration of selenium in the supernatant fractions was higher than the added 150 ppb, since the refinery effluent already contained selenite.

Selenium was determined with the supernatant fractions obtained by centrifuging samples at 15,000 × *g* for 20 min. Samples in the ppm concentration range were analyzed by Inductively Coupled Plasma Spectrometry using a Perkin-Elmer Plasma 40 Emission Spectrophotometer, Berkeley. Similarly, ppb concentrations of selenium were determined by hydride generation atomic absorption spectrophotometry on a Perkin-Elmer 2280 Atomic Absorption Spectrometer, Berkeley.

Cells were prepared for electron microscopy by fixing in 0.1 M sodium cacodylate buffer, pH 7.2, containing 1% glutaraldehyde and 1% paraformaldehyde. The fixed cells were washed in cacodylate buffer, dehydrated by a series of washes in aqueous ethanol solutions (30, 50, 70, 95, 100% v/v ethanol/water), and embedded in a low temperature resin (Lowicryl K4M) that was photopolymerized by long wavelength (360 nm) ultraviolet light at –35°C. Sections were examined in a JEOL 100CX transmission electron microscope. The designated inclusions were identified as selenium from the spectrum obtained by X-ray microanalysis using energy dispersive spectrometry in a JEOL JEM 200CX, Berkeley.

Results

After an average 48 h incubation of the TBAB plates at 37°C, ten bacterial strains were recovered independently from the Exxon plant samples. Of those ten, five were unable to grow on the chemically defined minimal glucose medium and were not studied further. The remaining five strains were identified as: *Bacillus* sp. 1, *Bacillus* sp. 2, *Pseudomonas* sp., *Enterobacter* sp. and *Aeromonas* sp., by the Biolog Microbial Identification System.

Our first objective was to determine whether the isolates were capable of growth in the presence of selenite, the oxidized form of selenium that predominates in oil refinery wastestreams. To test this possibility, cells were grown in defined MOPS medium containing different concentrations of selenite. Fig. 1 illustrates representative growth and selenite removal data from one of the Exxon isolates—namely, the Gram-positive *Bacillus* sp. 1—when cultures were supplemented with 1 mM sodium selenite (79 ppm selenium). Identical experiments were carried out with the Gram-negative *Aeromonas* sp. (data not shown).

Similar levels of growth were observed with both strains as determined by culture turbidity. In addition, both strains were capable of reducing the concentration of selenium in the super-

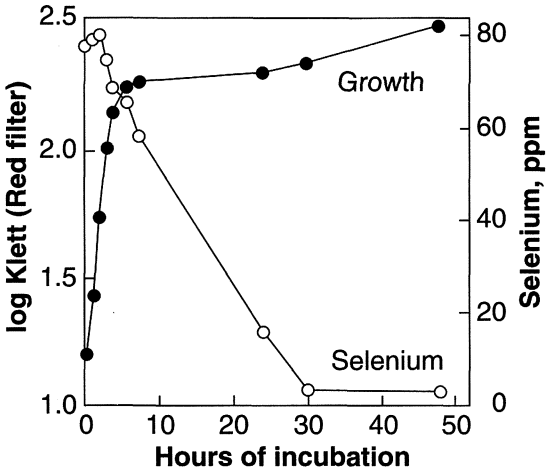

FIG. 1. Effect of 1 mM sodium selenite (79 ppm selenium) on growth of *Bacillus* sp. 1 and selenium concentration in the supernatant fraction.

natant fraction from 79 ppm to less than 4 ppm. The cultures turned red as growth progressed, which suggested the conversion of the soluble selenite into the insoluble red elemental form.

The data indicated that the two strains catalyzed a valence transformation of 1 mM selenite, but left open the question of the magnitude of the change at the much lower selenite concentrations typical of oil refinery wastestreams. When the *Bacillus* sp. 1 cultures were supplemented with concentrations of selenite representative of those occurring in the BIOX activated sludge bioreactor (ca., 150 ppb selenium as sodium selenite), the cells demonstrated the previously observed pattern of valence transformation (Fig. 2). Again, identical experiments were carried out with *Aeromonas* sp. (data not shown). Both strains were able to reduce the selenium concentration in the supernatant fraction to less than 3 ppb after 6 h of incubation.

In the experiments described, individual strains were grown using glucose as a carbon source. As mentioned above, in view of the importance of carbon substrates to remediation capability (4), it was of interest to study the response of the whole BIOX microbial community to different substrates—namely, maltose, acetate and propionate. The effect of various carbon sources on microbial growth (determined by total viable counts) is shown in Table 1. As observed previously (4), sugars (glucose and maltose) supported higher growth rates than organic acids, during the first 24 h of incubation. After 60 h of incubation, cultures supplemented with sodium propionate showed the greatest number of colony-forming units (CFU).

Sugars appear to be the most effective substrates not only for early growth but for selenite reduction (data not shown). When cultures were supplemented with either glucose or maltose, the selenium concentration in the supernatant fraction decreased to less than 10 ppb after 20 h of incubation. Although the other substrates tested were capable of decreasing the selenium concentration to a similar extent, much longer incubation times were required.

Additional treatability studies were carried out to assess the importance of the nitrogen or phosphorus source using a different sample of

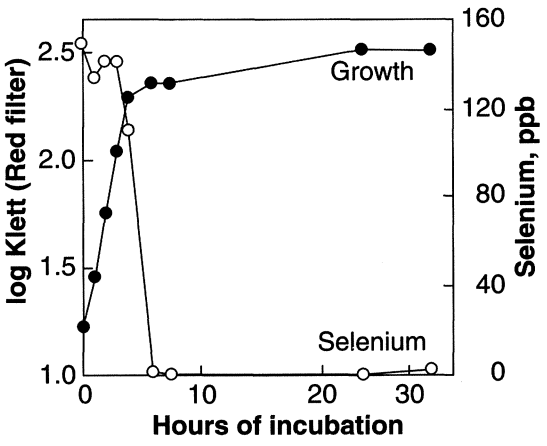

FIG. 2. Effect of 150 ppb selenium (as sodium selenite) on growth of *Bacillus* sp. 1 and selenium concentration in the supernatant fraction.

TABLE 1. Effect of various substrates on BIOX microbial community growth expressed as total viable cell count (CFU/ml [$\times 10^7$]). Cultures were supplemented with 150 ppb selenium as sodium selenite

	Incubation time		
Substrate	20 h	36 h	60 h
Glucose	195	164	92
Maltose	199	221	182
Acetate	12	120	101
Propionate	0	15	399

BIOX activated sludge. Growth and selenite reduction were monitored in the presence and absence of NH_4Cl and K_2HPO_4. In this set of experiments, the remaining components of the MOPS medium (i.e., MOPS 10× mixture and 70 mM glucose) were added to the BIOX activated sludge before supplementation with 150 ppb selenium as sodium selenite. As seen in Fig. 3A, the cultures lacking an external source of phosphorus grew considerably after 24 h of incubation, although a bit less than the control flasks containing both the nitrogen and phosphorus source. By contrast, addition of NH_4Cl as a nitrogen source appeared essential for significant growth (Fig. 3A).

Fig. 3B shows the removal of selenite from the supernatant fraction during growth of the BIOX sludge cultures under these conditions. Whereas the presence of phosphorus made little difference, the lack of an external source of nitrogen delayed the remediation of selenite. After 24 h of incubation, the cultures lacking NH_4Cl had only reduced 32% of the initial selenium concentration as compared to 81 and 97% for the control (nitrogen and phosphorus present) and the culture lacking added phosphorus, respectively.

Finally, the question arose as to the nature of the product formed by the BIOX isolates (i.e., whether it is elemental selenium as has been shown to be the case for other bacteria) (1, 4, 5). To this end, we examined cultures grown in 1 mM selenite by transmission electron microscopy and identified selenium by energy dispersive X-ray spectrometry analysis. As we previously reported for *Pseudomonas fluorescens* (5), electron dense intracellular elemental selenium granules were observed to accumulate within the cells (data not shown). Fig. 4 shows the spectrum

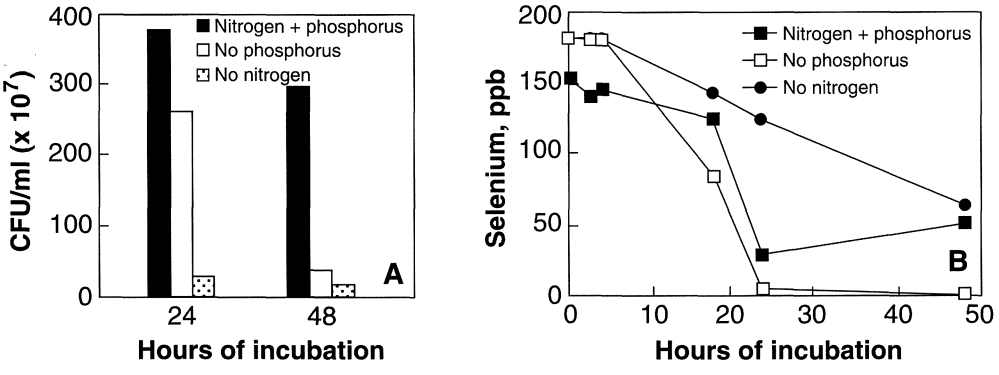

FIG. 3. (A) Effect of the presence of 10 mM NH_4Cl (nitrogen source) or 3 mM K_2HPO_4 (phosphorus source) on BIOX microbial community growth in cultures supplemented with 150 ppb selenium as sodium selenite. (B) Selenium removed from the supernatant fraction during growth of the BIOX sludge cultures supplemented with 150 ppb selenium as sodium selenite under those conditions.

obtained when applying energy dispersive X-ray microprobe analysis to one of those electron dense granules. In this respect, the organism studied most extensively, *Aeromonas* sp., appeared to resemble *Wolinella succinogenes* (12) and *Salmonella heidelberg* (9), which deposit selenium granules in the cytoplasm, and to differ from *B. subtilis* (4) and *Escherichia coli* (6), which deposit selenium as granules between the cell wall and the plasma membrane.

Discussion

The purpose of this study was to determine whether the microbial population of the BIOX system is capable of reducing selenite under altered culture conditions. The product formed, elemental selenium, is much less toxic than the selenite precursor, and it is insoluble.

Each of the five Exxon BIOX isolates capable of growing on the chemically defined minimal glucose medium was found to detoxify selenite with kinetics similar to the well characterized laboratory *B. subtilis* strain 168 Trp^+ (4), irrespective of whether it was a Gram-positive or Gram-negative. The selenite detoxifying phenotype of the Exxon isolates was stable to repeated transfer on defined or complex culture medium which did not contain selenium. The selenite reducing phenotype of *B. subtilis* strain 168 Trp^+ has been stable to repeated transfer from normal to selenite containing culture media over the past five years in our laboratory.

It is clear from our data that resident microorganisms in the Exxon BIOX reactor are capa-

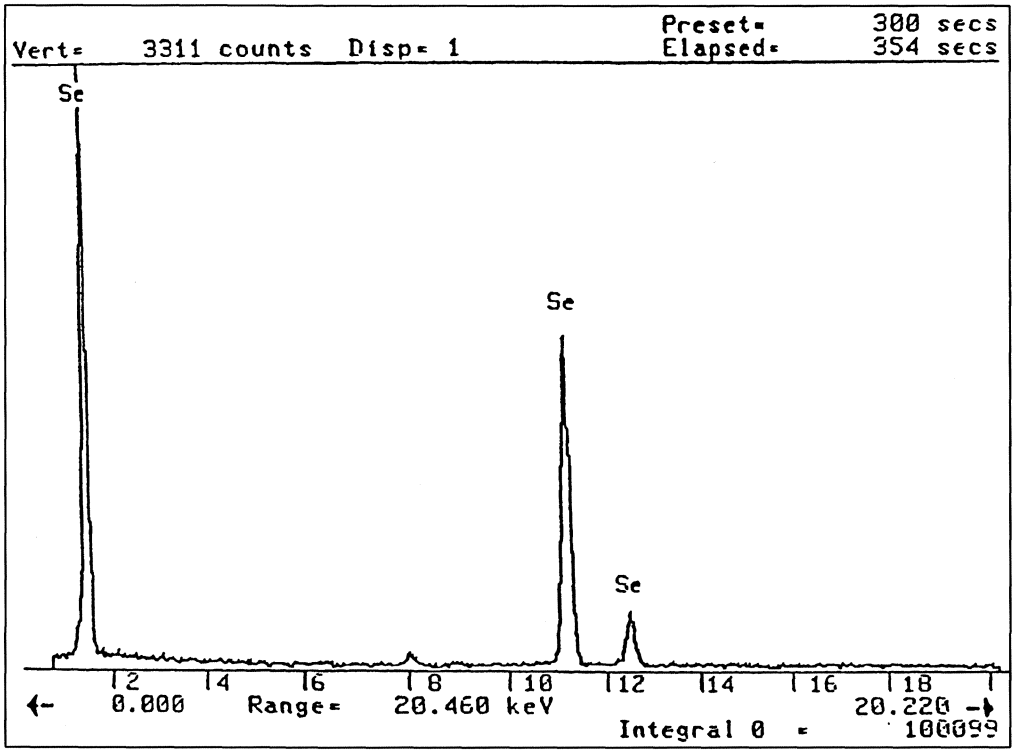

FIG. 4. Energy dispersive X-ray microprobe analysis of *Aeromonas* sp. cells grown in MOPS medium containing 1 mM sodium selenite. The spectrum was obtained with a single cellular granule.

ble of detoxifying selenite, the major toxic selenium compound present in refinery effluent. Our treatability studies suggest that the indigenous bacteria may be capable of remediating selenite from the incoming feed stream by modifying conditions of the system, notably by adding a source of carbon (such as glucose or maltose) and nitrogen (such as NH_4Cl). The other carbon substrates tested, acetate and propionate, were not as efficient for selenite remediation. The findings indicate that indigenous isolates from the Exxon BIOX activated sludge system may be useful for the remediation of sites polluted with selenite. Since the BIOX activated sludge system operates under conditions of nitrogen deficiency, it was not surprising to find that the absence of an external nitrogen source (NH_4Cl in this case) resulted in both growth and selenite reduction. Further research is in progress to test other nitrogen sources on selenite remediation by the BIOX microbial community.

Acknowledgements

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Sorption of metals by *Chlorobium* spp.

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Summary

The capacity of two species of green phototrophic sulfur bacteria, *Chlorobium limicola* and *C. phaeobacteroides*, to sorb several metal ions (Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+}) has been tested in laboratory batch cultures at increasing concentrations up to 2,000 µmol/l. Except for nickel—which was not sorbed to bacterial cells—the rest of metals tested were bound in a fast and passive process, which was mathematically described by means of Freundlich isotherms models. The sorption capacity of the two species studied were found to be dependent on the metal involved, whereas no differences were observed in the sorption intensity, suggesting that in all cases the sorption process proceeds in a similar way. Further, the comparison of the sorption intensity values as well as the metal recovery index (R_i), for both species, revealed that *C. phaeobacteroides* was more efficient that *C. limicola* to attach metal ions. The ecological significance of this ability in the water column of some stratified lakes, where coinciding maxima of ferrous iron and green photosynthetic sulfur bacteria are frequently found, is discussed.

Key words: *Chlorobium*, green sulfur bacteria, heavy metals, metal sorption, aquatic microbial ecology

Resumen

Se ha experimentado la capacidad de adsorción de diferentes iones metálicos (Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} y Pb^{2+}) por parte de dos especies de bacterias fototróficas verdes del azufre,

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Chlorobium limicola y *C. phaeobacteroides*, en cultivos discontinuos de laboratorio y a concentraciones de hasta 2000 µmol/l. Excepto el níquel, que no fue adsorbido por las células bacterianas, el resto de metales examinados se adsorbieron de forma rápida y pasiva a las paredes bacterianas. La adsorción se ha descrito matemáticamente mediante isotermas de Freundlich. La capacidad de adsorción de los diferentes metales por parte de las dos especies dependió del tipo de metal implicado, mientras que no se observaron diferencias en la intensidad de adsorción, hecho que sugiere que dicho proceso se produjo de manera similar en todos los casos. Por otra parte, la comparación de las capacidades de adsorción, así como del índice de recuperación de metales (R_i) entre las dos especies reveló que *C. phaeobacteroides* es más eficiente que *C. limicola* en la adsorción de cationes metálicos. Se discute el significado ecológico de esta relación en ambientes naturales, especialmente en la columna de agua de algunos lagos estratificados, donde es frecuente la coincidencia de máximos de bacterioclorofila *e* y de Fe^{2+}.

Introduction

Photosynthetic sulfur bacteria are commonly found in anaerobic layers of stratified lakes where light and sulfide occur together (14, 20, 22). In these parts of the water column, some metals, particularly iron and manganese, present active cycles where the different metallic species exhibit seasonal fluxes between the sediment and the water column (2, 8, 9, 21, 25). In some cases, other metals such as cadmium, chromium, copper, zinc, nickel or lead can also be detected (1, 3, 18).

Although the simultaneous presence of photosynthetic sulfur bateria and heavy metals in aquatic environments is frequent, studies focused on their potential interactions and ecophysiological implications are scarce in the literature (Garcia-Gil, L. J. 1990. Ph. D. Thesis, Autonomous University of Barcelona). The relationships between photosynthetic bacteria and metal ions may be significant in their biogeochemical cycles assuming two factors: first, the well known capacity of bacterial cell walls to sorb metals (4, 5, 7, 10, 13, 15), and second, the capacity of some *Chlorobium* species to photosynthetically oxidize MnS and FeS in a process that seems to be dependent on the ability of bacterial cells to attach Fe^{2+} and Mn^{2+} to their membranes (6).

The aim of this work was to test the capacity of two species of green photosynthetic sulfur bacteria to bind several metal cations in order to obtain further information about their potential eco-physiological role. For this purpose, green *Chlorobium limicola* and brown-colored *Chlorobium phaeobacteroides* were incubated in media containing increasing concentrations of Mn^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Pb^{2+}. Then, the cell-bound metallic fractions were quantified by atomic absorption spectrophotometry. Results show that, except for nickel—which was not sorbed to bacterial cells—, the rest of metals tested were bound to the cell membrane in a fast and passive process, that can be described by Freundlich isotherms. The observation that brown *C. phaeobacteroides* is more efficient that *C. limicola* in the recovery of metal ions from solution seems to be related to the adaptation of this bacterium to its natural habitat.

Material and methods

Bacterial strains and growth conditions. *C. limicola* and *C. phaeobacteroides* are obli-

gate anaerobic, photosynthetic anoxygenic, green sulfur bacteria, of the family Chlorobiaceae (16). *C. limicola* strain DSM249 was from the German Collection of Microorganisms and Cell Cultures. *C. phaeobacteroides* strain UdG6030 was isolated from lake Banyoles, located near Girona (NE of Spain), following the isolation methods of van Niel (24) and Kondratieva et al. (16). Batch cultures were grown at room temperature in 500 ml glass bottles completely filled with standard Pfennig mineral medium (16). The final pH was adjusted to 6.7. Since the bacterial metal uptake was very sensitive to changes in the pH, it was ensured to remain fairly constant over the experimental time by the CO_2/HCO_3^- buffering system present in the medium.

Batch cultures were continuously stirred and mantained under nitrogen pressure to ensure anaerobic conditions. Incubation was done at room temperature and under a saturating light intensity of 30 μEinstein m^{-2} s^{-1}. Illumination was provided by 2 × 100 W tungsten incandescent bulbs. Light intensity was measured using a quantometer Biospherical Instruments QSP-170.

Experimental procedures. The following metallic salt solutions: $MnSO_4 \cdot H_2O$; $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$; $CuSO_4$; $ZnSO_4 \cdot 7H_2O$; $Cd(NO_3)_2$; $NiCl_2 \cdot 6H_2O$, and $PbNO_3$, were added to sulfide-free bacterial cultures to avoid chemical precipitation of undesired metal sulfides. Concentrations tested ranged from 10 to 2,000 μmol/l. Thirteen min after each addition, 10 ml aliquots were removed from the cultures and centrifuged at 11,950 × *g* for 15 min. Pellets were resuspended in 10 ml of MilliQ ultrapure water by mild sonication with a B-Braun Labsonic 2,000, and acidified by adding 0.1 ml of 1 N HNO_3 to redissolve attached metal ions Cell-bound metal was then measured from these suspensions by atomic absortion spectroscopy using a Varian Spectra-300 model, equipped with hollow cathode lamps Photron AAS (for Ni and Mn) and Varian Spectra AA lamps (for Fe, Cd, Cu, Pb, and Zn). All analyses were performed under an air-acetylene flame. Concentrations of different metals were calculated after the calibration of the system with appropriate metal solution standards (Carlo Erba Reagents). Control samples, consisting of sterile medium plus correspondant metal ions, were processed in the same way than experimental bacterial cultures, to quantify either the possible precipitation or the quelation of unbound metals with media components (carbonates, sulfur, etc). For all cases, the chemical precipitation was estimated to be less than 3% of total metal concentration.

Biomass of the cultures was estimated from protein concentration at the end of the experimental time. A 10 ml aliquot was centrifuged at 11,950 × *g* for 15 min, and wet cell pellets were used for protein determinations. To avoid the interference of photosynthetic pigments, they were extracted from pellets according to Borrego and Garcia-Gil (6). Protein concentration was measured following the method by Lowry (17).

Isotherms models. For the mathematical description of the sorption process, experimental data were fitted to Freundlich isotherms (23, 27). The Freundlich equation can be expressed as follows,

$$q_e = K_f C^{1/n}$$

where C is the metal concentration of the medium, q_e is the metal sorbed (in μmoles) per gram of cell protein at concentration C, and K_f and n are constants.

Results

Freundlich isotherm model is commonly used to define the binding of metal cations to bacterial cell walls. The monophasic isotherms for both *Chlorobium* species and the constant values resulting from calculation are shown in Fig. 1 and

Table 1, respectively. For all metals tested, both bacterial species exhibited no saturation within the range of concentrations tested. However, two exceptions can be considered: nickel, which even at concentrations higher than 2,000 µmol/l did not bound to the cell walls, and manganese, which only adsorbed at concentrations higher than 100 µmol/l.

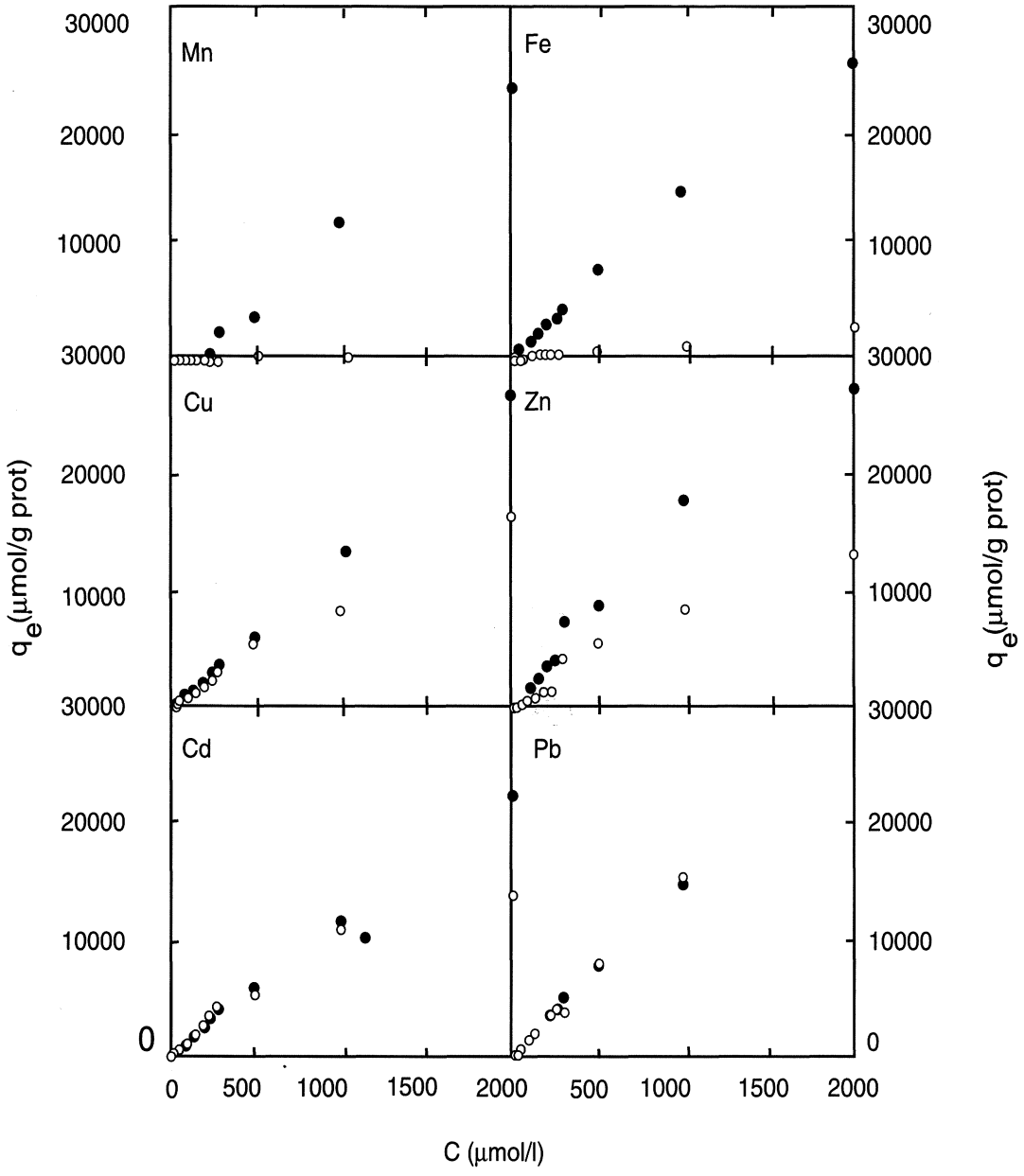

FIG. 1. Freundlich sorption isotherms for *Chlorobium limicola* (○) and *Chlorobium phaeobacteroides* (●) for the different metals tested.

TABLE 1. Freundlich constants for the different metals and species studied. The log K_f is the sorption capacity (SC) for C = 1. The sorption intensity (SI) was estimated from the slope value (n^{-1}) according to Shuttleworth and Unz (23)

Species	Metal	SC	SI	r
C. limicola	Mn	–0.03	1.15	0.997
	Fe	0.04	0.98	0.994
	Ni	–	–	–
	Cu	1.01	1.03	0.994
	Zn	1.11	1.05	0.964
	Cd	1.40	1.14	0.998
	Pb	1.29	1.03	0.999
C. phaeobacteroides	Mn	0.58	0.87	0.995
	Fe	1.35	1.08	0.999
	Ni	–	–	–
	Cu	0.89	0.93	0.999
	Zn	1.29	1.01	0.993
	Cd	1.22	1.05	0.999
	Pb	1.27	1.03	0.999

Both *Chlorobium* species exhibited differences on the sorption of the different metals. According to Shuttleworth and Unz (23), the metal sorption capacity (SC) can be calculated from the isotherm as the log K_f, whereas the slope of the isotherm (n^{-1}) could be used as an estimation of the sorption intensity (SI). Although SI values for both species were fairly similar for all metals tested, the SC values calculated for *C. phaeobacteroides* were generally higher than those of *C. limicola* (Table 1). This observation suggests that in all cases the sorption process proceeded in a similar way whereas the sorption capacity was found to be related to both the metal type and the species involved.

Another comparative variable used was an estimation of the metal recovery efficiency (recovery index, R_i), which was calculated as the percentage of the ratio between the cell-bound metal and total metal concentration. Except for lead, *C. phaeobacteroides* showed a higher efficiency than *C. limicola* in the removal of metal cations from the media (Fig. 2). This fact was especially apparent with Fe^{2+} and Mn^{2+}, which were poorly recovered by *C. limicola,* (with R_i of 10.1 and 1.1%, respectively) as compared to the recovering by *C. phaeobacteroides* (with R_i of 81.9 and 53.2%, respectively).

Discussion

The metal sorption capacity of *Chlorobium* spp. in the laboratory seems to be determined by the metal itself, as happens in other Gram-negative microorganisms (19). However, no effect of the metal concentration in the medium was detected in our case within the range of concentrations tested. The fact that no saturation was observed in any case at concentrations up to 2,000 µmol/l can be explained by an intense metal deposition around firstly chelated metal ions, that act as nucleation sites (7).

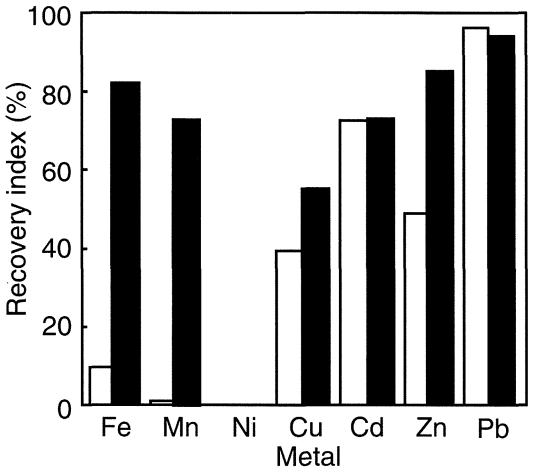

FIG. 2. Mean Recovery Index (R_i, in %) for the *Chlorobium* species studied. Symbols: ■ *C. phaeobacteroides*, ❑ *C. limicola*.

The inability of *Chlorobium* spp. to sorb nickel is difficult to understand since many microorganisms can easily bind this metal to their membranes (10, 15). The presence of different kinds of exopolymers and the different structures of the bacterial walls are reported as the main responsible factors for the selectivity of the metal binding process (7, 13). Differences in the structural properties of bacterial cell walls of the *Chlorobium* strains used in the experiment could account for the observed behavior, but no previous studies supporting this hypothesis have been found.

The different efficiencies of metal recovery exhibited by both *Chlorobium* species could arise from the different origin of the strains used. Whereas *C. limicola* DSM 249 is a collection strain, *C. phaeobacteroides* was isolated from a meromictic lake where coinciding maxima of BChl *e* and Fe^{2+} have been frequently found (11, 12). The ability of brown *Chlorobium* to photosynthetically oxydize FeS and MnS has been related to the capacity of *Chlorobium* cells to attach Fe^{2+} and Mn^{2+} to cell membranes and use them as S^{2-} traps in sulfide limited environments (Garcia-Gil, L. J. 1990. Ph. D. Thesis, Autonomous University of Barcelona; and 6). A similar relation was found by Wainwright and Grayston in fungi, where the metal sulfide oxidation process is strongly dependent on the capacity of fungal filaments to bind metal ions (26). According to these observations, it seems plausible that the isolated *C. phaeobacteroides* strain UdG6030 has a high affinity to metal cations, particularly Fe^{2+}, because this high affinity appears to be useful in natural conditions. Although other metals such as Cu^{2+}, Zn^{2+}, Cd^{2+}, or Pb^{2+} were efficiently removed from the medium, no evident benefit could be obtained from their sorption, since their metal sulfides are hardly oxidized (6). In these cases, metal binding appears as a simple chemical reaction rather than a controlled physiological process.

The study of the interaction between natural phototrophic sulfur bacteria and metal cycles in stratified lakes can provide further information about the factors that control metal sorption processes under natural conditions, and also about some additional physiological adaptations of these bacteria to their environments. Besides, studies focused on the ability of different *Chlorobium* species—and extensively of other anoxygenic photosynthetic bacteria—to recover metal ions from solution can be useful to develop new techniques for wastewater bioremediation and for other studies on applied microbial ecology.

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Gram-positive bacteria of marine origin: a numerical taxonomic study on Mediterranean isolates

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Summary

A numerical taxonomic study was performed on 65 Gram-positive wild strains of heterotrophic, aerobic, marine bacteria, and 9 reference strains. The isolates were obtained from oysters and seawater sampled monthly over one year, by direct plating on Marine Agar. The strains were characterized by 96 morphological, biochemical, physiological and nutritional tests. Clustering yielded 13 phena at 0.62 similarity level (S_j coefficient). Only one of the seven phena containing wild isolates could be identified (*Bacillus marinus*). A pronounced salt requirement was found in most isolates.

Key words: *Bacillus marinus*, Gram-positive marine bacteria, numerical taxonomy, oysters, Mediterranean Sea

Resumen

Se realizó un estudio mediante taxonomía numérica de 65 cepas ambientales de bacterias Gram positivas heterotróficas aeróbicas de origen marino, junto con 9 cepas de referencia. Los aislados se obtuvieron de muestras de ostras y agua de mar recogidas mensualmente a lo largo de un año, mediante siembra directa en Marine Agar. Las cepas se caracterizaron mediante 96 pruebas morfológicas, bioquímicas, fisiológicas y nutricionales. El análisis de agrupamiento resolvió 13

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fenones al nivel de semejanza 0,62 (coeficiente S_J). Sólo pudo ser identificado uno de los siete grupos que contenían aislados ambientales (*Bacillus marinus*). La mayoría de las cepas mostraron un marcado requerimiento de sal.

Introduction

The role of Gram-positive bacteria as autochtonous members of marine bacterial communities is not generally recognized, although there are some reports on high percentages of isolation of these microorganisms from specific habitats, such as the skin and mucoses of marine mammals (23), or from estuarine waters, where they are oligotrophic members (13). Recent studies suggest, however, that marine Gram-positive bacteria represent a large percentage of the culturable heterotrophs associated with algal surfaces and sediments (6, 7, 14), and that most of them require seawater to grow, a trait classically considered as definite for indigenous marine bacteria (12). Detailed taxonomic information on these Gram-positives of marine origin is also scarce, and deals mainly with fish pathogens (10) or selected genera (4, 5, 19, 28).

A study of bacteria associated with oysters and seawater samples from the Spanish Mediterranean coast yielded near six hundred bacterial strains. They were isolated by direct inoculation on Marine Agar plates, a general medium for marine, non-obligately oligotrophic bacteria. Gram-positive isolates represented about 10–15% of the total isolates, and they reached 40% in some samples (Ortigosa, 1995, Ph. D. Dissertation). All isolates were extensively characterized, and the data were submitted to numerical taxonomic analysis to determine the taxonomic diversity of the heterotrophic bacterial community. Numerical taxonomic studies of the Gram-negatives have been recently published (16, 17). The results of the taxonomic analyses of the Gram-positive strains are presented here.

Materials and methods

Bacterial strains. Sixty five wild strains were isolated during an annual survey of seawater and oysters cultivated off the Mediterranean coast at Valencia (Spain). Isolation was achieved by direct plating on Marine Agar 2216 (MA, Difco) of seawater or oyster flesh homogenates diluted in sterile seawater. Cultures were maintained on Marine Agar slants at room temperature and as suspensions in Marine Broth 2216 (MB, Difco) supplemented with 10% glycerol at –80°C. Nine reference strains were obtained from culture collections. They are shown in Table 1.

Characterization of strains. Unless otherwise indicated, the incubation temperature was 25°C, all commercial media were supplemented up to 1% (w/v) NaCl, and all strains were routinely cultured on MA or MB. The following phenotypic properties were recorded for all strains: Gram reaction by the KOH method (2), plus Gram stain (Hucker's method) when the reaction was dubious or positive; cell morphology, spore presence in wet and stained mounts (no attempt was made to induce sporulation in different culture media); arrangement of cells and motility in wet mounts examined under phase contrast microscopy; pigment production and swarming on MA; catalase (10 vol H_2O_2), Kovac's oxidase, O/F test (O/F medium, Difco, rehydrated with 1/2 strength Artificial Sea Water (ASW) plus 1% glucose); ability to reduce nitrate to nitrite in Nitrate Broth plus 1% NaCl (24); luminescence on MA and Seawater Agar (1% Bacteriological Peptone, Oxoid, 1% Meat Extract, 75% (v/v) aged, filtered seawater); Thornley's arginine dihydrolase (ADH) (1); alkalynization/

TABLE 1. Bacterial reference strains used

Species	Strains[a]	Origin
Bacillus marinus	CECT4272^T	Marine sediment
Bacillus marinus	DSM 1298	Id.
Bacillus licheniformis	LMG 7629	Seawater
Micrococcus luteus	CECT 51^T	–
Kytococcus (Micrococcus) sedentarius	DSM 20547^T	Seawater
Planococcus citreus	CECT 951^T	Seawater
Planococcus kocurii	DSM 20747^T	Skin of cod
Rhodococcus marinonascens	DSM 43752^T	Marine sediment
Dietzia (Rhodococcus) maris	LMG 5361^T	Soil

[a] CECT, Colección Española de Cultivos Tipo, Universitat de València, Burjassot, Spain; DSM, Deustche Sammlung von Mikroorganismen, Braunschweig, Germany; LMG, Col. Laboratorium voor Microbiologie, Ghent, Belgium.

decarboxylation of arginine in Moeller broth (Decarboxylase Medium Base, Difco); H_2S production from thiosulphate; motility and indole production in Sulphide Indole Motility (SIM) Medium (Difco); urease (Urea Broth, Pronadisa); xanthine and tyrosine clearing on MA plus 1% substrate (14 days); growth and acid production from sucrose on Thiosulphate Citrate Bile Sucrose (TCBS, Oxoid), hydrolysis of agar, esculin (11), casein, Tween-20 and 80, lecithin (Egg Yolk Emulsion, Oxoid), starch, sodium alginate (Sigma), chitin (Sigma), and sodium dodecyl sulphate (SDS, sulphatase), all of them by using MA as basal medium and 6 days incubation; DNAse activity on DNAse Agar (Oxoid), gelatin liquefaction on MB + 12% gelatin (Oxoid); growth at 4°C (7 days), 37, 40 and 42°C on MB, growth in Salt Tolerance Broth (1% Tryptone, 0.3% Yeast Extract, plus 0-6-8-10-12 or 20% NaCl, pH 7.2) and use as sole carbon sources, on Basal Medium plates (BM) (1), of the compounds listed in Table 2.

Numerical taxonomy. Results of the phenotypic characterization (excluding invariant characters) were coded as binary responses. The data matrix (82 OTUs × 87 characters) was submitted to numerical taxonomic analysis by the program NTSYS.pc version 1.70 of Dr. F.J. Rohlf (Exeter Software, NY). Both S_{SM} and S_J coefficients were used, clustering was performed by UPGMA and the corresponding cophenetic coefficients were calculated. Test reproducibility was evaluated by duplication of the nine reference strains and the corresponding error was calculated (25).

Results and discussion

The error due to lack of test reproducibility was evaluated to be 2.7%. Few tests accounted for the major part of this overall figure, especially temperature and salinity growth ranges.

Cluster analysis was performed independently of S_{SM} and S_J-based similarity matrix. Cophenetic correlation between similarities of resulting dendrograms and the corresponding original matrix were 0.75 and 0.88, indicating that the S_J-based analysis reflected more accurately the resemblance among the strains. Thus, we selected the S_J-based tree for phena definition and description.

TABLE 2. Characteristics of seven phena of Gram-positive marine bacteria[a,b]

Phenon (no. of strains)	1 /9/	2 /5/	3 /2/	4 /3/	5 /2/	6 /6/	7 /6/
Cocci	3	1	+	–	–	–	–
Cocobacilli	3	2	–	–	1	–	–
Rods	1	2	–	+	1	+	+
Spores	1	–	–	2	1	–	+
Motility	–	–	+	NT	+	–	+
Pigment production	1	–	+	–	–	–	2
NO_3^- reduction	4	+	–	+	1	–	+
Esculin hydrolysis	–	NG	NG	+	–	NG	3
Xanthine clearing	3	–	–	–	+	–	–
Acid from glucose	–	+	–	NG	NG	–	NG
Growth without NaCl	1	–	–	1	–	–	–
Growth at 6% NaCl	4	+	1	–	+	3	+
Growth at 8% NaCl	2	+	–	–	+	1	2
Growth at 10% NaCl	1	4	–	–	+	–	–
Growth at 20% NaCl	1	–	–	–	+	–	–
Growth at 4°C	1	–	1	–	+	–	–
Growth at 40°C	–	4	–	–	+	+	+
Growth at 42°C	–	–	–	–	+	–	4
Gelatin hydrolysis	1	–	1	+	–	–	2
Casein hydrolysis	5	–	–	+	–	–	5
Tween-20 hydrolysis	4	+	+	–	+	+	4
Tween-80 hydrolysis	2	+	–	1	–	1	+
Lecithin hydrolysis	1	–	–	–	–	–	+
DNA hydrolysis	1	NG	–	–	–	–	+
Use of sole carbon sources:							
D-Ribose	8	+	+	+	–	–	–
L-Arabinose	3	+	–	1	–	–	–
D-Xylose	8	–	–	+	–	–	–
D-Glucose	+	+	+	+	+	+	–
D-Fructose, D-mannose	+	+	+	+	+	–	–
D-Galactose	+	+	+	–	1	–	–
Maltose	7	2	+	+	1	–	–
Cellobiose	+	+	+	+	–	–	–
Sucrose	5	–	+	+	+	–	–
Lactose	1	–	+	+	–	–	–
Melibiose	7	–	+	+	–	–	–
Amygdalin	–	–	–	+	–	–	–
Salicin, arbutin	–	–	+	+	–	–	–

Continued on following page

TABLE 2.—*Continued*

Phenon (no. of strains)	1 /9/	2 /5/	3 /2/	4 /3/	5 /2/	6 /6/	7 /6/
D-Gluconate	2	+	+	+	+	–	–
D-Glucuronate	6	+	+	+	–	–	–
D-Galacturonate	–	+	–	1	–	–	–
N-Acetil D-glucosamine	+	–	–	+	1	–	+
Glycerol	+	–	+	+	+	+	–
i-Erythritol	1	+	–	–	–	–	–
D-Mannitol	8	+	+	+	–	–	–
D-Sorbitol	–	+	–	1	–	–	–
m-Inositol	6	1	–	+	1	–	–
Citrate	+	+	+	+	+	–	–
α-Ketoglutarate	+	+	+	1	–	+	–
Succinate, fumarate, DL-malate							
Acetate, DL-β-hydroxybutyrate	+	+	+	+	+	+	–
DL-Lactate	8	+	+	+	+	+	4
Glycerate	6	–	–	+	1	–	–
p-Hydroxybenzoate	5	+	–	–	–	–	–
Glycine	7	–	–	–	–	–	–
L-Leucine	8	2	+	–	1	–	–
L-Serine	+	3	+	–	–	+	–
L-Threonine	+	+	–	1	1	–	–
L-Arginine	+	–	–	+	1	+	–
L-Tyrosine, L-citrulline	8	–	–	–	1	–	–
L-Glutamate, L-alanine	+	4	+	+	+	+	–
γ-Aminobutyrate	7	+	+	–	+	+	–
L-Ornithine	+	+	–	1	+	+	–
Sarcosine	6	+	–	–	–	+	–
Putrescine	6	2	–	–	–	3	–

[a] +, All strains positive; –, all strains negative; NT, not tested; NG, no growth; numbers, indicate number of positive strains in each phenon.

[b] All strains included in the 7 phena were positive for the following characters: Gram reaction, catalase, oxidase (except for 1 strain in phenon 4), growth in Marine Broth and at 25 and 37°C. All were negative for: denitrification, luminescence, arginine dihydrolase, indole and H_2S production, Voges–Proskauer, urease, growth at 12% NaCl (except for one strain in phena 1 and 2, and the strains in phenon 5), growth on TCBS agar, hydrolysis of alginate, starch (except for one strain in phenon 4), chitin (except for one strain in phena 1 and 7), sulphatase, and use of aconitate and D-saccharate.

At 0.62 similarity level, 13 phena of two or more members were defined, but 6 of them contained only reference strains and their duplicates (Fig. 1). The remaining 7 phena were composed exclusively by fresh isolates, and thirty seven strains remained unclustered.

The seven phena have been numbered on Fig. 1, and their properties are shown in Table 1. All strains grouped in these phena were positive for catalase, grew in Marine Broth at 25°C, and were negative for: glucose fermentation in O/F medium, denitrification, luminescence,

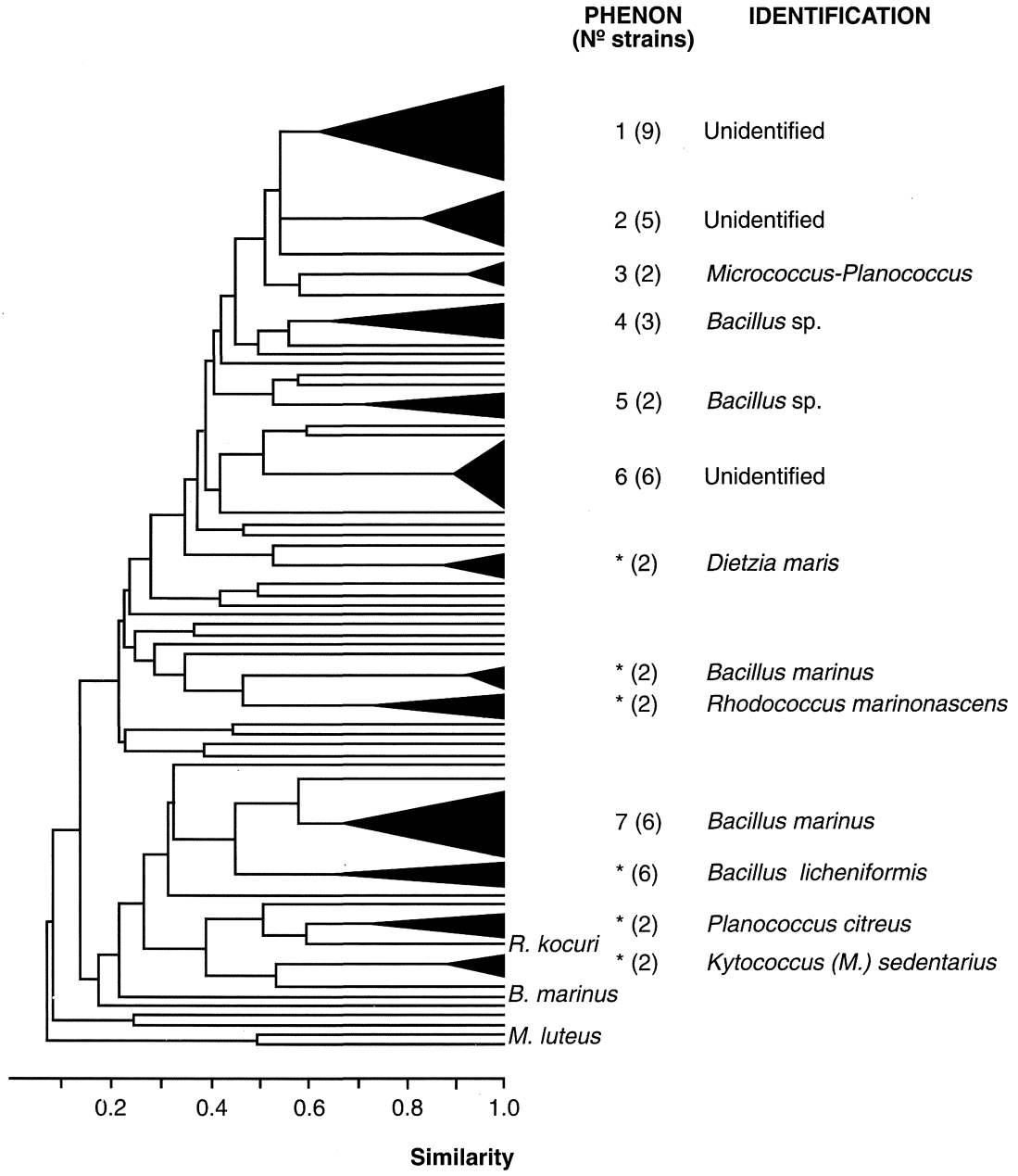

FIG. 1. Simplified phenogram showing relationships among Gram-positive bacteria of marine origin and 9 reference strains, based on UPGMA/S_J analysis. Asterisks indicate phena composed exclusively by reference strains and their duplicates. Unclustered reference strains are indicated.

Voges–Proskauer, arginine dihydrolase, urease, alginate hydrolysis, H_2S and indole production in SIM medium, and ability to growth on TCBS agar.

Phenon 1. The nine strains included in this phenon clustered at a similarity of 0.63; they were isolated both from seawater and from oyster samples. Most of them were cocci and

cocobacilli but one was a sporeforming rod. All strains were non-motile and unpigmented, most of them reduced nitrates and were oxidase positive. Because of the low similarity among the strains and the inclusion of different morphologies, salinity ranges and hydrolytic abilities, this phenon may be an heterogeneous assemblage of strains unified by the wide number of compounds serving as sole carbon sources: in fact, only 4 out of the 47 compounds tested were not used by any of the strains.

Phenon 2. It comprises 5 strains that join at high similarity level (0.83). All strains were isolated from seawater in autumn. They were non-motile and nitrate positive, showed diverse celular morphologies, did not grow without added NaCl, and most of them grew in media up to 10% NaCl. These were the only strains that produced acid aerobically from glucose in O/F medium.

Phenon 3. This phenon contains two yellow-pigmented strains unable to grow without NaCl and at high salinities. They were motile, oxidase positive cocci, unable to hydrolyze the tested polymers, and able to use a variety of carbohydrates and organic acids as sole carbon sources. Their properties seem intermediate between those of *Planococcus* and *Micrococcus* species (8, 9) but none of the four reference strains of these two genera jointed them. The former genus *Micrococcus* has been recently splitted and four new genera have been created to allocate some of their constituent species (27). *Planococcus* has also suffered a major revision, since a rod-shaped bacterium, formerly known as *Flavobacterium okeanokoites* has been transferred to *Planococcus* (15). None of the new generic descriptions corresponds to the strains included in this phenon.

Phenon 4. This phenon includes three rod-forming strains that merge at 0.64 similarity. Two of them produced oval spores with non-swelling sporangia. All strains were proteolytic, slight halophiles and used a wide range of the tested carbon sources. They can be assigned to the genus *Bacillus*, even when considering the new genera of endospore formers appeared in last years (i.e., *Alicyclobacillus, Amphibacillus, Aneurinibacillus, Brevibacillus, Halobacillus, Paenibacillus* and *Sporolactobacillus*) (21, 22, 26, 30). Catalase, temperature/pH range and sporangia shape exclude all but one of these genera: *Halobacillus*, a moderate halophile growing up to 25–30% salt (26). Strains in phenon 4, however, are non-pigmented, DNAse negative, nitrate positive and only slightly halophilic, precluding their assignement to genera other than *Bacillus*.

An unclustered isolate, near phenon 4, has been identified as a *B. globisporus* strain unable to grow at 4°C (3).

Phenon 5. It is formed by two strains, one of which produced spores. They were motile and showed a remarkable halotolerance, growing up to 20% NaCl. These strains could belong to the genus *Halobacillus*, as they behaved as moderate halophiles (26). However, they did not show pigment production, DNAase activity and proteolysis, and one of the strains reduced nitrate, in contrast with the description of the genus and of the two rod-shaped species included (26). The moderately halophilic species *B. halophilus* is similar to phenon 5 in its halophilic nature, but differs from it in DNAse and urease activities, unability to grow at 4°C, and in the range of sole carbon sources used: phenon 5 strains are able to use D-fructose, manose, sucrose, glycerol, citrate, fumarate, D-gluconate and L-alanine, but they cannot use amygdalin and cellobiose, in contrast with *B. halophilus* (29).

Phenon 6. Contains 6 strains of non-motile, unpigmented, regular rods, positive for oxidase and unable to reduce nitrate. Spores were not observed in any of the strains. They did not grow

without NaCl nor at 4°C, but grew at 40°C. They were negative for all the exoenzymatic activities tested except for Tween-20 hydrolysis. D-Glucose and glycerol were the only carbohydrates used, but the strains utilized several organic acids and aminoacids.

Phenon 7. The six strains that cluster in this phenon were motile rods that produced spores with non-swelling sporangia, reduced nitrate to nitrite, and grew at 6% NaCl but not without this salt. Except for their ability to grow at 40°C, lipase activity and the unability to use maltose, they behaved similarly to the description of *B. marinus*, a species described from marine isolates that had been originally classified as a subspecies of *B. globisporus* (20) and then as a new species (19). This is the sole Na-requiring *Bacillus* species recognized in *Bergey's Manual*, although several other have been described later (26, 29). However, in spite of the similarity with the description, none of the two reference strains of this species included in the study (DSM 1298 and CECT 4272^T) clustered in this phenon. Their nearest neighbour was, instead, the pair formed by a marine strain of *B. licheniformis* (LMG 7629) and their duplicate. It should be noted that the coefficient used for the numerical analysis (S_J) may fail to reveal a relationship between groups when dealing with strains unreactive in most tests. In this case, the calculation of similarity may be done on very few data, which lowers the similarity to unrealistic levels. Phenon 7 showed the lowest assimilatory capabilities, as revealed by the data on carbon source utilization (Table 2); a few number of discrepancies between its strains and the reference ones (or between the two references) may account for the long distance among them.

The results of the present work reveal a high diversity among Gram-positive strains collected from the studied marine environment, which is reflected by the fact that less than 50% of the strains were recovered in phena, even with the relatively high similarity level selected. In addition, none of the reference strains selected among species and genera reported to be of marine origin clustered together with the wild strains. Both findings stress the need of more geno- and chemotaxonomic studies to elucidate the position of the strains. Salt requirements shown by most of our isolates were in agreement with a recent report on similar environments (7).

Gram-positive marine bacteria have been reported as a source of new bioactive compounds, and they have been also considered candidates to describe new eucaryotic-eubacterial associations in marine habitats (6). This study, together with some previous ones on the same environment (16, 17), support the hypothesis that a considerable portion of culturable marine bacteria remains unknown, and more deep and extensive research on this subject is needed.

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Coliforms and *Salmonella* in seawater near to domestic sewage sources in Fortaleza (Ceará, Brazil)

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Summary

The bacteriological conditions of the coastal region of Fortaleza (Ceará, Brasil), including the coastal zones of the Ceará and Cocó rivers, were examined. The study was conducted during June, September and December 1993, and March 1994. The region was divided into two areas: (i) Direct Influence Area (DIA), consisting of 20 sampling stations located near to discharge zones of the submarine pipeline system, where collections were carried out at the surface, and (ii) Indirect Influence Area (IIA), located near to the coastal zone, including Barra do Ceará, Kartódromo, Volta de Jurema, Mucuripe, Farol and Caça e Pesca beaches, totalling 26 sampling stations. The most probable number (MPN) of both total and fecal coliforms in DIA was positive only in station number 6, near to the sewage discharge exit. The following bacteria were identified: *Citrobacter* sp., *Enterobacter aerogenes* and *Escherichia coli*. Kartódromo beach was contaminated throughout the sampling period. Results of total fecal MPN was essentially lower than 3.0×10^2 coliforms/100 ml at Caça e Pesca beach. In December, at both DIA and IIA, *Salmonella* was identified in several samples. In DIA, the spatial distribution for *Salmonella* suggests that there should be a coastal sea current from east to west along the coastline. In IIA, *Salmonella* was identified at Kartódromo and Farol beaches throughout the sampling period.

Key words: *Salmonella*, coliforms, marine pollution, sewage disposal system, water analysis

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Resumen

Se examinaron las condiciones bacteriológicas de la región costera de Fortaleza (Ceará, Brasil), incluyendo las zonas de los ríos Ceará y Cocó. Dicho estudio se llevó a cabo durante los meses de junio, septiembre y diciembre de 1993, y marzo de 1994. La región se dividió en dos áreas: (i) Área de Influencia Directa (AID), con 20 puntos de muestreo situados cerca de las zonas de descarga de emisarios submarinos, donde las muestras se tomaron de la superficie, y (ii) Área de Influencia Indirecta (AII), situada cerca de la costa, que incluye la Barra de Ceará, Kartódromo, Volta de Jurema, Mucuripe, Farol y la playa de Caça y Pesca, con un total de 26 puntos de muestreo. El número más probable (MPN) de coliformes totales y fecales en el AID resultó positivo solamente en el punto 6, cercano a la salida de aguas residuales. Se identificaron las siguientes bacterias: *Citrobacter* sp., *Enterobacter aerogenes* y *Escherichia coli*. La playa de Kartódromo estaba contaminada durante el período de muestreo. En la playa de Caça e Pesca, los resultados de MPN fecales totales fueron considerablemente más bajos que $3,0 \times 10^2$ coliformes/100 ml. En diciembre, *Salmonella* se aisló de diversas muestras, procedentes tanto de AID como de AII. En AID la distribución espacial de *Salmonella* sugiere la existencia de una corriente que se movería de este a oeste a lo largo de la costa. En AII, *Salmonella* fue aislada en las playas de Kartódromo y Farol durante todo el período de muestreo.

Introduction

Most sewage from coastal cities is discharged into rivers and lakes, which are normally linked to estuarine and coastal regions. The ocean is the final receptor of untreated wastewater. Sewage can be a vehicle of pathogenic bacteria. In Brazil, the disposal of domestic sewage through submarine pipeline systems is classified as "preliminary grade". This is because domestic sewage does not undergo any pretreatment instead of having rough solids removed by grating, sand by mechanic sand-removal devices and floating solids through fine sieving (9). Therefore, it is necessary to analize sea water at beaches close to those submarine pipeline systems in order to evaluate pathogenic bacteria both qualitatively and quantitatively. Two possible pollutant sources in beaches are: disposal systems and wastes which reach the beaches through rivers and drain pipes. The most probable number (MPN) of total and fecal coliforms and the presence of *Salmonella* were investigated along the coast of Fortaleza.

Materials and methods

Water samples were collected in June, September and December 1993 and March 1994. Two areas were chosen, namely: Direct Influence Area (DIA) and Indirect Influence Area (IIA). DIA consisted of 20 sampling stations. IIA samples were collected near to the coast, totalling 26 collecting sites, on the following beaches: Barra do Ceará, Kartódromo, Volta da Jurema, Mucuripe, Farol and Caça e Pesca (Fig. 1).

The MPN of total and fecal coliforms per 100 ml of water was determined in both areas. Isolated strains were cultured on EMB agar and identified by conventional methods (3, 12, 19).

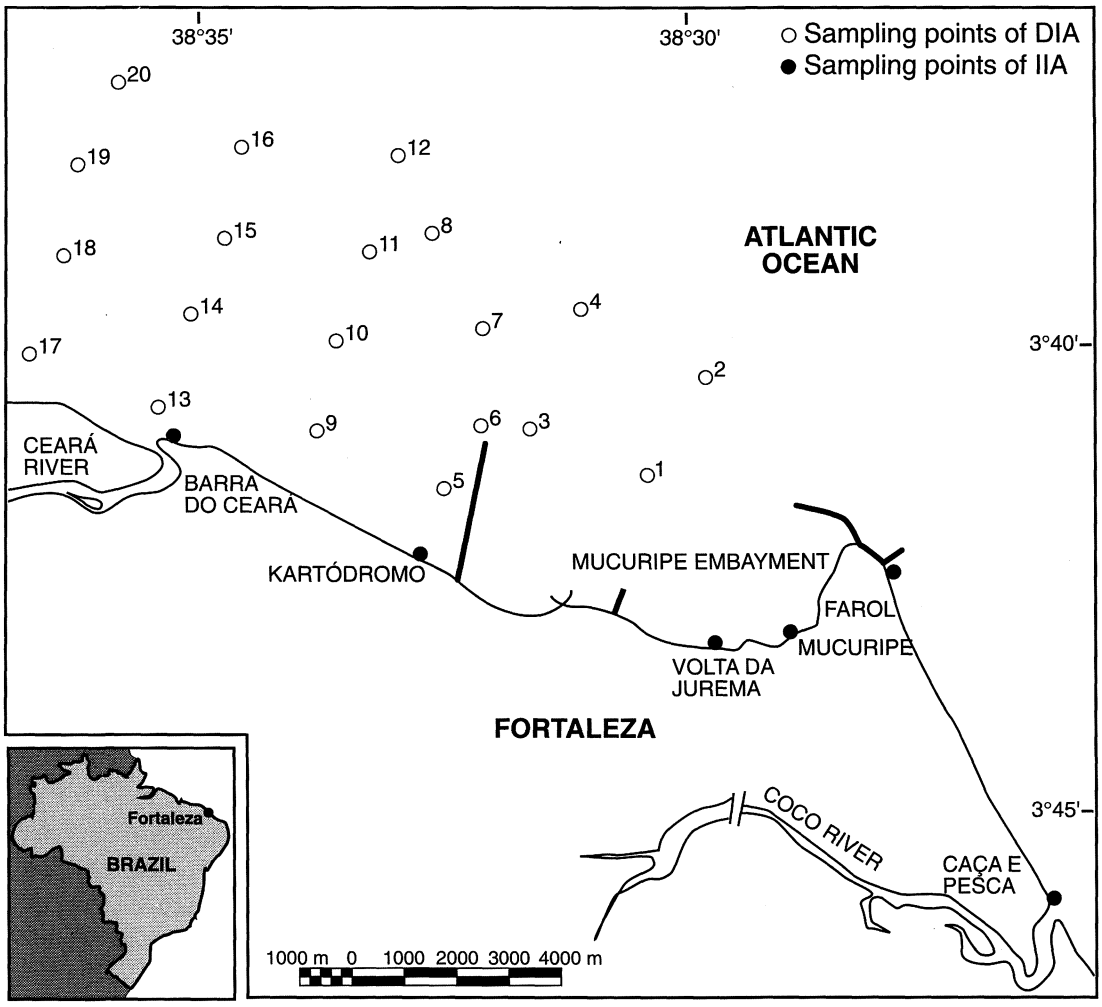

FIG. 1. Spatial distribution of the collection stations at Mucuripe embayment.

Salmonella pre-enrichment was carried out by filtering two-liter samples through HA 0.45 μm Millipore membrane filters (Millipore, Bedford, USA). These membranes were divided into two pieces and put into tubes with 10 ml Lactose Broth (Difco) and incubated at 37°C for 24 h (10).

Salmonella enrichment was prepared by inoculating 1 ml aliquots of the pre-enriched into tubes of Rappaport medium (7) and Tetrathionate Broth, which were incubated at 41–43°C for 24 h. Then, they were inoculated on MacConkey Agar and Enteric Hektoen Agar (Difco) and incubated for 24 h at 37°C. From each plate, three colonies suspected to be *Salmonella* were isolated using Kligler culture medium (Difco). Tubes which presented red top and yellow bottom, with or without H_2S production, were selected for further characterization, which consisted of serological tests using the rapid sero-agglutination technique for *Salmonella* (2).

For species identification, the strains were sent to the laboratories of the Oswaldo Cruz Foundation, Rio de Janeiro.

TABLE 1. Total and fecal coliform counts in MPN/100 ml in IIA collection stations and in DIA collection station No. 6, in Fortaleza (Ceará, Brazil)

Samples	Total coliforms				Fecal coliforms			
	Jun. 93	Sep. 93	Dec. 93	Mar. 94	Jun. 93	Sep. 93	Dec. 93	Mar. 94
Barra do Ceará	4.3×10^3	9.0×10^2	9.0×10^2	7.0×10^2	2.3×10^3	4.0×10^2	9.0×10^2	7.0×10^2
Kartódromo	9.3×10^3	2.4×10^4	4.3×10^7	9.3×10^6	9.3×10^3	4.3×10^3	1.5×10^6	4.3×10^6
Volta da Jurema	9.0×10^2	2.3×10^3	2.3×10^3	2.4×10^4	4.0×10^2	9.0×10^2	9.0×10^2	2.4×10^4
Mucuripe	4.3×10^3	2.3×10^3	9.0×10^2	2.3×10^3	9.0×10^2	9.0×10^2	4.0×10^2	2.3×10^3
Farol	2.4×10^4	2.3×10^3	9.0×10^2	2.3×10^3	2.4×10^4	2.3×10^3	9.0×10^2	2.3×10^3
Caça e Pesca	4.0×10^2	4.0×10^2	$<3.0 \times 10^2$	$<3.0 \times 10^2$	$<3.0 \times 10^2$	$<3.0 \times 10^2$	$<3.0 \times 10^2$	$<3.0 \times 10^2$
DIA station No. 6*	2.4×10^7	4.3×10^6	2.4×10^4	9.3×10^3	2.4×10^7	4.3×10^6	2.4×10^4	9.3×10^3

* Coliform bacteria were not found at any other DIA station.

Results and discussion

DIA station number 6 was the only one to show detectable total and fecal coliforms during the water sampling period. Results of MPN for both total and fecal coliforms were similar, the highest having been found in June (2.4×10^7/100 ml) and the lowest in March (9.3×10^3/100 ml) (Table 1). This may be due to high rainfall levels recorded in this particular month of the year, increasing the dilution of water. *Enterobacter aerogenes*, *Escherichia coli* and *Citrobacter* sp. were identified at this station (Table 2). Finding *E. coli* and *E. aerogenes* was quite predictable considering the site of sample collection.

Coliform bacteria were not found in any other sampling station at DIA. This may be due to these microorganisms being allocthonous in sea water (6) and local currents, which enhance the efficiency of the submarine system as a waste disposal device (15). To evaluate the efficiency of sewage treatment system it is necessary to observe whether the coliforms numbers are reduced (5). However, it is clear that continuous discharge of untreated sewage is responsible for faecal pollution shown by the presence of *E. coli* and *Salmonella* (16, 18).

Both total and fecal coliforms found in IIA samples ranged from less than 3×10^2/100 ml to 4.3×10^7/100 ml (Table 1). In June, Barra do Ceará beach showed 4.3×10^3/100 ml and 2.3×10^3/100 ml for total and fecal coliforms, respectively. In September, December and March, MPN results were much lower. Melo et al. (13) reported MPN values for total and fecal coliforms in the estuary of the Ceará river higher than 2.4×10^4/100 ml, both in water and sediment samples, during April and June. In September, MPN for both types of coliforms was reduced to 3.0×10^1/100 ml, and in December there was a slight increase of both total and fecal coliforms (9.3×10^1/100 ml sediment and 4.3×10^1/100 ml water).

Vasconcelos (20) reported that the highest pollution level in the Ceará river from May to August, when there is less water because of lack of rainfall.

Kartódromo beach showed the highest fecal MPN (4.3×10^6/100 ml) in March and the lowest (4.3×10^3/100 ml) in September. *E. coli* was identified in all samples (Table 2). Carneiro et

TABLE 2. Identification of isolated strains in IIA collection stations and in DIA collection station No. 6 in Fortaleza (Ceará, Brazil)

Samples	Identification of coliforms isolates			
	Jun. 93	Sep. 93	Dec. 93	Mar. 94
Barra do Ceará	*Citrobacter* sp.	*Enterobacter aerogenes*	*Citrobacter* sp.	*Enterobacter aerogenes*
Kartódromo	*Citrobacter* sp.	*Escherichia coli*	*Enterobacter aerogenes*	*Escherichia coli*
	Escherichia coli		*Escherichia coli*	*Enterobacter aerogenes*
	Enterobacter aerogenes			
Volta da Jurema	*Citrobacter* sp.	*Escherichia coli*	*Escherichia coli*	*Citrobacter* sp.
Mucuripe	*Enterobacter aerogenes*	*Enterobacter aerogenes*	*Citrobacter* sp.	*Citrobacter* sp.
Farol	*Citrobacter* sp.	*Escherichia coli* *Enterobacter aerogenes*	*Escherichia coli* *Citrobacter* sp.	*Escherichia coli* *Enterobacter aerogenes*
Caça e Pesca	–	–	–	–
DIA station No. 6	*Citrobacter* sp. *Enterobacter aerogenes*	*Citrobacter* sp. *Enterobacter aerogenes*	*Citrobacter* sp. *Enterobacter aerogenes*	*Citrobacter* sp. *Enterobacter aerogenes*
	Escherichia coli	*Escherichia coli*	*Escherichia coli*	*Escherichia coli*

al. (4) also found that Kartódromo beach presented the poorest water quality along Fortaleza city coast. This occurs because of the highly polluted discharge of the Jacarecanga river, as previously observed by Vasconcelos (20).

Volta da Jurema beach showed an increase in fecal pollution levels in March (Table 1). It is believed that there are sewage pipes connected illegally to the water drainage system before discharging into the sea.

The maximum value of fecal coliforms at Mucuripe beach was 2.3×10^3/100 ml, recorded in March 1994 (Table 1).

At Farol beach, *E. coli* was identified throughout the whole research period, except in June (Table 2). The occurrence of *E. coli* is probably due to the high frequency of pigpens and illegal sewage linkages.

Caça e Pesca beach, where the Cocó river's mouth is located, showed values lower than 4.0×10^2 fecal coliforms/100 ml (Table 1). Vieira and Façanha (21), analyzing different areas in the same river, found that close to the river's mouth MPN of coliforms was very low; some times, even apparently absent. Similar results were reported also by Mavignier and Frischkorn (11). According to them, pollution in this particular river is markedly evident from 3 km upstream because of the discharge of the Maracanaú Industrial District effluent.

Table 3 shows the incidence of *Salmonella* at DIA stations during June, September and December 1993 and March 1994. *S. give,* which belongs to serum group E_1, was isolated at station number 9 in June 1993. In September, *S. lexington* was isolated from station number 6,

TABLE 3. *Salmonella* identification in both DIA and IIA samples collected in June, September and December 1993 and March 1994 in Fortaleza, Ceará, Brazil

Site of sample collection	*Salmonella* isolates			
	Jun. 93	Sept. 93	Dec. 93	Mar. 94
DIA station No. 2	ND*	ND	*S. anatum*	ND
DIA station No. 4	ND	ND	ND	*S. anatum*
DIA station No. 5	ND	ND	*S. anatum*	ND
DIA station No. 6	ND	*S. lexington*	*S. urbana*	ND
DIA station No. 9	*S. give*	ND	ND	ND
DIA station No. 13	ND	ND	ND	ND
IIA-Barra do Ceará	*S. virchow*	ND	*S. djugu*	*S. saint-paul*
IIA-Kartódromo	*S. sinstorf*	*S. djugu*	*S. lexington*	*S. saint-paul*
	S. lexington	*S. lexington*	*S. saint-paul*	
IIA-Volta de Jurema	ND	ND	ND	ND
IIA-Mucuripe	ND	ND	*S. lexington*	ND
IIA-Farol	*S. lexington*	*S. djugu*	*S. djugu*	*S. saint-paul*
	S. djugu		*S. saint-paul*	*S. fyris*
IIA-Caça e Pesca	ND	ND	ND	ND

* ND, not detected.

which is close to the submarine disposal system exit, and *S. muenster* was isolated at station number 13. The latter serotype is very common in water from sewage (8). In December, *S. anatum* was identified both at stations 2 and 5, and *S. urbana* at station number 6. This particular *Salmonella* species also belongs to serological group E_1 and was identified also among the ten more frequent serotypes in the sewage from São Paulo city in Brazil (17). *S. anatum* was the most frequently found serotype at the DIA stations (Table 3). No other station of collection showed the presence of *Salmonella* during the period studied.

Note that the occurrence of *Salmonella* may be linked to local currents, which move from East to West parallel to the coast. This pattern has been shown by Morais (14).

At the IIA samples, the most common serotypes of *Salmonella* were: (i) *S. virchow*, *S. djugu* and *S. saint-paul* isolated from Barra do Ceará beach; (ii) *S. sinstorf*, *S. lexington*, *S. djugu* and *S. saint-paul* from Kartódromo beach; (iii) *S. lexington* from Mucuripe beach; and (iv) *S. djugu*, *S. lexington*, *S. saint-paul* and *S. fyris* from Farol beach. No *Salmonella* was detected in water samples collected either at Volta da Jurema or Caça e Pesca beaches (Table 3). It seems reasonable to believe that the occurrence of *Salmonella* in samples collected at IIA is due to sewage discharges.

Lisboa (10) indicated that the evaluation of water quality upon bacteriological basis is related to the presence of coliform bacteria and to the frequency of *Salmonella*. However, Hagler and Mendonça-Hagler (6) indicate that the methodology used to detect such bacteria (*Salmonella*) in water samples is laborious and of little value as an indicative of contamination.

Although *Salmonella* has its habitat in the intestinal tract of warm-blooded animals, it can

survive for long periods of time on several types of food, soil and in water (1). Rodrigues et al. (18) reported the presence of Enterobacteriaceae on garden soil at leisure public places, as well as in organic fertilizers collected at a sewage treatment plant in the Ilha do Governador, Rio de Janeiro. They showed that some *Salmonella* serotypes can survive for several months when soil conditions are ideal.

Paula and Hofer (16) suggested that the most frequent *Salmonella* serotypes occurring in Niterói, Rio de Janeiro, should be studied as well as those people who are asymptomatic carriers. This would be of much help to establish management measures and law enforcement for environmental protection.

Once more, it can be showed the presence of *Salmonella*, as well as other pathogenic microorganisms, in coastal seawater of large cities in Brazil, mainly where there is not effective sewage treatment. Therefore, beaches close to submarine pipeline systems may be potential sources of contamination and a vehicle for diseases.

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Usefulness of phage typing and "two-way ribotyping" to differentiate *Salmonella enteritidis* strains

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Summary

The capacity to differentiate *Salmonella enteritidis* strains by phage typing and "two-way ribotyping" performed with *Pst*I and *Sph*I was evaluated. The typeability was 96.8% in phage typing and 100% in ribotyping. The series was differentiated into 13 phage types, 19 combined ribotypes, and 39 subtypes or clonal lines by combining results from both methods (of which 11, 13, and 35, respectively, were represented by natural strains). Ribotyping differentiated strains ascribed to PTs 1, 4, 6a, 7, 8, RDNC and UPT. Conversely, some strains of PTs 1, 4, 5a, 6, 6a, 7, 34, RDNC and UPT fall into the most frequent combined ribotype. A dendrogram of genetic similarity generated from the combined ribotypes was traced, and, at a 0.82 similarity level, it showed a major cluster (including 17 combined ribotypes, 88.4% strains ascribed to all PTs tested except PT11), a minor cluster, and four additional lines more loosely related.

Key words: *Salmonella enteritidis,* phage typing, ribotyping, ribosomal DNA, epidemiological markers

Resumen

Se evaluó la capacidad para diferenciar *Salmonella enteritidis* mediante fagotipificación y "ribotipificación a dos vías", utilizando *Pst*I y *Sph*I. La capacidad de tipificación fue 96,8% en

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fagotipificación y 100% en ribotipificación. La serie fue diferenciada en 13 fagotipos, 19 ribotipos combinados y 39 subtipos o líneas clonales mediante la combinación de resultados de ambos métodos (11 fagotipos, 13 ribotipos combinados y 19 líneas clonales estaban representados por cepas naturales). Mediante ribotipificación se diferenciaron cepas adscritas a los fagotipos PT 1, 4, 6a, 7, 8, RDNC y UPT. Recíprocamente, algunas cepas PT 1, 4, 5a, 6, 6a, 7, 34, RDNC y UPT pertenecían al ribotipo combinado más frecuente. El dendrograma de relación genética de los ribotipos combinados mostraba, a un nivel de similaridad de 0,82, una agrupación mayor (17 ribotipos combinados, 88,4% de las cepas representando a todos los PTs ensayados excepto PT11), una agrupación menor, y cuatro líneas con menor relación.

Introduction

Salmonella enterica serovar Enteritidis (*Salmonella enteritidis)* is a major source of human salmonellosis in Spain (3, 23), as well as in many other industrial countries (17, 19, 22). Although *S. enteritidis* incidence in Spain is decreasing (3, 23), it still represents an important percentage of human *Salmonella* isolates. In fact, in 1996, 41.5% of *Salmonella* isolates reported to the "Centro Nacional de Microbiología" (CNM), were *S. enteritidis* (unpublished data). Over the last two decades, different phenotypic and genetic techniques have been reported as useful typing methods for *S. enteritidis* (4–7, 10–12, 14, 19, 20, 22, 24). Among the phenotypic methods, phage typing performed following Ward et al. (24) is playing a central role in epidemiological studies. The CNM has been using this scheme routinely since October 1996, finding that five phage types represented 87% of *S. enteritidis* strains phage typed, these being: PT4 (45.8%), PT6a (19%), PT1 (12.1%), PT34 (5.1%), PT6 (5%). As reported in other European countries (6, 7, 19, 20, 22, 24), PT4 is the most common, whereas in the USA are PT8 and PT13a (11, 22). All these phage types are associated with food poisoning and with the concurrent widespread infection of commercial poultry meat and egg dishes (15). Among the genetic techniques, ribotyping is playing a key role in epidemiological studies. Ribotyping reveals the sequence divergence around and within rRNA gene loci, which are organized as polycistronic transcriptional units called *rrn* ribosomal operons (2). The appropriate enzyme must be determined for each bacterial species and serotype, and the use of two or more enzymes can increase the discriminating power of the method, but also the work load. A ribotyping procedure, performed separately with *Pst*I and *Sph*I, and the posterior combination of the results (termed "two-way ribotyping"), has been reported as the most useful genetic method to differentiate *S. enteritidis* pathogenic strains (10).

The aim of the present work was to evaluate phage typing and the "two-way ribotyping", alone and in combination, in the differentiation of *S. enteritidis* strains collected from different sources in Spain. In addition, a dendrogram of the genetic relationship between combined ribotypes was traced, and the resulting branches and clusters were correlated with phage types.

Materials and methods

Bacterial strains. This study included 93 randomly selected strains of *S. enteritidis;* 53 were isolated in Asturias and 40 in other regions of Spain. Of these, 68 strains were associated with 68 unrelated human infections, 15 were

collected from water or sewage and 10 from food. In addition, 11 reference strains (ATCC 13076 ascribed to PT1; CNM 8, a German strain ascribed to PT8; type strains of PTs 4, 6, 6a, 8, 13a and 9, from the Central Public Health Laboratory of London (CPHL); and of PTs 1, 7 and 34 from the CNM) were tested by ribotyping. *Salmonella typhimurium* LT2 was used as an outgroup strain in ribotyping.

Phage typing. Phage typing was carried out following Ward et al. (24). Ten selected phages from different sources (sewage, lysogenic strains and adapted phages) were used in the final scheme. In brief: problem strains were cultivated in nutrient broth with 0.8% NaCl for 2 h, poured onto nutrient agar with 0.8% NaCl, allowed to dry for 15 min at room temperature, and a drop of each diluted phage was added. After 24 h at 37°C, plates were examined for lysis as recommended by the CPHL. The strains showing a lytic pattern that did not correspond to any recognized phage types were referred to as RDNC (reaction does not conform), and the phage untypable strains as UPT (untypable by phage typing).

DNA isolation and ribotyping. The plasmid pKK3535 which carries the *rrnB* operon (2, 12) was the source for the DNA probe. This consisted of a 7.5 kb *Bam*HI fragment collected as in (18) and labelled with 11-dUDP-digoxigenin, according to the instructions of the kit's manufacturers (Boehringer-Mannheim, Germany).

Chromosomal DNA was obtained and purified using a phenol isolation method, and samples of about 2 µg of DNA were digested with 3U of *Pst*I or 5U of *Sph*I (Amersham Ibérica, S.A.) for 6 h, and then tested by Southern blot hybridization performed as previously described (5, 13), using the above cited non-radioactive DNA labelling and detection kit (Boehringer-Mannheim, Germany). In the analysis of each enzyme, isolates representing the different banding patterns were tested at least three times to evaluate the reproducibility of the method. When the DNA was not completely digested some artefactual fragments appeared. These fragments were not taken into account to define the ribotypes. The polymorphic restriction sites (PRSs) within the rDNA region were deduced by the presence or absence of bands among the total ribotypes from each enzyme as described previously (9, 13). Strains showing identical ribotypes with *Pst*I and *Sph*I were ascribed to the same combined ribotype (CRT).

Statistical analysis methods. The discrimination index (DI), i.e. the probability that two unrelated strains sampled from the population would be placed into different typing groups, was calculated by Simpson's index of diversity (8). To calculate the DI of phage typing, all RDNC strains were considered as organisms belonging to a single phage type, and the UPT strains as another phage type.

For genetic relationships of the rDNA regions of *S. enteritidis,* the banding profiles or ribotypes generated with *Pst*I and *Sph*I, also using data from *S. typhimurium* LT2 as the outgroup strain, were processed as described elsewhere (14). Therefore, clustering analysis between CRTs was carried out using Dice's similarity coefficient and the unweighted pair group method with arithmetic averages (UPGMA) in the software programme MVSP (Multivariate Statistics Package, version 2.0a).

Results and discussion

Phage typing. Eighty-one of the 93 strains tested (the reference strains are not included) could be ascribed to eight defined phage types: PT1 (8 strains), PT4 (37 str.), PT 5a (1 str.), PT6 (12 str.), PT6a (15 str.), PT7 (3 str.), PT8 (2 str.), PT11 (2 str.), PT34 (1 str.). Seven strains were

considered as RDNC, and the five remaining strains as UPT. The stability and reproducibility of this method were good; the typeability was 96.81%, and the DI = 0.77. Comparing results from this series (which includes mainly strains from Asturias) with data obtained in a larger series phage typed in the CNM, the following findings were revealed: PT4 and PT6a were the most frequent in both series, although the percentages were different (39.8% and 16.1% vs. 45.8% and 19%, respectively). The PT6 percentage found in our study was more than twice than the percentage in CNM series (12.9% vs. 5%). On the contrary, PT34 was less frequent in this series; it was represented by a single clinical strain collected in Madrid, whereas this phage type represented 5.1% in the CNM series. When our data were compared with data from other European countries, the only similarity found between different countries was that PT4 is the most common phage type that causes human salmonellosis, whereas its frequency in this study, and in general in Spain, was lower than in other European countries. The frequency of the other PTs varies greatly from one country to another (1, 19, 24).

Phage types are generally considered to be stable and definitive epidemiological markers. However, several different kinds of PT conversions have been reported (1, 16, 21). Despite these conversions, both the increasing internationalization of the use of the phage typing scheme of Ward et al. (24), and its easy performance, make this scheme an accurate general procedure for the epidemiological survey of *S. enteritidis*. A major inconvenience of this scheme is that it has to be performed in special National Reference Laboratories.

Two-way ribotyping. *S. typhimurium* LT2 and the 53 *S. enteritidis* strains from Asturias have been analyzed by ribotyping performed with *Pst*I and *Sph*I in a previous work (10). The remaining 40 strains, as well as the 11 reference strains, were ribotyped using each one of the enzymes in the present work. The strains could be differentiated into 8 ribotypes with *Pst*I and into 18 with *Sph*I (labelled P and S, respectively, and shown in Fig. 1 and 2). Since *Salmonella* contains seven *rrn* operons distributed throughout the chromosome, its genomic DNA will show a maximum of seven DNA hybridizing fragments when digested with an enzyme which does not cut into the *rrn* operons, such as *Pst*I. The P ribotypes (Fig. 1) included seven or less fragments, no fragment was considered as common in all the P ribotypes, and 20 PRSs were found. The differences between some ribotypes affected only one fragment (P1 vs. P8, P14 or PZ), although differences affecting three or more fragments were also revealed (P1 vs. P4 or P15; P4 vs. P2, P5, P7 or P15), which are shown in Fig. 1. The distribution of strains into P ribotypes was as follows: P1 (88 strains), P2 (7 str.), P8 (4 str.); five others were represented by one only strain. The S ribotypes included a different number of DNA fragments, between 5 and 13, all except one in the region from 6.5 to more than 40 kb (Fig. 2). These data support the fact that *Sph*I cut into some of the *rrn* operons. No fragment was common in all the S ribotypes, and a large number of RPSs were revealed. The distribution of strains into S ribotypes was: S1-S1a (67 strains); S23 (8 str.); S7 (7 str.); S9 and S12 (3 str.); S2, S5, S8 and S11 (2 str.); the other eight types were represented by one only strain.

When results from *Pst*I and *Sph*I were combined, a different distribution of the strains into 25 CRTs was found (Table 1, Fig. 3). Only nine CRTs grouped two or more strains, P1-S1 (63 strains); P1-S7 (6 str.); P1-S23 (5 str.); P1-S12 and P2-S23 (3 str.); P1-S9, P1-S11, P2-S8 and P8-S2 (2 str.); the other 16 CRTs were represented by one only strain and they were categorized as uncommon. Type strains of PTs 6a, 8, 9

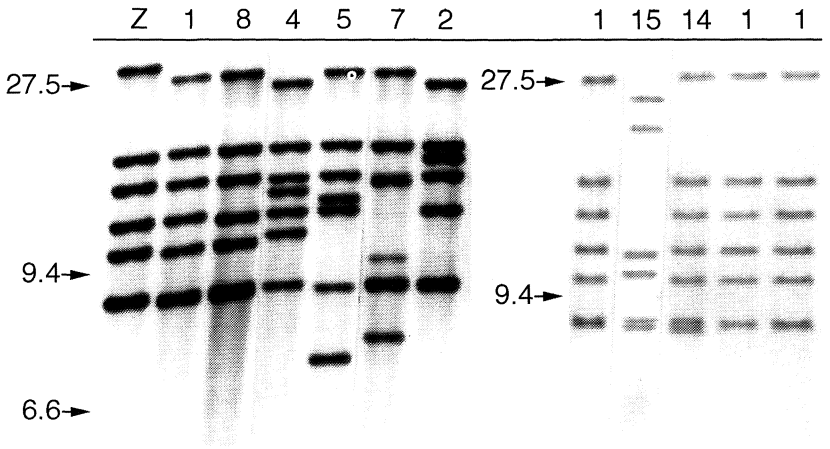

FIG. 1. *Pst*I ribotypes of *Salmonella enteritidis* strains. Lane Z, P ribotype of *Salmonella typhimurium* LT2; the other lanes show representative P ribotypes of *Salmonella enteritidis*, which have been labelled with the numbers shown at the top of the lane. The arrows indicate standard fragments size (in kb).

and 13a (from CPHL) and the German strain, labelled CNM 8 (ascribed to PT8), exhibited CRTs that were not found among the Spanish strains tested (Table 1).

The typeability of "two-way ribotyping" reached 100%, the reproducibility (with completely digested DNA) reached 100% with *Pst*I, but not with *Sph*I; because some fragments, of certain *Sph*I banding patterns, were sometimes not revealed. When the patterns included one to three fragments (in the region between 14 and 8 kb) they were labelled S ribotypes, and when these fragments were not revealed they were labelled Sa ribotypes (Fig. 2). The discrimination index reached values of 0.28 with *Pst*I, 0.59 with *Sph*I, and 0.63 by combining the types from both enzymes. The interpretation of banding patterns and the comparison of results from different experiments were better achieved in *Pst*I-ribotyping than in *Sph*I-ribotyping. *Sph*I has been previously used in *S. enteritidis* ribotyping and high differentiation into types was reported (6, 10–12, 20). However, we must note that *Sph*I can cause some problems, which seem to be related to the "quality" of its commercial form. The problems observed were: (i) Some commercial forms of *Sph*I can cleave *Salmonella* DNA only partially, yielding banding patterns with artefactual bands. In fact, DNAs of *S. enteritidis* that were repeatedly resistant to digestion with this enzyme have been reported in previous studies (5, 22); in other articles the *Sph*I ribotypes are labelled but the banding patterns are not shown (11, 20). As indicated above, some ribotypes appearing in previous papers (6, 10, 12), as well as in the present work, included one to three fragments which were not revealed in all experiments, this fact being more frequent among the reference strains than among clinical, food or environmental strains. We considered that these fragments could be generated by partial digestions and that they should not be taken into account to study the sequence divergence of rDNA regions. (ii) The interpretation of the number and size of the fragments located in the upper region of the banding patterns (higher than 20 kb, which differentiate several types) could not be accurately defined (6, 10, 12), it being necessary to repeat the assays in order to ascribe organisms to ribotypes. In Fig. 2 a schematic representation of the banding patterns of the upper region of the gel is shown. We considered

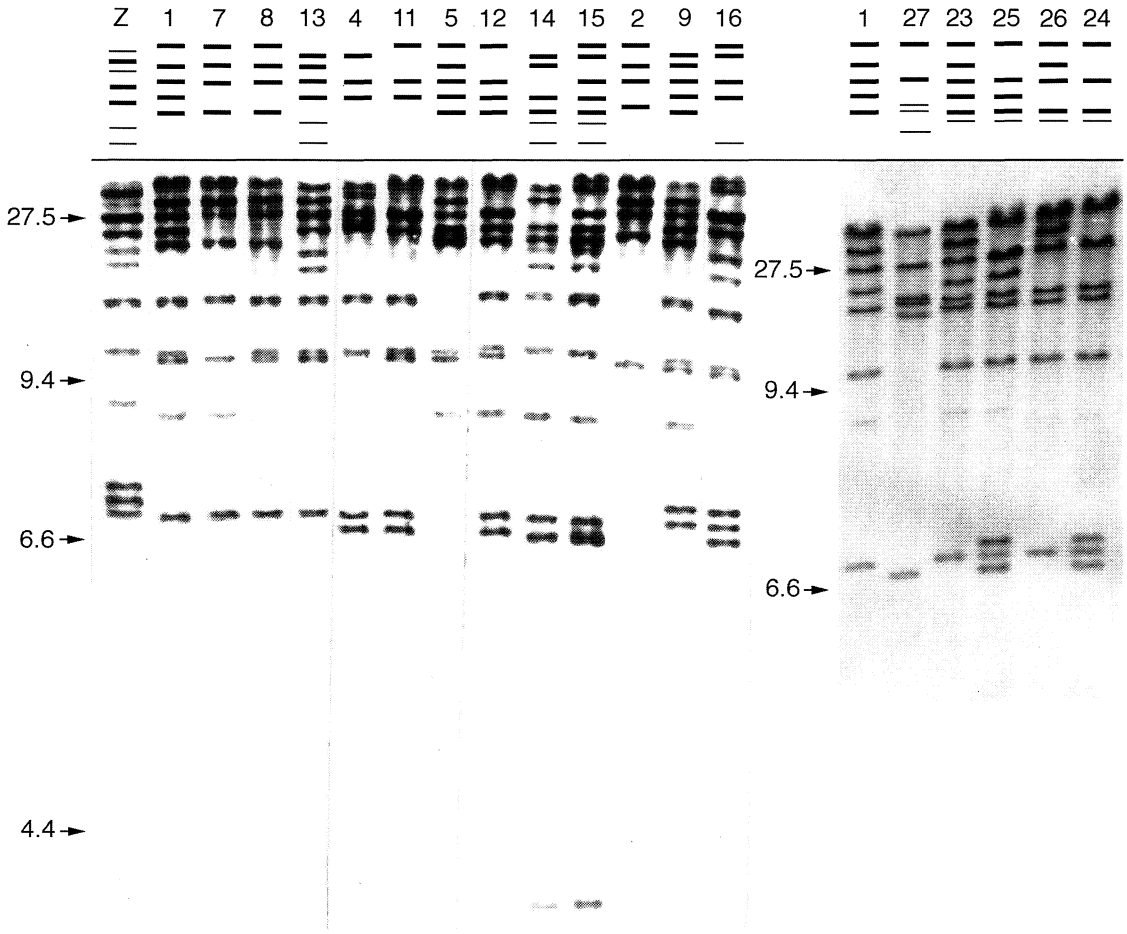

FIG. 2. *Sph*I ribotypes of *Salmonella enteritidis* strains. At the top of the figure appears a schematic representation of the banding patterns of the upper region of the gel. Lane Z, S ribotype of *Salmonella typhimurium* LT2; the other lanes show representative S ribotypes of *Salmonella enteritidis,* which have been labelled with the numbers shown at the top of the lane. The arrows indicate standard fragments size (in kb).

these fragments to define the S ribotypes. In addition, in any typing method useful for epidemiological routine, features such as ease of operation, rapidity, and cost, are also important. However, these features do not apply to the "two-way ribotyping", the major disadvantages of which are their time-consuming and labour intensive nature.

On the other hand, the sequence divergences of the rDNA regions (which include the *rrn* operons and adjacent sequences) of the 104 strains under study were analyzed by comparing the fragment restriction length polymorphisms observed in their *Pst*I and *Sph*I ribotypes (in this case, the fragments between 14 and 8 kb were not taken into account, as above was proposed). The resulting dendrogram (Fig. 3) showed a high degree of genetic heterogeneity in rDNA regions of *S. enteritidis*, the similarity between CRTs ranging between 95 and 32%. At a significance level of 0.8, the following groupings can be identified: a major grouping, labelled "cluster A", including seventeen CRTs (88.4% of strains), a minor grouping "cluster B", including four CRTs (6.7% of strains), and four other additional branches represented by each one of the

TABLE 1. Differentiation of phage types into combined ribotypes within *Salmonella enteritidis*

PT [strains]	CRT		[strains]*	PT [strains]	CRT		[strains]*
	*Pst*I	*Sph*I			*Pst*I	*Sph*I	
1[10]	1	1	[4]	7[4]	1	1	[3]
	1	23	[1], ST, ATCC 13076		1	23	ST
	1	13	[1]	8[4]	1	15	[1]
	2	1	[1]		1	16	[1]
	8	9	[1]		1	14	CNM 8
					1	25	ST
4[38]	1	1	[25], ST				
	1	5	[1]	9[1]	14	24	ST
	1	9	[1]				
	1	11	[1]	11[2]	8	2	[2]
	1	12	[3]				
	1	23	[1]	13a[1]	1	26	ST
	2	5	[1]				
	2	23	[2]	34[2]	1	1	[1], ST
	4	4	[1]				
	8	1	[1]				
				RDNC[7]	1	1	[4]
5a[1]	1	1	ST		2	23	[1]
					2	8	[1]
6[13]	1	1	[12], ST		7	7	[1]
6a[16]	1	1	[8]	UPT[5]	1	1	[2]
	1	7	[6]		1	9	[1]
	2	8	[1]		1	11	[1]
	15	27	ST		5	1	[1]

* Numbers in brackets only include natural strains. The reference strains are indicated by "ST" or by their reference number.

Abbreviations: CRT, combined ribotype; PT, phage type; ST, strain type of the corresponding phage type; CNM, Centro Nacional de Microbiología, Spain; RDNC, "react with the typing phages but did not conform" to a designate type; UPT, untypable by phage typing.

other four CRTs. *S. typhimurium* LT2 was situated between A and B clusters.

Correlation between phage types and combined ribotypes. The analysis of the strains distribution into PTs and CRTs showed correlation in only a few cases (Table 1, Fig. 3). The thirteen PT6 strains fall into CRT:P1-S1, and the six CRT:P1-S7 strains belonged to PT6a; both groups of strains are included in the cluster A. The two PT11 strains fall into CRT:P8-S2, which is situated as an independent branch in the dendrogram, loosely related to cluster A. PTs 1, 4, 6a, 7, 8, and RDNC, as well as the UPT strains, were differentiated into several CRTs, P1-S1 being the most frequent. Conversely, some or all the strains of PTs 1, 4, 5a, 6, 6a, 7, 34, RDNC and UPT fall into CRT:P1-S1; and some strains of PTs 1, 4 and 7 into CRT:P1-S23. The five strains representing prevalent phage types in the USA (PTs 8 and 13a) showed the same P ribotype (P1, which was the most frequent in the series), but each strain yielded a different S ribotype which

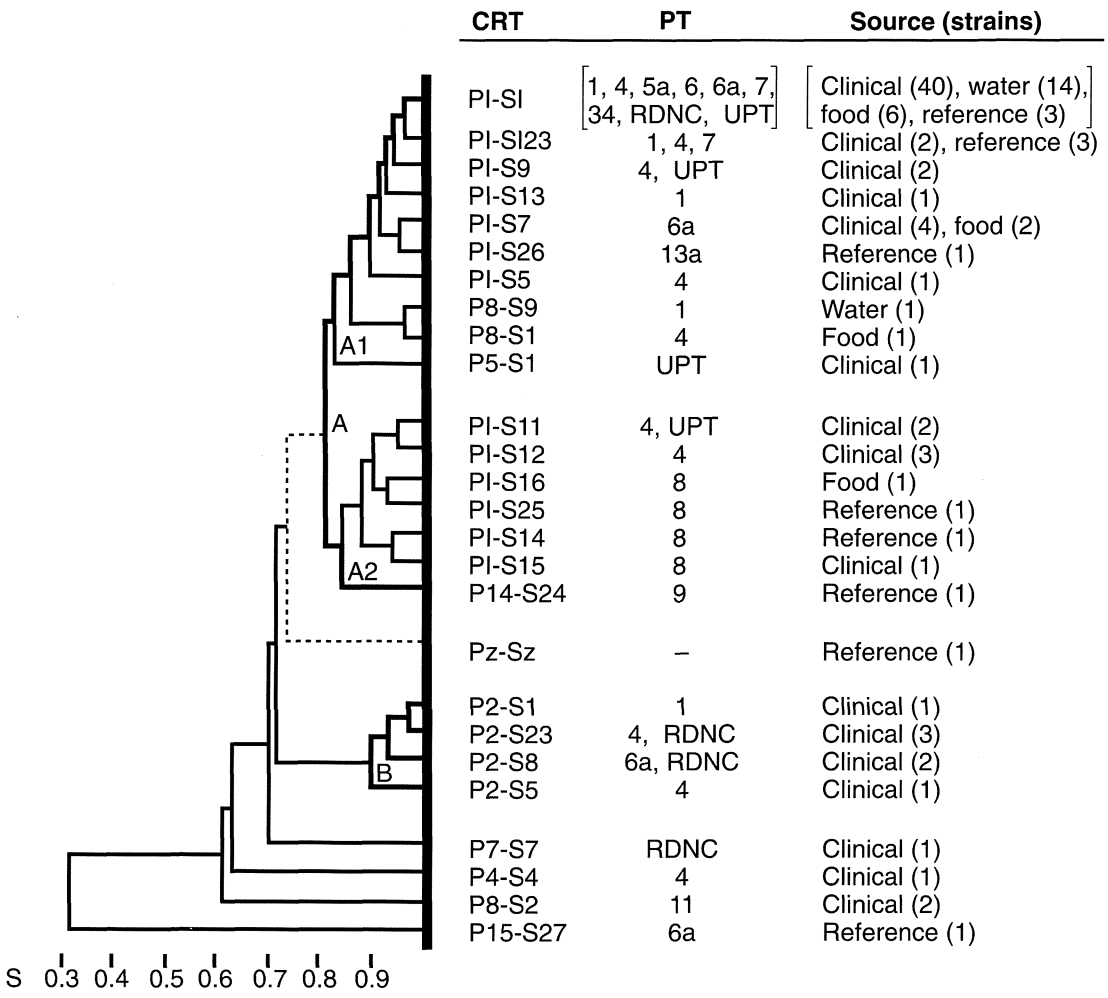

FIG. 3. Single linkage dendrogram showing the results of the cluster analysis on the basis of the ribotypes of the *Salmonella enteritidis* strains obtained with *Pst*I and *Sph*I. Relation with phage types and sources. Abbreviations: CRT, combined ribotype defined by combination of P ribotypes and S ribotypes; PT, phage type. The clusters and subclusters are framed by dark lines. CRT of *Salmonella typhymurium* LT2 is indicated by the dotted line; UPT, untypable by phage typing; RDNC, "react with the typing phages but did not conform" to a designated type; S, similarity coefficient.

was not present among the other strains analyzed. In the dendrogram it can also be observed that cluster A, includes strains of different origins grouped into two subclusters, labelled A_1 and A_2. All strains ascribed to PTs 5a, 6, 7 and 34, as well as most of the strains ascribed to PT4, PT1, RDNC and UPT fall into subcluster A_1 (82 strains, 78.8% of the series); whereas the single PT9 and four PT8 strains, as well as one UPT and four PT4 strains, fall into subcluster A_2 (10 strains, 9.6% of the series). Cluster B includes all the 7 strains showing P2 ribotype, but differentiated into S1, S8, and S13 ribotypes, and ascribed to PTs 1, 4, 6a, and RDNC, all of which have been collected from clinical samples (Fig. 3).

This heterogeneous of relationships between PTs and CRTs could be due either to changes in

the PT of isolates from the same clone or to mutations affecting the rDNA regions, both of which could have taken place among natural *S. enteritidis* populations in different environments. The first presumption is supported by findings from previous works: Rankin and Platt (16) demonstrated that the PT can be changed as a result of phage conversion by the temperate phages 1, 2, 3 and 6 from the phage typing scheme of Ward et al. (24). In fact, they reported that PTs 4, 6a, 13 and 15 were converted to PTs 8, 4, 7, 13a and 11. Threlfall et al. (21) have shown interrelationships between PTs 4, 7, 7a, 8, 13, 13a, 23, 24 and 30, caused by the loss or acquisition of an *Inc*N plasmid, respectively. Baggesen et al. (1) reported that PT7 organisms may be derived from PTs 1, 4, and 6, which appeared to be associated with the loss of lipopolysaccharide. On the other hand, the separation of strains of the same PT into different ribotypes was based on differences in one or more DNA fragments. Such differences can be caused by single genetic events, including insertion or deletion of DNA fragments or gain or loss of restriction sites. The genetic changes yielding different ribotypes might have taken place in the sequences adjacent to rRNA genes more frequently than in conserved rRNA genes which is supported for the polymorphisms by *Pst*I and *Sph*I ribotyping.

The above seems to be appropiate to consider the different PTs and CRTs as separate clones of *S. enteritidis*. Thus, organisms of the same PT and CRT can be considered as members of the same clonal line. Obviously, single events can be accumulated in natural *S. enteritidis* populations, generating evolutionary lineages, some of which could be confined to defined geographical areas for a certain time, but could then be dispersed and appear as prevalent and endemic in neighbouring countries or geographical areas. The rapidly growing international food trade could favour the fact that some lineages which are well adapted to both animal and human hosts are being dispersed worldwide.

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Distribution of the trehalase activation response and the regulatory trehalase gene among yeast species

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Summary

In *Saccharomyces cerevisiae* and other yeasts the activity of regulatory trehalases increases in response to the addition of glucose and to thermal changes in the extracellular medium. We have performed an screening on the extent of this response among different representative yeast species and the results show that this ability is displayed only by a few members of the Saccharomycetaceae family. However, all yeasts examined contain a gene related to that coding for regulatory trehalase in *S. cerevisiae*. This finding reveals that the operational distinction between regulatory and nonregulatory trehalase in yeasts is not a property of the enzyme by itself but relays on the expression of accompanying mechanisms able to modulate trehalase activity.

Key words: regulatory trehalases, trehalase gene, enzyme activation, glucose signal, signalling pathways

Resumen

En algunas levaduras, incluyendo *Saccharomyces cerevisiae*, la actividad de las trehalasas reguladoras aumenta en respuesta a cambios nutricionales o térmicos en el medio extracelular. Hemos realizado un estudio sobre la distribución de esta respuesta entre diferentes levaduras representativas y los resultados indican que las trehalasas con propiedades reguladoras sólo se presentan en algunos miembros de la familia Saccharomycetaceae. Sin embargo, todas las levaduras examinadas contienen un gen similar al que codifica la trehalasa reguladora en *S. cerevisiae*. Este

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hallazgo revela que la distinción entre trehalasas reguladoras y no reguladoras en levaduras no depende de propiedades intrínsecas de la enzima sino más bien de que se expresen o no las rutas de activación que modulan su actividad.

Introduction

The nonreducing disaccharide trehalose accumulates in yeast cells as a reserve carbohydrate. In addition to its role as a carbon and energy source, this sugar serves as a stress metabolite able to protect cells under adverse environmental conditions (7). The endogenous trehalose is synthesized by the trehalose-6-P synthase complex and mobilized by specific trehalases (E.C. 3.2.1.28). Two types of trehalases have been described in yeasts: regulatory and nonregulatory trehalases (25). Regulatory (or neutral) trehalases are found in the cytoplasm and display an hydrolytic activity, which is controlled by reversible phosphorylation of the enzyme protein (15, 28). In contrast, nonregulatory (or acid) trehalases show either vacuolar or external location, and lack known mechanisms of activation (6, 10). Regulatory trehalases have been found only in a few yeast species, including *Saccharomyces cerevisiae* (15), *Candida utilis* (2) and *Schizosaccharomyces pombe* (18–20).

The activity of regulatory trehalases increases markedly in the presence of fermentable sugars and by a heat shock (25, 29). In *S. cerevisiae*, the sugar-induced activation response is likely due to post-translational phosphorylation of the enzyme, whereas that induced by a heat shock appears to be due to increased transcription of the regulatory trehalase gene. In yeasts with neutral trehalase activity, both the synthesis and the hydrolysis of trehalose are targets of regulatory mechanisms involved in signalling pathways which control major cellular responses (27). Consequently, regulatory trehalases are viewed as cellular sensors which respond to environmental changes.

We considered of interest to determine whether the observations performed in particular yeast models were relevant to other unrelated species of yeasts in which trehalase activity had not been described. We studied the occurrence and distribution among yeasts of the response signals which can cause activation of trehalase induced by either glucose or heat shock. Such a goal was equivalent to the detection of potential regulatory trehalases among ascosporogenous, basidiosporogenous and deuteromycetous yeasts. We also looked for genes related to that coding for the regulatory trehalase in *S. cerevisiae.*

Materials and methods

Yeast strains and culture conditions. The organisms employed in this study are listed in Table 1. The yeast strains were cultured in YPD medium as previously indicated (8, 21), except that *Citeromyces matritensis*, *Metschnikowia pulcherrima*, *Brettanomyces bruxellensis* and *Cryptococcus albidus* were incubated at 25°C.

Analysis of the trehalase response systems. Derepressed cells from stationary phase cultures after glucose exhaustion, or repressed cells from exponentially growing cultures, were used to analyze the existence of either glucose-induced signal or heat skock-triggered trehalase activation. Experimental conditions have been described previously (3, 4). Trehalase activity was expressed as nmol of glucose produced per min (enzyme units) per mg protein. Experiments were

TABLE 1. Distribution of the glucose-induced signal for trehalase activation among yeasts

	Trehalase activity[1] (units/mg protein)	
	Control	+Glucose
Positive signal[2]:		
Candida utilis ATCC 60459	5.40	35.50
Citeromyces matritensis CECT 1066	3.91	8.65
Pachysolen tannophilus CECT 1426	1.58	25.71
Schizosaccharomyces pombe L972	4.02	31.98
Saccharomyces cerevisiae ATCC 26786	37.10	113.40
Zygosaccharomyces rouxii CECT 1229	0.01	1.18
Negative signal:		
Brettanomyces bruxellensis CECT 1009	7.14	1.56
Candida albicans CECT 1472	29.40	34.00
Cryptococcus albidus CECT 1069	0.18	0.25
Geotrichum lactis ATCC 48590	8.94	11.10
Kluyveromyces aestuarii TGV-1	1.52	2.98
Kluyveromyces lactis MW-103-1A	3.80	6.68
Kluyveromyces marxianus TGV-2	11.30	4.94
Metschnikowia pulcherrima CECT 1691	23.40	32.74
Pichia canadensis CECT 1466	7.73	4.90
Pichia fermentans CECT 1455	5.90	5.51
Pichia membranaefaciens CECT 1155	18.92	25.20
Rhodotorula rubra ATCC 2510	1.30	1.30
Sporobolomyces salmonicolor CECT 1274	4.70	3.90

[1] Derepressed cells from stationary phase cultures.
[2] A positive signal produced at least a 100% activation of trehalase under the assay conditions.

repeated at least three times. Representative results are shown in each case.

DNA manipulations. Total DNA preparations from different strains were obtained essentially as described by Moreno et al. (14). Southern (DNA)-hybridization analysis were performed as described in (22). DNA probe was labelled by digoxigenin as indicated by the suppliers (Boehringer-Mannheim). The following oligonucleotides corresponding to two conserved regions were prepared on the basis of sequence similarity of the cloned neutral trehalase genes (9, 11, 16, 23, 24): 5'GTT CCT GGT GGT AGA TTC 3' and 5'GAA TGG GGG CTG TGA TCT 3'. These oligonuclotides were used as primers (100 pmoles each) in PCR with 500 ng of the particular genomic DNA, 20 pmoles of each deoxynucleotide and 1.5 units of *Taq* polymerase (Perkin Elmer) in a final volume of 100 µl. The samples were run through several cycles in a Perkin Elmer thermocycler according to the following program: 1 min at 94°C to denature, 2 min at 45°C to anneal, and 2.5 min at 72°C for elongation. The same pattern was obtained when annealing was performed at 50°C. The products of the amplifications were separated by agarose gel electrophoresis and ligated into PCR™II. Sequencing was performed by the dideoxy-chain termination method (17).

Results

Presence of regulatory trehalase among yeasts. The addition of 100 mM glucose to cells

suspended in buffer brings about a marked increase in regulatory trehalase activity (25). We performed an analysis of the glucose-induced response of trehalase in various yeasts, and included *S. cerevisiae*, *Candida utilis* and *Schizosaccharomyces pombe* as positive controls (3, 4, 26), whilst *Rhodotorula* was used as a negative control (13). In addition, we considered that a response to the glucose signal was positive when the basal level of trehalase activity increased by at least 2-fold after adding the sugar. According to this criterion, three out of the fourteen species newly analysed gave a clear glucose-induced positive response (Table 1). Hence, the trehalases of these three species, namely *C. matritensis*, *Pachysolen tannophilus* and *Zygosaccharomyces rouxii*, can be added to the list of regulatory trehalases already known to occur in *S. cerevisiae*, *C. utilis* and *S. pombe* (3, 4, 26). In the three new cases the increase triggered by glucose was maximal 40–60 min after the addition of the sugar and ranged from about a 5-fold increase in *C. matritensis* to above a 100-fold increase in *Z. rouxii*. The trehalase response to glucose was negative is most yeasts although that of *Kluyveromyces lactis*, in particular, was rather ambiguous, with final values in trehalase around the limit of a 2-fold increase (Table1).

As indicated previously, the activity of trehalases of the regulatory type also increases in vivo under a thermal stress (4, 20, 25). Accordingly, the trehalase activity increased in *S. cerevisiae*, *C. utilis* and *S. pombe* following an incubation at 40°C for 1 h. (Table 2). However, this enzyme activity was insensitive to the temperature upshift in most species, and only two out of the three species mentioned above as giving positive the glucose-induced response, namely, *Pachysolen tannophilus* and *Z. rouxii*, were able to increase trehalase after a heat treatment (Table 2). These results sustain that these species, together with *S. cerevisiae*, *C. utilis* and

TABLE 2. Heat shock-induced trehalase activation among yeasts

	Trehalase activity[1] (units/mg protein)	
	Control	40°C, 60 min
Brettanomyces bruxellensis CECT 1009	3.80	1.22
Candida albicans CECT 1472	29.40	23.60
Candida utilis ATCC 60459	30.00	63.20
Citeromyces matritensis CECT 1066	0.61	0.97
Geotrichum lactis ATCC 48590	32.16	27.83
Kluyveromyces aestuarii TGV-1	4.74	5.25
Kluyveromyces lactis MW-103-1A	2.09	5.83
Kluyveromyces marxianus TGV-2	21.16	34.64
Metschnikowia pulcherrima CECT 1691	26.33	16.69
Pachysolen tannophilus CECT 1426	10.23	34.50
Pichia canadensis CECT 1466	15.09	3.89
Pichia fermentans CECT 1455	5.63	5.50
Pichia membranaefaciens CECT 1155	11.60	10.71
Saccharomyces cerevisiae ATCC 26786	30.00	108.00
Schizosaccharomyces pombe L972	3.50	50.00
Zygosaccharomyces rouxii CECT 1229	0.05	3.25

[1] Repressed cells from exponentially growing cultures.

S. pombe, have signalling pathways for trehalase activation which can be triggered either by glucose or heat shock, while *C. matritensis* lacks the second type of transduction pathway. Again, under heat shock, the yeast *K. lactis* gave results that were close to those shown by species with regulatory trehalases (Table 2).

Evidence of genes coding for regulatory trehalases in yeasts. A search on databases (GenBank, EMBL) revealed that genes coding for regulatory trehalases from various origins show conserved domains that are related to the catalytic centre (9, 11, 16, 23, 24). Therefore, an alternative, more direct way to establish the presence of regulatory trehalases among yeasts was to analyze the presence of DNA sequences corresponding to such conserved regions. We used the oligonucleotides outlined in Materials and methods to amplify by PCR the conserved fragment in the different species under study. Taking into account the size of the DNA region found between the oligonucleotides designed, a product of about 190 bp was expected to appear by PCR in positive samples. As shown in Fig. 1, when we used DNA of *S. cerevisiae* as a positive control, a product of 189 bp was obtained. However, a product of identical size was also present when genomic DNA from any other yeast species analyzed was used as template. This was extensive to species not included in Fig. 1, in which the presence of a gene coding for regulatory trehalase was not suspected because they showed trehalase unresponsive to glucose or heat shock, as described above. Cloning and sequencing of the PCR product confirmed that the 189 bp product was a rather conserved fragment flanked by the two oligonucleotides which are present in the neutral trehalase gene of *S. cerevisiae* (11). As an example, Fig. 2A illustrates the sequence of the 189 bp product which was obtained by using the *S. pombe* genome as template. As indicated, it shows 70% identity

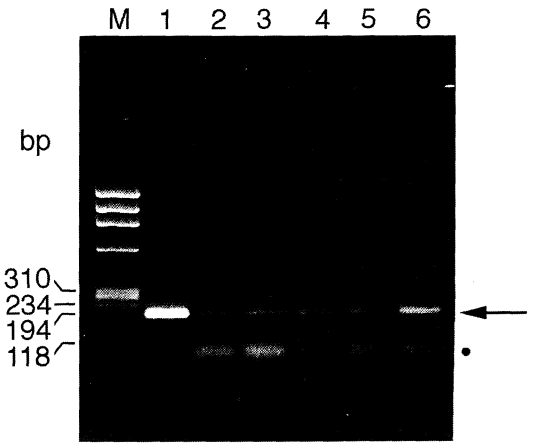
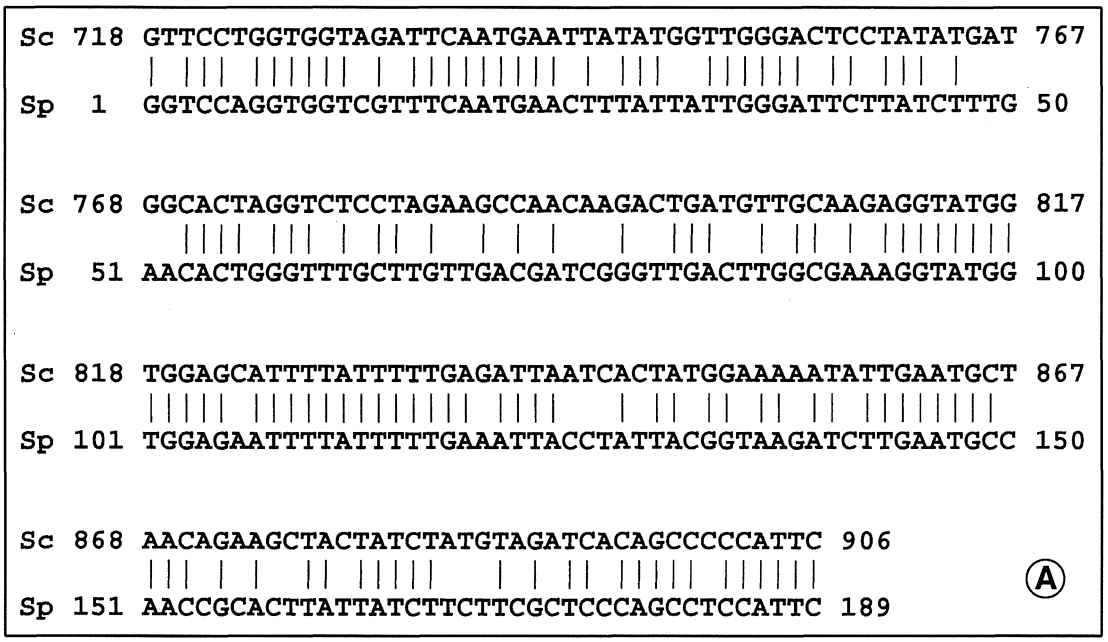


FIG. 1. Presence of the regulatory trehalase gene in several yeast species. A 189 bp DNA fragment (arrow), which was amplified by PCR, is conserved in the regulatory trehalase gene from *Saccharomyces cerevisiae* (control, lane 1), *Schizosaccharomyces pombe* (lane 2), *Candida albicans* (lane 3), *Candida utilis* (lane 4), *Zygosaccharomyces rouxii* (lane 5), and *Pachysolen tannophilus* (lane 6). Lane M shows molecular weight markers (DNA from ØX174 after digestion by *Hae*III). The point indicates the position for dimers of the primer oligonucleotides.

with the nucleotide sequence of the same fragment obtained from *S. cerevisiae,* and shares 78% identity of predicted aminoacid sequence (Fig. 2B). Hence, these results may be interpreted as an evidence for the presence of genes coding for regulatory trehalases even in those species where the levels of such enzyme activity remains relatively constant after drastic changes in the external conditions. Besides, studies by Southern-blot hybridization, using digoxigenin-labelled 189 bp fragment from *S. cerevisiae* as a probe and genomic DNAs from other yeast species after digestion by *Eco*RI y *Bam*HI, were consistent with this interpretation (data not shown).

Discussion

In yeasts, the distinction between regulatory and nonregulatory trehalases is mainly based on



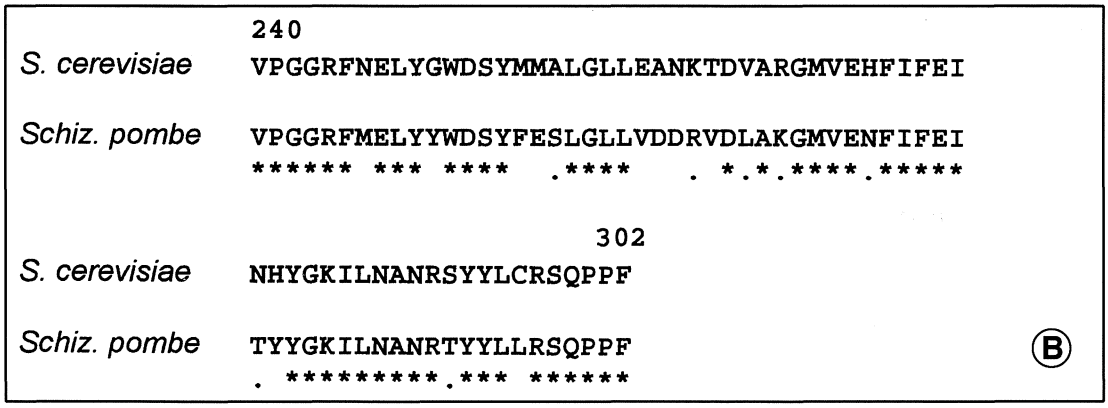


FIG. 2. Panel A: Comparison of the nucleotide sequence of the 189 bp DNA fragment amplified by PCR from genomic DNA from *Schizosaccharomyces pombe* (Sp) and the homologous region in the gene coding for regulatory trehalase in *Saccharomyces cerevisiae* (Sc). Panel B: Comparison of the deduced amino acid sequence. Amino acid identities (*) and conservative differences (.) are shown. Numbers refer to the corresponding *Saccharomyces cerevisiae* published sequences (see text, ref. 11).

functional properties of the trehalase enzymes. The enzyme activity in the first type of trehalases increases markedly in response to changes in the nutrient composition of the medium and during a physical stress (25). Moreover, at least in *S. cerevisiae*, the two types of trehalase are coded by two entirely different genes (11).

This study, together with results obtained earlier (3, 4, 13, 25), reveals that all yeasts with positive signal for glucose-induced or heat shock-induced activation of trehalase belong to the Saccharomycetaceae family (12). Although the deuteromycete *C. utilis* appears to be an exception to this rule, a more close analysis indicates

that such species corresponds to one of the sexual types of *Hansenula jadinii* and, hence, that this yeast can be also included within the Saccharomycetaceae family (12). However, this does not mean that all members of this ascosporogenous family, as for instance *Pichia*, show trehalase with regulatory properties. No functional regulatory trehalase activity was found among the ascosporogenous members of the Spermophthoraceae family (as *Metschnikowia*), the basidiomycetous yeasts (as *Rhodotorula* and *Sporobolomyces*) or among truly imperfect yeasts (as *C. albicans* or species of the Cryptococcaceae family).

Following this study, we have reported elsewhere the properties of the trehalase activity in *Z. rouxii* and in *P. tannophilus* in more detail (8, 21). We found that trehalase activity in the two species not only increased markedly *in vivo* after addition of glucose and by heat shock, but also decreased by in vitro treatment with phosphatase, suggesting that these trehalases are regulated by phosphorylation (8, 21). Related to the present study, it should be mentioned that while this work was in progress, a paper (1) has indicated that in *K. lactis*, trehalase undergoes glucose-induced changes in activity, although such response was slower and much less pronounced (about 2-fold increase) than in *S. cerevisiae*. The recent consideration that this species shows regulatory trehalase activity is not at variance with our conclusions, since we find that the trehalase activity in some species included in the *Kluyveromyces* genus is just in the borderline which we established arbitrarily to distinguish between regulatory and nonregulatory enzymes on the basis of their glucose-induced activation

We also show here that, independently of a responsive (i.e., regulatory) trehalase, all yeast examined bear a gene similar to that coding for regulatory trehalase in *S. cerevisiae*. It is very unlikely that the amplified bands shown in Fig. 1 might correspond to the acid trehalase gene, since no homology exists at the active center nor at any other region between the two types of trehalase (5, 11). Our results demonstrate that some yeasts have regulatory trehalases which, nevertheless, lack the coupled regulatory mechanisms by which these enzymes are operationally recognized. This represents a limitation to the classification of yeast trehalase enzymes into two groups (regulatory and nonregulatory) based only on the existence of accompanying control pathways. The possibility of comparing different species carrying similar genes for trehalase, without expressing similar regulatory properties, might reveal novel principles in physiological studies of the yeast cells.

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Comparison between the polypeptide profile of halophilic bacteria and salt tolerant plants

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Summary

Changes in the polypeptide profile induced by salt stress in halotolerant and halophilic bacteria, isolated from the Atacama desert (northern Chile), were compared with those in the cotyledons of *Prosopis chilensis* (Leguminoseae) seedlings, a salt tolerant plant. SDS–PAGE analyses show the presence of four predominant polypeptides, with molecular weights around 78, 70, 60 and 44 kDa respectively, both in bacteria and in cotyledons from *P. chilensis* seedlings raised under salt stress conditions. Moreover, the 60 and 44 kDa polypeptides seem to be salt responsive, since their concentration increases with increasing NaCl in the growth medium. Our results suggest a common mechanism for salt tolerance in prokaryotes and in eukaryotes.

Key words: *Prosopis chilensis* (Leguminoseae), halophilic bacteria, salt tolerance, salt stress, polypeptide profile

Resumen

Se compara el perfil polipeptídico en respuesta a la salinidad entre bacterias halotolerantes y halófilas, aisladas del desierto de Atacama (norte de Chile), con el perfil de cotiledones de plántulas de *Prosopis chilensis* (Leguminoseae), una planta halotolerante. El análisis mediante SDS–PAGE muestra cuatro polipéptidos predominantes, con pesos moleculares aproximados de 78, 70, 60 y 44 kDa, tanto en bacterias como en cotiledones de plántulas de *P. chilensis,* en respuesta al estrés salino. Además, los polipéptidos de 60 y 44 kDa se sintetizan en respuesta a concentraciones

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crecientes de salinidad. Nuestros resultados sugieren que organismos eucariontes y procariontes presentan un mecanismo común de tolerancia a la salinidad.

Introduction

Salt stress is a major factor restricting life. Although saline environments may contain a variety of salts, sodium chloride is the most commonly source of salinity (6), and one of the main factors that limit the growth of bacteria and plants in arid zones. High salt concentrations in the environment are responsible for osmotic stress, because water availability is low and there is a tendency for water to flow out of the cells. Ionic stress also occurs due to specific ion toxicity. These two types of stresses are related, and they affect adversely the development of organisms in saline environments.

Salt tolerant organisms have evolved mechanisms to cope with salinity. These mechanisms may be underlaid by the biosynthesis of specific polypeptides which are involved in survival under salt stress conditions (2, 8). Basically, salt responsive proteins are related with membrane stabilization and with the synthesis of osmoprotectant compounds, which is one of the most important adaptive mechanisms that assure the maintenance of turgor by reducing the adverse effects of salinity and drought (7).

To study the protein synthesis response of prokaryotic and eukaryotic salt tolerant organisms, we compared the polypeptide profile of two bacterial strains (halotolerant and halophilic, respectively) isolated from the Atacama Desert in northern Chile with that of *Prosopis chilensis*, a native plant from saline environments. *P. chilensis* (Leguminoseae, subfam. Mimosoideae) is a leguminous tree native from the arid and semiarid regions in central and northern Chile (4), which is resistant to drought, heat and salt stress (10). The primary effect of salt stress is experienced by seeds, germination being a critical stage of plant development during which salt effects are most prominent (14).

Materials and methods

Halotolerant (strain LTC99) and halophilic (strain LT6) bacteria were isolated from the Tebenquiche Lake in the Atacama desert, north-

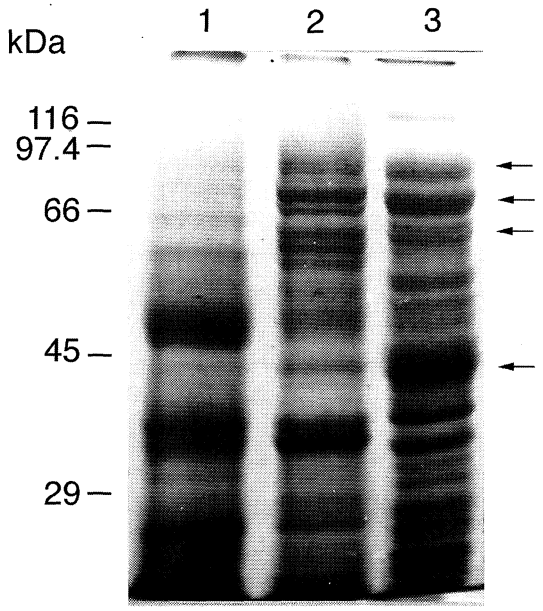

FIG. 1. Polypeptide profiles of *Prosopis chilensis* and halophilic bacteria grown in saline conditions. Proteins were resolved by SDS–PAGE 10%. Lane 1: *P. chilensis* cotyledons from seedlings grown in seawater; lane 2: plant cotyledons from seedlings grown in 2.3% NaCl; lane 3: culture from bacterial strain LT6 grown in 15% NaCl. Arrows stands for changes in the polypeptide profile in response to saline conditions, indicating the appearance of 78, 70, 60 and 44 kDa polypeptides.

ern Chile (22°54'S, 69°49'W), and grown in MH medium (12), supplemented with 0, 5, 10 and 15% NaCl. The cultures were grown for 24 h at 32°C, with agitation. Seeds of *P. chilensis* were collected in Putaendo, central Chile (33°09'S, 70°39'W). To break out dormancy, the seeds were scarified with concentrated H_2SO_4 for 10 min (11), washed several times with H_2O and finally germinated in distilled water supplemented with 0, 0.6, 1.2, 1.8 and 2.3% NaCl (1). Cell extracts were obtained from bacteria and from cotyledons of 5-day-old seedlings. Samples were frozen at –20°C and ground in a mortar and pestle with buffer Tris 10 mM pH 7.2 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO). The resulting macerates were centrifuged at 10.000 × *g* for 10 min at 4°C, and the supernatant was recovered. Protein concentration was determined using the Bradford method (3). Proteins were analyzed by one-dimensional gel electrophoresis, essentially as described by Laemmli (9). Slabs of 10 polyacrylamide with SDS were stained with Coomassie Blue R-250 (Sigma). Molecular weight values of the polypeptides were determined from mobility on SDS–PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis) and then compared with the following protein standards run in the same gel: β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; albumin bovine, 66 kDa; albumin egg, 45 kDa and carbonic anhydrase, 29 kDa.

Results and discussion

The selection of *P. chilensis* and halotolerant and halophilic bacteria as model systems for studying the effect of salt stress in protein synthesis is based on the fact that these organisms inhabit highly stressing ecosystems in which salinity and high temperatures are predominant.

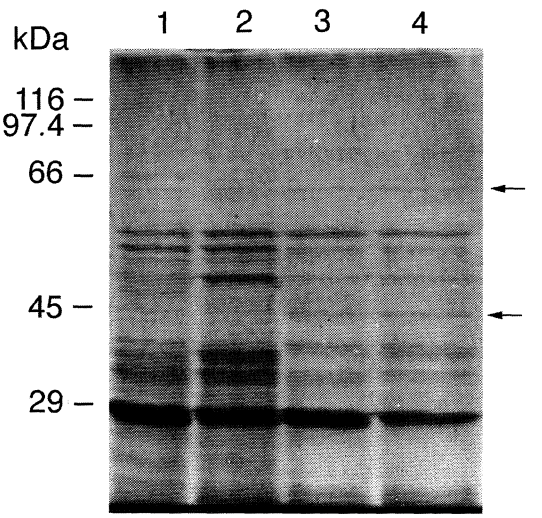

FIG. 2. Effect of saline stress in the polypeptide profile of strain LTC99, halotolerant bacteria. Proteins were analyzed by SDS–PAGE 10%. Positions of molecular weight standards are shown on the left. Lane 1: 0% NaCl; lane 2: 5% NaCl; lane 3: 10% NaCl; lane 4: 15% NaCl.

The protein content of cotyledons was analyzed in plants, since seed germination and seedling stage are most sensitive to saline conditions (13). Our

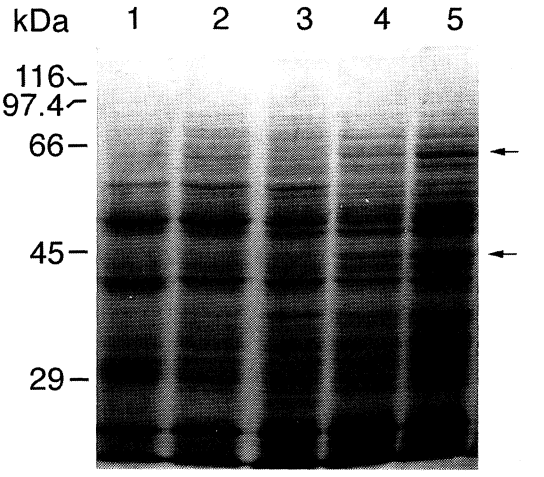

FIG. 3. Polypeptide composition of *Prosopis chilensis* cotyledons from seedlings grown with increasing concentrations of sodium chloride. Proteins were resolve by SDS–PAGE 10%. Lane 1: 0% NaCl; lane 2: 0.6 NaCl; lane 3: 1.2% NaCl; lane 4: 1.8% NaCl; lane 5: 2.3% NaCl.

results show the appearance of common polypeptides in both bacterial strains and *P. chilensis* grown under saline conditions. This could be due to a common response to salt tolerance in both highly separated biological organizations.

Fig. 1 shows the polypeptide profiles of *P. chilensis* cotyledons from seedlings grown in distilled water (lane 1) and NaCl (lane 2), and that of halophilic bacteria (lane 3). Under salt stress conditions common responses are observed, both in cotyledons and in the two bacterial strains which are related mainly to the synthesis of four predominant polypeptides with apparent molecular weights of 78, 70, 60 and 44 kDa. The 60 and 44 kDa polypeptides were found to be specifically induced in halotolerant bacteria (Fig. 2) and in *P. chilensis* cotyledons (Fig. 3) by increasing NaCl concentration in the growth medium. A similar 60 kDa polypeptide has also been described as a salt-induced protein in the halotolerant alga *Dunaliella* (5). A 70 kDa salt stress responsive polypeptide has been reported in protein extracts from 3-day-old Indica rice (*Oryza sativa*) seedlings (13).

At present we do not know yet whether the proteins detected in response to salinity in bacteria and *P. chilensis* are the same, or if they share common amino acid sequences. Further research must be done to answer this question.

We can conclude that tolerance or resistance to salinity stress induced by NaCl in *P. chilensis* and in both halotolerant and halophilic bacteria is related to the synthesis of specific polypeptides, and showing that this two highly diversified salt tolerant organisms might have evolved common protein synthetic responses.

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Development of a selective culture medium for *Fusarium moniliforme*

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Summary

Nash and Snyder medium and malachite green agar 2.5 ppm medium, a new selective culture medium designed in our laboratory, were challenged with pure cultures of *Fusarium moniliforme* strains and two different mixed-conidium suspensions, which included rapidly spreading fungi, for their utility in the isolation and enumeration of *F. moniliforme*. From the results of this comparative study, malachite green agar 2.5 ppm allowed only the selective growth of *F. moniliforme* whereas Nash and Snyder medium allowed both the growth of *F. moniliforme* and other species not belonging to *Fusarium* spp. The enumeration of *F. moniliforme* propagules was similar in both culture media.

Key words: *Fusarium moniliforme*, selective culture medium, enumeration of fungi, isolation of fungi, malachite green agar

Resumen

Los medios de cultivo de Nash y Snyder y el agar verde de malaquita 2,5 ppm, un nuevo medio de cultivo selectivo diseñado en nuestro laboratorio, fueron ensayados con cultivos puros de cepas de *Fusarium moniliforme* y con dos suspensiones distintas de mezclas de conidios, que incluían hongos de crecimiento invasivo, para valorar su utilidad en el aislamiento y recuento de

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F. moniliforme. Los resultados de este estudio comparativo muestran que el medio agar verde de malaquita 2,5 ppm permite sólo el crecimiento selectivo de *F. moniliforme,* mientras que el medio de Nash y Snyder permite el desarrollo de *F. moniliforme* y de otras especies no pertenecientes al género *Fusarium*. El recuento de las colonias de *F. moniliforme* fue similar en ambos medios de cultivo.

Introduction

Fusarium moniliforme (Sheldon) is a common contaminant of maize and other cereals. This fungus occurs worldwide and it is implicated in human and animal toxicoses. The interest in this species has more and more increased over the last decade. In fact, novel mycotoxins that have been called fumonisins were isolated and characterized from *F. moniliforme* present in maize cultures (1, 6). Fumonisins are produced mainly by this species, and they are implicated in leucoencephalomalacia disease in equines (10), and are also associated with pulmonary oedema syndrome in swine (8). Moreover, it has been statistically correlated with oesophageal cancer in humans (14). Besides, experimentally fumonisin B_1 has proved to induce liver damage and cancer-promoting activity in rats (6) and some pathological effects in poultry (4, 5).

On the other hand, selective media for the isolation and enumeration of one or more species of the genus *Fusarium* spp. have been studied by many authors. Nash and Snyder medium (11) and several modifications (7, 9, 12) are recommended for these purposes. However, these media contain pentachloronitrobenzene, a potential carcinogen. This fact prompted us to investigate the effect of some dyes as fungal inhibitors (2, 3), and particularly the dye malachite green on the colony enumeration of *F. moniliforme* in pure and mixed cultures. In previous studies we tested malachite green at different concentrations: 2.5, 5 and 7 ppm (Castellá, G., 1997, Ph. D. Thesis, Autonomous University of Barcelona). We found statistically significant differences among these media, 2.5 ppm being the lowest concentration of dye with the highest counts for *F. moniliforme*. The objective of the present study was to compare Nash and Snyder medium and malachite green agar 2.5 ppm medium, a new selective culture medium designed in our laboratory, for their use in the isolation and enumeration of *F. moniliforme*.

Materials and methods

Malt extract agar (MEA) (malt extract, 20 g; peptone, 1 g; glucose, 20 g; agar, 20 g; distilled water, 1 liter), and Nash and Snyder medium base (NSMB) (peptone, 15 g; KH_2PO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; agar, 20 g; distilled water, 1 liter) (11) were used as control media. Malachite green agar (MGA) (NSMB plus malachite green, 2.5 ppm) and Nash and Snyder medium (PCNB) (NSMB plus pentachloronitrobenzene [PCNB], 1 g/l) were used as selective media for *Fusarium*.

Culture media were challenged with pure cultures of strains of *F. moniliforme* (7 strains from different sources), and with two different spore suspensions which had the following species composition: mixture A (*Aspergillus flavus*, *Penicillium aurantiogriseum* and *F. moniliforme*) and mixture B (*F. moniliforme* and *Rhizopus stolonifer*). The final concentrations of the spore suspensions were ca. 10^3 spores/ml in the case of individual enumeration of *F. moniliforme* strains, and ca. 10^5 spores/ml in the case of mixtures A and B. Concentrations of the

TABLE 1. Mean colony counts in CFU/ml of each *Fusarium moniliforme* strains used in the three media assayed at 7 days of incubation. (Figures are CFU/ml)

Strain	NSMB	PCNB		MGA		
F2	136	190	(41.3)[a]	234	(85.5)	
F6	148	160	(28.1)	170	(55.9)	
F13	228	304	(18.4)	470	(84.5)	
F19	70	104	(38.4)	96	(85.2)	
F27	156	126	(15.2)	102	(15.2)	
F28	652	698	(0)	718	(69.8)	
F35	408	480	(41.4)	456	(84.6)	
	256.8	294.6	(26.1)	320.8	(68.6)	NS

[a] In parenthesis, mean percentages of reduction of colony diameters compared with the colony diameters in the NSMB medium.

Abbreviations: NSMB, Nash and Snyder medium base; PCNB, the former (NSMB), plus pentachloronitrobenzene; MGA, malachite green agar; NS, no significant differences ($p > 0.05$) among *Fusarium moniliforme* counts in the media assayed.

suspensions were determined by a counting chamber.

The mixed-spore suspensions were prepared taking equal volumes of each individual spore suspension. At least five plates per dilution and medium were inoculated using the surface-spread method. Plates were incubated at 28°C and lectures were made after 7, 10 and 14 days of incubation in the case of *F. moniliforme* pure cultures tested, and after 3, 5, 7, 10 and 14 days in the cases of the mixtures A and B. Only plates with 10–100 colony forming units (CFU) were used for counting. Total CFU/ml was determined as well as the number of CFU/ml for *F. moniliforme* in mixtures A and B, according to macroscopic and microscopic studies. In addition, percentage of reduction of the colonies diameters was determined when assaying pure cultures of *F. moniliforme*. The data obtained were statistically analyzed by analysis of variance.

Results

Table 1 shows the mean colony counts (CFU/ml) of each *F. moniliforme* (7 pure culture strains) tested in NSMB, PCNB and MGA media, after 7 days of incubation, and also the mean percentages of reduction in colony diameters obtained in PCNB and MGA media compared with the colony diameters in the control medium NSMB. Higher counts were recorded in decreasing order in MGA, PCNB and NSMB media, but no significant differences among them were observed ($p = 0.7760$). Cultural characteristics of the colonies were similar in MGA and PCNB, although mean percentages of reduction in colony diameters of *F. moniliforme* strains were higher in MGA than in PCNB media.

Table 2 shows the mean values of both total and partial counts (CFU/ml) of all strains of *F. moniliforme* assayed (Mixture A). No statistically significant differences were found ($p = 0.1030$) in the *F. moniliforme* counts in the four culture media used. Nevertheless, in MGA only colonies of *F. moniliforme* were grown, whereas the other media allowed colony development of both *A. flavus* and *P. aurantiogriseum* strains.

Table 3 shows the mean values of both *F. moniliforme* and *R. stolonifer* colony counts

TABLE 2. Spores mixture A. Mean values of total counts (TC) and partial counts of each strain of *Fusarium moniliforme* (PC) observed in the four media assayed, after 7 days. (Figures are CFU/ml)

Strain	MEA	NSMB	PCNB	MGA	
	TC/PC	TC/PC	TC/PC	PC	
F2	6,780/3,240	7,440/4,180	7,480/4,240	4,760	
F6	17,600/9,800	13,400/7,000	15,600/8,600	9,400	
F10	5,860/3,260	5,420/4,520	6,180/4,060	3,920	
F13	4,160/2,120	3,980/2,160	4,100/2,540	2,780	
F19	5,940/2,500	5,880/2,840	5,760/2,420	2360	
F21	8,280/5,960	5,900/5,240	7,840/6,500	5,560	
F24	3,840/1,960	5,520/1,320	7,180/1,620	2,080	
F27	4,700/940	5,150/1,050	4,580/1,080	540	
F28	4,580/2,940	4,300/3,100	5,280/3,040	3,080	
F35	2,400/560	1,940/760	1,780/500	720	
	6,414/3,328	5,893/3,261	6,578/3,460	3,520	NS

Abbreviations: MEA, malt extract agar; NSMB, Nash and Snyder medium base; PCNB, the former (NSMB), plus pentachloronitrobenzene; MGA, malachite green agar; NS, no significant differences (p >0.05) among *Fusarium moniliforme* counts in the media assayed.

(CFU/ml) on PCNB and MGA media (Mixture B). As it could be expected, neither isolation nor identification of *F. moniliforme* were possible in NSMB and MEA media because of the overgrowth of *R. stolonifer*. Higher counts were obtained on MGA for *F. moniliforme*, although

TABLE 3. Spores mixture B. Mean values in CFU/ml for *Rhizopus stolonifer* (RS) and *Fusarium moniliforme* strains (FM) in Nash and Snyder and Malachite Green media, after 7 days

Strain	PCNB	MGA	
	RS/FM	RS/FM	
F2	3,880/6,340	0/7,940	
F6	1,560/9,820	0/9,060	
F10	440/4,620	0/4,480	
F13	1,950/3,420	0/3,300	
F19	18,260/2,360	0/6,300	
F2	980/8,460	0/9,200	
F24	2,340/3,540	0/3,660	
F28	3,160/4,060	0/4,320	
F35	1,620/900	0/920	
	3,790/4,835	0/5,464	NS

Abbreviations: PCNB, Nash and Snyder medium base plus pentachloronitrobenzene; MGA, malachite green agar; NS, no significant differences (p >0.05) among *Fusarium moniliforme* counts in the media assayed.

no significant differences in relation to PCNB were observed ($p = 0.9840$). In this case, MGA was a very useful selective medium because colonies of *R. stolonifer* did not grow on it, in contrast to PCNB where a high number of colonies of *R. stolonifer* developed.

Discussion

Singh and Nene (13) studied the positive selective effect of malachite green and captan on the growth of *Fusarium* spp. from plant tissues. However, Papavizas (12) indicated the excessive inhibitory effect of the malachite green concentration (50 ppm) assayed by the above mentioned authors in *Fusarium* spp., and suggested the use of both Nash and Snyder medium (PCNB) and one modification of this medium (VDYA-PCNB) for effective isolation of *Fusarium* spp. from soil samples.The inhibitory effect of malachite green had been also observed in our previous studies, the best malachite green concentration having been observed at 2.5 ppm (Castellá, G., 1997, Ph. D. Thesis, Autonomous University of Barcelona). In fact, lower concentrations allow the development of other fungal genera is allowed (3).

The results of the preliminary comparative study showed that MGA allowed only the selective growth of *F. moniliforme,* whereas modified Nash and Snyder medium allowed the growth of both *F. moniliforme* and the other species not belonging to *Fusarium* spp. The enumeration of *F. moniliforme* propagules was similar in both medium. We can conclude that MGA is a very useful media for the isolation and enumeration of *F. moniliforme*. Further studies will be made on the suitability of this selective medium for other species of *Fusarium* and it will be tested with some natural substrates and food and feed samples.

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MICROBIOLOGÍA SEM **13** (1997), 499–508 **PERSPECTIVES**

European journals on microbiology

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Summary

A survey on the scientific journals dealing with microbiology published in Europe has been carried out. Eighteen European countries publish microbiological journals with the United Kingdom, Netherlands and Germany leading in number of journals on this speciality. Most of the European journals on microbiology are published bimonthly (27%), and English is the most common language used (54%). Most of these journals (86%) are included in some database, but only 36 (25%) are indexed in the six databases studied. Out of the 146 journals registered, 71 (49%), published in 11 European countries, are included in the 1995 *Journal Citation Reports* (ISI, Philadelphia).

Key words: microbiology journals, citation reports, scientific language

Resumen

Se han recopilado y analizado las revistas de microbiología que se editan en Europa. Son 18 los países europeos que publican revistas de esta especialidad, destacando por el número de publicaciones el Reino Unido, Holanda y Alemania. La mayoría aparecen cada dos meses (27%), y el idioma más utilizado es el inglés (54%). La mayor parte (86%) de las revistas están incluidas en alguna de las bases de datos que cubren esa especialidad, con 36 (25%) que están recogidas en todas las bases de datos examinadas. De las 146 revistas registradas, 71 (49%), publicadas en 11 países europeos, están incluidas en el *Journal Citation Reports* (ISI, Philadelphia) de 1995.

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Introduction

Due to the large number of scientific journals published on the main scientific disciplines, researchers try to select the best both to catch up with scientific advances and to have the results of their own work published in them.

Some criteria to make this selection take into account factors that guarantee reliability of the publication, assuring the quality of the articles they publish. Other criteria are the impact the article may have in the journal, by their inclusion in databases and the international impact or predictable references the articles are likely to have, based on the impact factor of the journals. For researchers to be able to select the most suitable journal, it is of great interest to have the journals in the discipline make their work available.

Microbiology is a discipline of interest in different scientific fields such as biology, medicine, pharmacology or food chemistry. In fact, the interaction between microbiology and other scientific areas holds most of the best prospects of scientific development in the near future are foreseen. Due to the interdisciplinary interest that they arouse, scientific articles of potential interest for microbiologists are found dispersed in different thematic sections in journal directories. Besides, there is no uniformity among them. Thus, the same journal might be classified either as a medical journal, a pharmacology journal or a chemistry journal in different indexes.

We present here a compilation of journals published in Europe that partially or totally include microbiology in their scope. For every journal studied the following aspects are included in the final appendix, European Journals of Microbiology: title, sponsoring institution/publisher, country, periodicity, language. A more complete information resulting from such an exhaustive study includes: Title of the journal. ISSN. Country. Periodicity. Language of publication. Sponsoring institution/Publisher. Board address (Postal address, phone and fax numbers, e-mail, URL). Impact factor. Circulation. Price in the currency of the country where the journal is published. Databases which include the journal. The report is completed with Internet addresses of editors wherever instructions to potential authors can be obtained via Internet. People interested in having this information may ask it to the author of this work.

Material and methods

All journals published in Europe classified under the epigraph "Biology–Microbiology" (or containing this term in the description of their coverage) in the Ulrich's Periodicals Directory (1) and Serials Directory (2) journal directories have been selected. These directories contain the major repertories of existing journal, and both are published in printed and CD-ROM format. The CD-ROM version is updated quarterly, and the printed version once a year.

Although most journals in both directories coincide, this coincidence is not complete in the number of journals they contain, or in the classification topics used, or in the data offered by every journal. So, the information of the two directories has been complemented and we have created a database in Microsoft Access.

Results

Distribution by countries of the European journals on microbiology is shown in Fig. 1. The United Kingdom publishes the most journals on this subject, which means more than 25%. The Netherlands follows, with 22%; and Germany is third, with 20%. The journals published in these three countries make up more than 50% of all

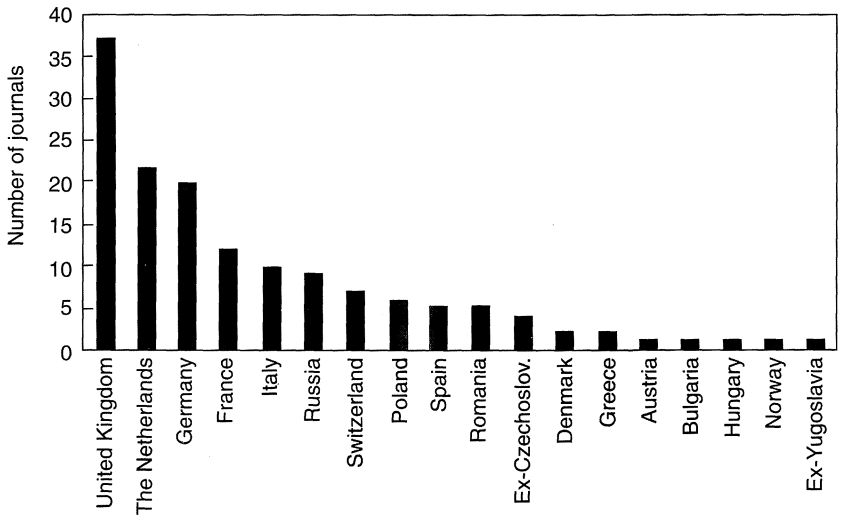

FIG. 1. Number of European journals on microbiology, distributed by countries.

journals on microbiology published in Europe. While five countries publish only one journal on this speciality, Spain and Rumania are in the ninth with five. Interestingly enough, Belgium and Sweden do not publish any.

Sponsoring institution/publisher. 80 out of the 146 journals on microbiology published in Europe indicate their being sponsored by any kind of institution or scientific society. Forty-two of these journals are official publications of scientific societies in the field of microbiology, 15 of which are published by general microbiology societies, 6 by federations of microbiology societies, and 6 by infectious diseases societies; the other fifteen are published by parasitology associations, virology, bacteriology, mycology, etc. Other group of sponsoring entities include government research institutions, which are responsible for the publication of 38 journals.

The other journals are published by companies of great prestige, as Elsevier Science (Netherlands), Blackwell Science (United Kingdom), Springer and Gustav Fischer Verlag (Germany), and S. Karger A. G. (Switzerland).

Periodicity. Frequency of publication is related to the number of articles submitted (Fig. 2). Most journals are published bimonthly (40 titles), quarterly (37 titles) and monthly (32 titles). More than 73% of the journals studied belong to one of these three periodicities. The periodicity of 11 journals ranges from 8 to 33 issues per year, and have been enclosed in a group called "other periodicity". The group of "irregular periodicity" is composed of major series-type publications, most of which are devoted to reviews or updating.

Language. Most of the journals (79 titles) are published exclusively in English, i.e. all journals published in the United Kingdom and most of those published in the Netherlands and in Switzerland. The second group (22 titles) accept a number of different languages (including English). Almost all other are in the national languages and also accept English. Besides, most of the journals state that they include summaries in English.

Diffusion in databases. International scope is important since scientists tend to publish in high visibility journals. To assess objectively this criterion, only six of all the databases that include subjects on microbiology were surveyed. They were selected because of their prestige and

international scope: *Biosis* (BA), *Chemical Abstracts* (CA), *Excerpta Medica* (EM), *Medline* (IM), *Index Veterinarius* (IV), *Sci Search* (SCI).

There are 36 journals included in the studied databases; 29 appear in 5 of them; 21 journals are not included in any of the databases that are been considered in our study. We must point out that Austria and Norway publish each one only journal on microbiology, and both are included in the six databases selected; Bulgaria and ex-Yugoslavia publish each one only journal, both of which are included in three databases. Of the five Spanish microbiology journals, two are included in three databases; one is included in two databases, and the other appear in only one (Table 1).

Impact factor. The impact factor (IF) for a specific year is defined as the total number of citations made that year for articles published in the two previous years divided by the number of citable articles published in those two years. I.e.,

$$IF = \frac{\text{Citations in 2 years}}{\text{Total No. of articles in 2 years}}$$

Because of the method used to obtain it, IF is quite variable and favors "classical" journals, and not recent ones. Also, IF favors journals with fewer issues per year, and is detrimental to journals with a larger number of articles (e.g., in our field, *Journal of Bacteriology*, or *Microbiology*). The IF currently used does not distinguish between review and research (containing primary results) articles; therefore, journals or book series devoted to reviews get a higher IF (e.g., *Annual Review in Biochemistry* or *Annual Review in Microbiology*). Those reviews are written usually by invitation, in which case peer-reviewing may not be so strict.

For this study, values corresponding to the last results appearing in the 1995 *Journal Citation Reports* (JCR-95; published by the Institute for Scientific Information) were used (3).

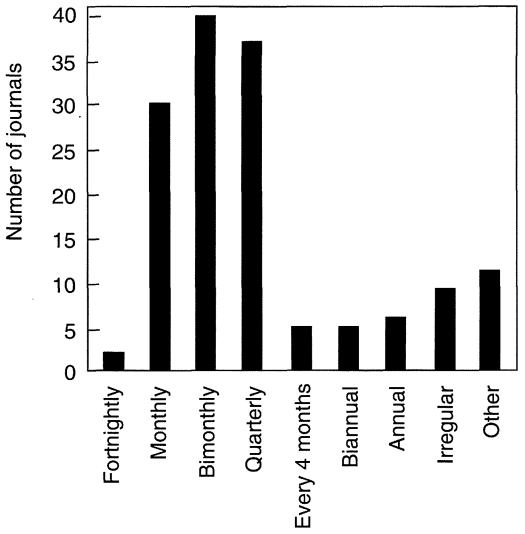

FIG. 2. Periodicity of European journals on microbiology.

Table 2 shows the European journals on microbiology with an IF ≥ 2. *Molecular Microbiology* has the highest IF: 5.024. This journal is ranked fourth place in the thematic classification of microbiology from the JCR-95, following *Microbiological Reviews*, *Annual Review in Microbiology* and *Clinical Microbiology,* all three, published in the United States.

There are 71 journals, approximately half of the total, coming from 11 countries (Table 3). Although journals recorded in more databases are among those included in the JCR, there are some exceptions.

Interestingly, *Acta Microbiologica Polonica* is the only journal recorded in the six databases that is not included in the JCR. The following journals recorded in five databases are not included in the JCR: *Acta Biologica Iugoslavica.* Serija B. Mikrobiologija, *Acta Microbiologica Bulgarica, Acta Microbiologica et Inmunologica Hungarica, Contributions to Microbiology and Inmunology* (Switzerland), *Medycyna Doswiadczalna i Mikrobiologia* (Poland), *The New Microbiologica* (Italy), *Progress in Medical Vi-*

TABLE 1. Number of databases in which are included the European journals on microbiology, distributed by countries

Country	Number of data bases							
	Six	Five	Four	Three	Two	One	None	Total
United Kingdom	10	9	2	5	1	2	8	37
Netherlands	8	4	1	1	3	2	3	22
Germany	7	3	2	1	2	–	5	20
France	3	1	2	2	1	2	1	12
Russia	1	1	2	1	1	–	3	9
Italy	–	1	3	2	1	3	–	10
Switzerland	1	3	–	1	1	1	–	7
Poland	1	2	1	1	–	1	–	6
Rumania	–	2	–	–	2	–	1	5
Spain	–	–	–	2	1	2	–	5
Ex-Czechoslovakia	2	–	2	–	–	–	–	4
Denmark	1	–	–	–	–	1	–	2
Greece	–	–	–	–	2	–	–	2
Austria	1	–	–	–	–	–	–	1
Bulgaria	–	1	–	–	–	–	–	1
Hungary	–	1	–	–	–	–	–	1
Norway	1	–	–	–	–	–	–	1
Ex-Yugoslavia	–	1	–	–	–	–	–	1

rology (Switzerland), *Revue Roumaine de Virologie,* and *Romanian Archives of Microbiology and Immunology.* Most of these journals are from Mid and Eastern Europe (and formerly dependent on the USSR). However, one of the journals analyzed by the JCR is only recorded in one of the databases, namely *Oral Microbiology and Immunology*, from Denmark.

Circulation and prices. Only rarely do the directories consulted provide information about the circulation of the journals, provided that the journals themselves accept to give this information. Only 62 journals include it. The circulation figures range from 22,000 copies (*Microbiology Europe*) to 173 copies (*Microbial Ecology in Health Diseases*). Two of the Spanish journals are among those with the highest circulation: *Enfermedades Infecciosas y Microbiología Clínica*, with 3500 copies, and *Microbiología SEM*, with 2500 copies. Prices are those indicated in the last edition of Ulrich's directory and might have changed.

Conclusions

To sum up, we can conclude:

(i) European journals on microbiology are published by 18 countries, the United Kingdom being the major publishing country with 37 journals; five countries publish each one only journal in the field. This fits in with the scientific tradition of microbiological research in the countries, and the significance given to the diffusion of research in appropriate media. On the other hand, it is surprising that Belgium and Sweden, both countries with high scientific development, do not publish any journal on microbiology.

(ii) Many journals (80 out of 146 in our study) are sponsored and/or published by scientific associations and research institutions. Among the commercial publishers, some of the most prestigious and well known in Europe can be found.

(iii) The most frequent publishing periodicity is bimonthly (27%), followed closely by

TABLE 2. Impact Factor (IF) ≥ 2 of the European journals on microbiology. (Data from the 1995 *Journal Citation Reports*, JCR-95.)

Title	IF (JCR-95)	Country
Molecular Microbiology	5.024	United Kingdom
FEMS Microbiology Reviews	3.988	Netherlands
Seminars in Virology	3.625	United Kingdom
Journal of General Virology	3.410	United Kingdom
Parasitology Today	3.296	United Kingdom
Molecular Ecology	2.992	United Kingdom
Molecular and Biochemical Parasitology	2.803	Netherlands
Systematic and Applied Microbiology	2.216	Germany
Virus Research	2.161	Netherlands
Medical Microbiology and Immunology	2.145	Germany
Microbiology	2.132	United Kingdom
Journal of Antimicrobial Chemotherapy	2.105	United Kingdom
Glyoconjugate Journal	2.065	United Kingdom
Yeast	2.000	United Kingdom

quarterly and monthly journals (25% and 20%, respectively).

(iv) The most used publication language is English. Practically all journals published in other languages accept also collaborations in English.

(v) More than 85% of the European journals on microbiology are listed at least in one database from the six considered here, and 65% are included in at least three of them.

(vi) Out of a total of 146 European journals on microbiology, 71 appear in the Journal Citation Reports and are classified by their impact factor, but they come only from 11 countries. The only journals published in Austria and in Norway are both in these classifications and in all databases considered.

TABLE 3. Number of European journals on microbiology included in the 1995 *Journal Citation Reports* (JCR-95), distributed by countries

Country	JCR-95	Total
United Kingdom	26	37
Netherlands	16	22
Germany	12	20
France	5	12
Switzerland	3	7
ex-Czechoslovakia	2	4
Denmark	2	2
Russia	2	9
Austria	1	1
Norway	1	1
Poland	1	6

Acknowledgements

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2. The Serials Directory. 1996. EBSCO Publishing.
3. Science Citation Index: Journal Citation Reports. 1995. Institute for Scientific Information. Philadelphia.

APPENDIX: EUROPEAN JOURNALS ON MICROBIOLOGY

P: periodicity (number of journals per year); Q, quarterly; Ir, irregular; L, language; B, Bulgarian: C, Czech; E, English; F, French; G, German; Gr, Greek;I: Italian; P, Polish; Pg, Portuguese; Ru, Ruman; R, Russian; S, Spanish; Sl, Slovak; NS, not stated.

Acta Biologica Iugoslavica. Serija B: Mikrobiologija (Biological Sciences, Yugoslavia) [P: 1. L: NS]

Acta Microbiologica Bulgarica (Bulgarian Academy of Sciences) [P: 2. L: B, R]

Acta Microbiologica et Immunologica Hungarica (Magyar Tudomanyos Akademia, Hungary) [P: Q. L: E]

Acta Microbiologica Polonica (Polskie Towarzystwo Mikrobiologow) [P: Q. L: P, E]

Acta Parasitologica (Polish Academy of Sciences) [P: Q. L: E, F, G, P]

Acta Protozoologica (Polish Academy of Sciences) [P: Q. L: E]

Acta Virologica (Slovak Academy of Sciences) [P: 6. L: E]

Actinomycetes (Centro per l'Ecologia Teorica e Applicata, Italy) [P: 3. L:NS]

Alpe Adria Microbiol. Journal (Assoc. Microbiologici Clinici Italiani) [P: Q. L: E]

Anaerobe (Academic Press, United Kingdom) [P: 6. L: E]

Annali di Igiene, Medicina Preventiva e di Comunità (Istituto di Igiene Guiseppe Sanarelli, Italy) [P: 6. L: I]

Annali di Microbiologia Ed Enzimologia (Univ. degli Studi di Milano, Dipart. di Scie. e Tec. Alimentari e Microbiol., Italy) [P: 2. L: E, F, I]

Antimicrobial Agents Annual (Elsevier, Netherlands) [P: 1. L: E]

Antiviral Research (Internat. Soc. for Antiviral Research, Netherlands) [P: 12. L: E]

Antonie Van Leeuwenhoek (Stichting Antonie van Leeuwenhoek, Netherlands) [P: 8. L: E]

Acta Pathologica, Microbiologica et Immunologica Scandinavica (Denmark) [P: 12. L: E]

Applied Biochemistry and Microbiology (Russian Academy of Sciences) [P: 6. L: R, E]

Applied Microbiology and Biotechnology (Springer-Verlag, Germany) [P: 12. L: E]

Aquatic Microbial Ecology (Germany) [P: 6. L: E]

Archives of Microbiology (Springer-Verlag, Germany) [P: 12. L: E]

Archives of Virology (Springer-Verlag, Austria) [P: 12]

Bacteriologia, Virusologia, Parazitologia, Epidemiologia (Societatea Romana de Microbiologia, Rumania) [P: 4]

Binary: Computing in Microbiology (Society for General Microbiology, United Kingdom) [P: 6. L: E]

Bioforum (Germany) [P: 10. L: G]

Clinical Microbiology and Infection (Eur. Soc. of Clinical Microb. and Infec. Dis., France) [P: Q. L: F, E]

Comparative Immunology, Microbiology and Infectious Diseases (Elsevier, United Kingdom) [P: Q. L: E]

Contributions to Microbiology and Immunology (Karger, Switzerland) [P: 1. L: E]

Current Advances in Applied Microbiology & Biotechnology (Elsevier, United Kingdom) [P: 12. L: E]

Developments in Molecular Virology (Netherlands) [P: Ir. L: E]

Diseases of Aquatic Organisms (Germany) [P: 12. L: E]

Enfermedades Infecciosas y Microbiología Clínica (Soc. Española de Enfermedades y Microbiol. Clínica) [P: 12. L: S]

Epidemiologie, Mikrobiologie, Immunologie (Czech Medical Society) [P: 6. L: Cz, Sl]

Epidemiology and Infection (Society for the Study of Infectious Diseases, United Kingdom) [P: 6. L: E]

European Journal of Clinical Microbiology & Infectious Diseases (Germany) [P: 12. L: E]

European Journal of Protistology (Centre National de la Recherche Scientifique, France) [P: Q. L: F, E, G]

FEMS Immunology and Medical Microbiology (Federation of European Microbiological Societies/Elsevier, Netherlands) [P: 12. L: E]

FEMS Microbiology Ecology (FEMS/Elsevier, Netherlands) [P: 12. L: E]

FEMS Microbiology Letters (FEMS/Elsevier, Netherlands) [P: 24. L: E]

FEMS Microbiology Reviews (FEMS/Elsevier, Netherlands) [P: 12. L: E]

Folia Microbiologica (Czech Academy of Sciences, Institute of Microbiology, United Kingdom) [P: 6. L: E]

Folia Parasitologica (Ceskoslovenska Akademie Ved, Czech Republic) [P: Q. L: E]

Food, Chemie, Mikrobiologie, Technologie (Tecnische Universite Muenchen, Germany) [P: Q. L: G, E, F]

Fortschritte Der Medizinischen Mikrobiologie/ Progress in medical Microbiology (Germany) [P: Ir. L: G,E]

Giornale di Batteriologia, Virologia ed Immunologia (Italy) [P: 2. L: I]

Giornale Di Microbiologia (Istituto Microbiologia, Italy) [P: Q. L: E, F, G, I, S]

Hellenike Mikrob. Kaihygienologike Hetaireia. Deltion/Acta Microb. Hellenica (Greek Society of Microbiology) [P: 6. L: Gr]

Innovation in Microbiology Series (United Kingdom) [P: Ir. L: E]

Institut Pasteur Hellenique. Archives (Institut Pasteur Hellenique, Greece) [P: 1. L: F]

Institut Pasteur. Annales. Actualites (Institut Pasteur, France) [P: Q. L: F]

Institut Pasteur. Bulletin (Institut Pasteur, France) [P: Q. L: E, F]

International Journal Of Antimicrobial Agents (Elsevier, Netherlands) [P: 8. L: E]

International Journal of Food Microbiology (Internat. Union of Microbiology Societies/ Elsevier, Netherlands) [P: 21. L: E]

Intervirology (Karger, Switzerland) [P: 6. L: E]

Journal of Antimicrobial Chemotherapy (British Soc. for Antimicrobial Chemotherapy, United Kingdom) [P: 12. L: E]

Journal of Applied Microbiology (Society for Applied Bacteriology, United Kingdom) [P: 12. L: E]

Journal of Applied Phycology (Netherlands) [P: 6. L: E]

Journal of Basic Microbiology (Akademie-Verlag, Germany) [P: 6. L: G, E, F]

Journal of Fish Diseases (United Kingdom) [P: 6. L: E]

Journal of General Virology (Society for General Microbiology, United Kingdom) [P: 12. L: E]

Journal of Infection (British Society for the Study of Infection, United Kingdom) [P: 6. L: E]

Journal of Medical Microbiology (Pathological Soc. of Great Britain and Ireland, Scotland) [P: 12. L: E]
Journal of Microbiological Methods (Elsevier, Netherlands) [P: 12. L: E]
Journal of Virological Methods (Elsevier, Netherlands) [P: 12. L: E]

Lactic Acid Bacteria (Elsevier, United Kingdom) [P: Ir. L: E]
Letters in Applied Microbiology (Soc. for App Bacteriol., United Kingdom) [P: 12. L: E]

Medical Microbiology and Immunology (Springer-Verlag, Germany) [P: 3. L: G, E]
Medical Microbiology Letters (Birkhauser, Switzerland) [P: 8. L: E]
Medycyna Doswiadczalna i Mikrobiologia (Panstwowy Zaklad Higieny, Poland) [P: Q. L: NS]
Microbial Clean-Up (United Kingdom) [P: 10. L: E]
Microbial Ecology in Health & Diseases (United Kingdom) [P: 6. L: E]
Microbial Pathogenesis (United Kingdom) [P: 12. L: E]
Microbiologia Medica (Associazione Microbiologici Clinici Italiani) [P: Q. L: I]
Microbiología SEM (Soc. Española de Microb./ Garsi-Masson) [P: Q. L: S, E]
Microbiology Research (Germany) [P: Q. L: G, E, F]
Microbiology (Society for General Microbiology, United Kingdom) [P: 12. L: E]
Microbiology Europe (Germany) [P: 6. L: E]
Microbiology Newsletter (England, United Kingdom) [P: 12. L: E]
Microbios (United Kingdom) [P: 12. L: E, F, G]
Mikrobiology (Russian Academy of Sciences) [P: 6. L: R]
Mikrokosmos (Germany) [P: 6. L: G]
Molecular and Biochemical Parasitology (Elsevier, Netherlands) [P: 14. L: E]
Molecular Genetics, Microbiology and Virology (Biotech. Akademiya, Russia) [P: Q. L: R]
Molecular Microbiology (Blackwell, United Kingdom) [P: 23. L: E]
Molekulyarnaya Genetika, Mikrobiologiya i Virusologiya (Biotechnologicheskya Akademiya, Russia) [P: Q. L: R, E]
Monografie Parazytolgiczne (Polskie Towarzystwo Parazytologiczne, Russia) [P: Ir. L: NS]
Monographs in Virology (Karger, Switzerland) [P: 1. L: E]
Mycologia Helvetica (Systematisch-Geobotanisches, Switzerland) [P: 2. L: G, E, F,I]
Mycopathologia (C A B Internat. Mycological Institute, United Kingdom) [P: 12. L: E]
Mycoses (Deutschsprachige Mykol. Gesellschaft, Germany) [P: 6. G: E]
Mycotoxin Research (Germany) [P: 2. L: E]
Nanobiology (United Kingdom) [P: Q. L: E]
Nederlands Tijdschrift Voor Medische Microbiologie (Dutch Association of Medical Microbiology, Netherlands) [P: Q. L: NS]

Oie Bulletin (Office International des Epizooties, France) [P: 6. L: F, E, S]
Oie Rev. Scient. et Technique (Office International des Epizooties, France) [P: 3. L: E, F, S]
Oral Microbiology and Immunology (Denmark) [P: 6. L: E]

Parasite (Société Française de Parasitologie) [P: Q. L: E, F]
Parasitologia Hungarica (Hungary) [P: 1. L: E]
Parasitology Research (Deustschen Gesellschaft fuer Parasitologie/Springer-Verlag, Germany) [P: 8. L: E, G, F]
Parasitology Today (Elsevier, United Kingdom) [P: 12. L: E]
Parazitologiya/Parasitology (Rossiiskaya Akademiya Nauk, Russia) [P: 6. L: NS]

Pathobiology (Switzerland) [P: 6. L: E]
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Progress in Medical Virology (Karger, Switzerland) [P: Ir. L: E]

Research and Reviews in Parasitology (Asociación de Parasitólogos Españoles, Spain) [P: 4. L: S]
Research in Microbiology/Formerly *Annals of the Institut Pasteur* (Institut Pasteur, France) [P: 9. L: E]
Research in Virology (Institut Pasteur, France) [P: 6. L: E]
Retrovirus (Société Française de Microbiologie, France) [P: Q. L: E, F]
Reviews in Medical Microbiology (Pathol. Society of Great Britain and Ireland, United Kingdom) [P: Q. L: E]
Reviews in Medical Virology (Wiley, United Kingdom) [P: Q. L: E]
Revista Española de Quimioterapia (Sociedad Española de Quimioterapia) [P: Q. L: S, E]
Revista Iberoamericana de Micologia (Asociación Española de Micología, Spain) [P: Q. L: S, E, Pg]
Revue Roumaine de Virologie (Acad. Romana, Instit. de Virologie St. Nicolau, Rumania) [P: Q. L: E, F, G, Ru]
Rivista di Parassitologia (Istituto di Parassitologia, Italy) [P: 3. L: E, F, G, S]
Romanian Archives of Microbiology and Immunology (Institutul Cantacuzino, Rumania) [P: Q. L: E, F]
Russian Progress in Virology (Academy of Medicine, Russia) [P: 6. L: R]

Scandinavian Journal of Infectious Diseases (Norway) [P: 6. L: E]
Studii si Cercetari de Inframicrobiologie (Academia Republicii Populare Romine Institutul de Inframicrobiologie, Rumania) [P: 6. L: Ru]
Systematic and Applied Microbiology (Germany) [P: 3. L: E, G, F]
Systematic Parasitology (Netherlands) [P: 9. L: E]

The New Microbiologica (Institute of Microbiology, Italy) [P: Q. L: E]
Trends in Microbiology (Elsevier, United Kingdom) [P: 12. L: E]

Veterinary Microbiology (Elsevier, Netherlands) [P: 24. L: E]
Veterinary Parasitology (Elsevier, Netherlands) [P: 24. L: E]
Virus Research (Elsevier, Netherlands) [P: 12. L: E]
Voprosy Virusologii/Problems of Virology (Russia) [P: 6. L: R]

World Journal of Microbiology and Biotechnology (Internat. Union of Microbiol. Soc., United Kingdom) [P: 6. L: E]

Yeast (Wiley, United Kingdom) [P: 16. L: E]

Zentralblatt Fuer Bakteriologie (Germany) [P: Ir. L: E, G]
Zentralblatt Fuer Hygiene Und Umweltmedizin (Germany) [P: Ir. L: E]
Zhurnal Mikrobiologii, Epidemiologii Immunobiologii (Izdatel'stvo Meditsina, Russia) [P: 6. L: R]

MICROBIOLOGÍA SEM **13** (1997), 509–516 **PERSPECTIVAS**

Cuatro años de *Microbiología SEM* (1994–1997)

Jordi Mas-Castellà

Redacción de la revista Microbiología SEM, *Barcelona, España*

Summary

Different aspects of *Microbiología SEM* editorial process over the years 1994-1997 are analyzed: number of originals received, the process leading to their publication, rates of accepted and refused papers, time needed for each step of the editorial process —which comprises scientific, language editing, if needed—, as well as some characteristics that can define the patterns of the articles, such as number of authors, institutions where the authors work and mean number of references. The contents of the different sections (editorial, research and review articles, perspectives, opinion, books review) are commented on, as well as the role played by some of them as forums for the discussion of topics of current scientific interest, especially the editorials focusing on the state-of-the-art of microbiological research in Latin American countries. Characteristics and frequency of monographic issues are also presented. The information is complemented with data about the circulation and distribution of the journal, its inclusion in international indexes and its current electronic publication on the world wide web.

Resumen

En este trabajo se analizan aspectos relativos al proceso editorial de la revista *Microbiología SEM* en el período 1994-1997: número de originales recibidos, proceso seguido hasta su publicación, porcentaje de aceptación o rechazo, tiempo de cada fase del proceso (que comprende correción lingüística y edición científica, si es necesario), así como algunas características que definen el artículo tipo, entre las cuales, número de autores, instituciones a las que pertenecen y promedio de referencias. Se comenta el contenido de las diferentes secciones (editorial, artículos de investigación

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y de revisión, perspectivas, opinión, revisiones de libros) y la función de algunas de ellas como foro de discusión sobre temas de interés actual, especialmente los editoriales dedicados a la situación actual de la investigación microbiológica en países latinoamericanos. La información se complementa con datos sobre la tirada y distribución de la revista, su inclusión en los diferentes índices internacionales y su publicación en version electrónica en Internet.

Introducción

Desde 1985, dentro de lo que puede considerarse la segunda etapa de *Microbiología SEM*, han sido tres los editores que han llevado las riendas de la publicación oficial de la Sociedad Española de Microbiología. Rubens López de1985 a 1989 y Juan Antonio Ordóñez de 1990 a 1993, con la realización de una excelente labor editorial cada uno de ellos, precedieron a Ricardo Guerrero, quien en 1994 asumió esta tarea. Desde la perspectiva de cuatro años formando parte del Comité de Redacción hay que agradecer la colaboración de estos compañeros que han mantenido a flote nuestro, podríamos llamarlo así, buque insignia. Han sido cuatro años de intenso trabajo, apoyado en la tenacidad y tesón del editor, Ricardo Guerrero, acompañado por un eficaz equipo de colaboradores.

Ya en 1995, en el XV Congreso Nacional de Microbiología, celebrado en Madrid, se presentó un primer balance del funcionamiento de la revista, que se publicó en forma de artículo en marzo de 1996 [véase *Microbiología SEM* 12(1), pp. 117–125]. En él se trataba, entre otros aspectos, los inicios de la revista, la temática de los artículos publicados, la evolución de la utilización del inglés, etc.

Dos años después podemos perfilar aún más algunos detalles del funcionamiento de la revista. Los artículos publicados se pueden clasificar en dos tipos: en primer lugar, los de investigación y de revisión (AI) y en segundo lugar los artículos generales (AG). En este segundo tipo de artículos (AG) se incluyen las contribuciones a cargo de expertos que cubren las secciones de Editorial, Opinión, Perspectivas, Revisiones de Libros, que han ido aumentando en los últimos números como se muestra en la Tabla 1.

De las más de dos mil páginas publicadas desde 1994, una quinta parte aproximadamente corresponde a estas secciones (AG), mientras que el resto son específicamente artículos de investigación y revisiones (AI). Este hecho personaliza mucho el tipo de publicación. Por una parte incluye resultados científicos objetivos, fruto de la experimentación de los autores. Por otra, expone diferentes puntos de vista y genera opinión. Es bueno que una publicación científica sea también foro de debate de una masa crítica en diferentes aspectos de la ciencia, su política, desarrollo e interacción social. En muchos casos, las revisiones y artículos de opinión proceden de reconocidos expertos, lo cual contribuye a aumentar el prestigio de la revista, cuyo contenido y estructura comentaremos a continuación.

Artículos de investigación y revisiones

Recepción y curso de los originales. En estos últimos cuatro años se han recibido en la Redacción de *Microbiología SEM* un promedio de un centenar de originales por año, que comprende artículos de investigación, y revisión y artículos generales. Resulta un total de unos 400 trabajos recibidos desde 1994, de los cuales se han aceptado y publicado 228. El porcentaje global de aceptación es de un 60%. Sin embargo, un análisis más detallado de este valor revela que

TABLA 1. Detalle de los números de *Microbiología SEM* publicados en el período 1994-1997

Año	Vol. (Núm.)	Núm. pág. AI*	Núm. pág. AG*	Núm. artículos	Núm. pág.
1994	10(1–2)	194	8		
	10(3)	94	12		
	10(4)	98	26	44	462
1995	11(1)	118	10		
	11(2)	132	12		
	11(3)	100	22		
	11(4)	62	30	59	532
1996	12(1)	98	44		
	12(2)	134	34		
	12(3)	90	28		
	12(4)	96	60	48	692
1997	13(1)	68	38		
	13(2)	84	42		
	13(3)	90	28		
	13(4)	94	42	42	560
		1572	458	193	2238

*AI: artículos de investigación. AG: artículos generales /Editoriales, Opinión, perspectivas, Revisiones de Libros).

el porcentaje de aceptación medio de originales se sitúa alrededor del 40%. Ello se debe a que algunas contribuciones de AG que responden a invitación del editor o miembros del Comité Editorial, suelen ser aceptadas desde el principio.

Aunque, como es lógico por el tipo de publicación, la procedencia de los autores es mayoritariamente española, también se reciben artículos de otros países (Tabla 2), especialmente de Oriente medio y América Latina.

Cada original es sometido por lo menos a dos revisores. Se envía a un tercero cuando existe una acusada discrepancia de criterios. Hay que reconocer que, con pocas excepciones, los revisores suelen atender la petición de evaluar el original con relativa rapidez. Cierto es que solemos caer en la tentación de dar más originales para evaluar a los revisores que responden con más prontitud. Durante estos cuatro años, han colaborado en el proceso de revisión unos 60 investigadores nacionales e internacionales, expertos en los temas de los artículos. El tiempo medio de aceptación de un artículo, considerando las diferentes fases (revisiones, modificaciones de los autores, comprobaciones, etc.), ha sido de unas 12 semanas. Es éste un tiempo más que aceptable dentro del margen en que se sitúan otras revistas científicas de la misma materia. En lo que quizá destaca *Microbiología SEM* es en la rapidez de la publicación una vez que ha sido aceptado el artículo. En este caso, los tiempos de publicación pueden ser extraordinariamente cortos, de varias semanas a un mes.

Ofrecemos seguidamente algunos detalles que proporcionan información sobre los artículos publicados en estos cuatro años. El 28% llevan la firma un solo autor. El número medio de páginas por artículo es de 9,2 (para artículos de

TABLA 2. Países, además de España, de los que se han publicado artículos (período 1994–1997)

Alemania	Francia
Argentina	Holanda
Austria	Iraq
Bélgica	Israel
Canadá	México
Cuba	Polonia
Chequia	Puerto Rico
Chile	Reino Unido
Dinamarca	Rusia
Egipto	Suecia
Estados Unidos	Venezuela

investigación y revisiones) y de 5,1 para los artículos generales mencionados al principio. El número medio de referencias por artículo es de 30,3.

El 90% de artículos ha sido firmado por un grupo de 1 a 5 autores. Aunque no mayoritariamente, la proporción de artículos firmados por un solo autor, es destacada. Hay que considerar que las contribuciones diversas suelen ser de un solo autor, lo que aumenta ese valor.

Una gran mayoría de trabajos (82%) proceden de un único centro, mientras que sólo en un 1% los autores proceden de tres centros diferentes. En cuanto al tipo de centros, la mayoría de artículos (72%), y por tanto también sus autores, provienen de universidades (Véase Fig. 1). Sorprendentemente, no alcanza el 10 % el número de artículos cuyos autores son investigadores del CSIC, y lo mismo ocurre en el caso de hospitales.

Artículos publicados y números monográficos

Desde 1994 se han publicado 171 artículos de investigación y revisión, con un total de 1572 páginas. La media ha sido de 11,4 artículos por número. En cuanto a los artículos generales, han sido 89 a una media de 5,9 artículos por número. Este tipo de contribuciones suma un total de 458 páginas y le corresponde (en número de artículos) una tercera parte de los publicados.

Desde 1994 se han publicado cuatro números monográficos: *Microbiología de los alimentos*, marzo 1995, editado por J. A. Ordóñez y V. Sanchis; *Origin and evolution of life*, junio1995, editado por Joan Oró y Antonio Lazcano; *Molecular pathogenesis of bacterial infections*, enero 1996, editado por Vázquez Boland y *Frontiers in antimicrobial resistance*, septiembre1997, editado por Miquel Viñas. Para el próximo año está previsto un monográfico sobre *Mechanisms of action of non-conventional fungi* cuyos editores serán Rafael Sentandreu y Enrique Herrero. Cada número monográfico ha de tener uno o más editores, responsables de coordinar la recepción de los artículos, y de mantener los contactos con los autores y con la Redacción de la revista, hasta que todos los originales están en condiciones de pasar a la fase de fotocomposición.

Secciones de la revista

El bloque final de la revista lo componen las Secciones de Opinión, Perspectivas y Revisio-

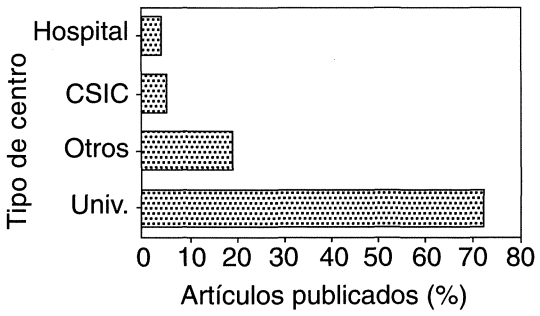

FIG. 1. Procedencia de los artículos

nes de libros, donde se incluyen los artículos generales. Estas secciones suelen escribirse mayoritariamente en castellano. En general suelen ser textos sobre temas que se consideran de interés, que se piden a autores con experiencia en ellos, aunque en algunos casos también se reciben directamente.

Editoriales

En el apartado de editoriales hay que destacar los publicados en el curso de los dos últimos años; son comentarios escritos por invitación, sobre la situación de la ciencia y, en especial, la microbiología, en los países latinoamericanos. Investigadores de gran prestigio en sus respectivos campos, norteamericanos y latinoamericanos, con un conocimiento directo de la situación, nos han ofrecido una visión clara del desarrollo y perspectivas de la microbiología y de la ciencia en general en México, Chile, Cuba, Brasil, Venezuela y en otros países. Se esperan nuevas aportaciones para los próximos números. Nos ha parecido que era un tema que necesariamente había que tratar en profundidad por múltiples razones, entre las cuales están los vínculos que nos unen con aquellos países. Pero hay mucho más, como son las colaboraciones e intercambios científicos practicados durante muchos años y las posibilidades de ampliarlo en el futuro. Por otra parte, *Microbiología SEM* nos parece un instrumento adecuado para poner en marcha esas colaboraciones en el ámbito de la microbiología y de las propias publicaciones, y para explorar las formas en que puede hacerse.

Opinión

Esta sección recoge las aportaciones de investigadores sobre temas no necesariamente microbiológicos, pero sí relacionados con aspectos de la ciencia que interesa conocer o sobre los cuales es adecuado entablar una discusión. Pueden ser temas de política científica, determinadas aplicaciones de la ciencia, comparaciones de diversos sistemas de investigación, cuestiones terminológicas y lingüísticas muy relacionadas con nuestro quehacer. Recordemos el artículo de Hans G. Trüper proponiendo medidas para evitar errores frecuentes en la designación de procariotas de descubrimiento reciente (véase 12(3) 473–475, 1996). Y también los de Frances Luttikhuizen "The ins and outs of scientific writing" [en 12(3) pp. 477–480, 1996] y "Scientific writing: revising basic communication strategies" [en 13(3) 371–374, 1997] Con estas colaboraciones, *Microbiología SEM* está en condiciones de paticipar en los foros internacionales de edición y publicación científica con conocimiento de causa. Críticas y comentarios diversos tienen igualmente cabida en ésta y en la siguiente Sección.

Perspectivas

Una aportación a la historia de la microbiología. Aun dedicando el máximo esfuerzo al avance de la revista, la Sociedad como motor de la misma ha sido siempre un elemento de atención. Fruto de ello fueron los capítulos dedicados a la historia de la SEM, publicados en la sección de Perspectivas, a lo largo de seis números, durante 1995 y 1996, con un total de 56 páginas. Lo consideramos un documento de interés no sólo por la vinculación de la revista a la SEM, sino por lo que tiene de perfil histórico de la microbiología en nuestro país. Queremos reconocer desde aquí, y una vez más, el mérito de nuestra compañera, Concepción García Mendoza en la recopilación y organización del fondo documental de la SEM.

Con este sentido de dejar constancia histórica de la SEM, de la microbiología en España y de rendir homenaje a figuras que han contribuido a su avance con el trabajo de muchos años, se han publicado en esta sección de la revista las noticias de aniversarios, premios o fallecimientos de

maestros y colegas. No todos eran microbiólogos, ni todos españoles o miembros de la SEM, pero consideramos adecuada la inclusión de todos ellos por su relación con la materia científica que nos es común. Y lo seguiremos haciendo, por ese deseo integrador y multidisciplinario que ha de regir la labor científica, si se quiere obtener el máximo rendimiento y satisfacción en todos los ámbitos. La Tabla 3 ofrece una síntesis de esta documentación.

Revisiones de libros

En los más de ochenta libros revisados se cuentan todos aquellos directamente relacionados con la microbiología de los que se ha tenido noticia y también otros títulos más generales. Quien haya seguido esta sección habrá podido observar que, aunque se haga hincapié en los defectos que, a juicio del revisor, pueda tener un libro, no aparecen comentarios destructivos. Se ha tomado como norma no escrita que, si un libro recibido para revisión no merece la pena por su escaso interés o calidad, no se comenta en la revista y se devuelve a la editorial o empresa que lo proporcionó para este fin. Esto nos ha parecido más adecuado que hacer críticas destructivas.

Idioma

La revista publica los trabajos de investigación en inglés y en castellano. Todos los artículos incluyen un sumario en las dos lenguas. El equipo editorial, convencido de que la mayor difusión de la revista está directamente relacionada con la publicación en inglés, recomienda a los autores el empleo de dicha lengua. De esta tendencia los primeros beneficiados son los propios autores, que tienen la posibilidad de que su trabajos sean leídos por un número mayor de investigadores. En la revista colaboran de forma desinteresada personas que realizan una profunda corrección lingüística, terminológica y de estilo, tanto en español como en inglés. También se dan facilidades al autor, cuando lo solicita, para ponerse en contacto con algún servicio de traducción. En cualquier caso, en los criterios de admisión de artículos no interviene el idioma en el que están escritos y se respeta la decisión del autor.

La edición científica

Un tema al que cada día se está dedicando más atención tenía que tener su comentario en la revista. Baste decir que son diversos los congresos que se realizan cada año en torno a las revistas biomédicas, que se publica una cantidad considerable de libros sobre el tema y que existen varias asociaciones internacionales que, además de congresos, organizan seminarios, cursos y talleres dirigidos a editores y personal interesado en la edición científica.

Entre las asociaciones más conocidas figuran la European Association of Scientific Editors (EASE), la World Association of Medical Editors (WAME) y el Council of Biology Editors (CBE). Relacionado con la edición y las publicaciones científicas, en este mismo número aparece el artículo de C. Ronda y M. Vázquez "European journals on microbiology", y la nota de C. Chica "Tres reuniones sobre publicaciones científicas". También hay que mencionar la completa información sobre la producción científica española en biomedicina contenida en el estudio realizado por I. Gómez y J. Camí, que fue comentado en el número de diciembre de 1996 12(4), pp. 663–666. Un nuevo campo de la edición científica lo constituyen las publicaciones electrónicas, tema que ha sido comentado por M. Piqueras en el número de junio de este año (13 [2]) pp. 229–236.

TABLA 3. Artículos sobre hechos que guardan relación con la historia reciente de la microbiología, publicados en *Microbiología SEM* en el período 1994–1997

Título*	Autor	Vol (N°)	Año	Páginas
La Sociedad Española de Microbiología				
Breve historia de la SEM:	C. García Mendoza			
Parte I. De 1946 a 1971		11(3)	1995	359–368
Parte II. De 1971 a 1977		11(4)	1995	491–498
Parte III. De 1977 a 1983		12(1)	1996	107–116
Parte IV. De 1983 a 1987		12(2)	1996	307–316
Parte V. De 1987 a 1991		12(3)	1996	457–464
Parte VI. De 1991 a 1995		12(4)	1996	621–630
Veíamos ayer	F. Uruburu	10(1–2)	1994	205–206
The Spanish Collection of Microganisms	F. Uruburu	10(3)	1994	311–314
La Revista de la Sem				
La revista de la SEM, de 1947 a 1995	D. Isamat et al.	12(1)	1996	117–125
Cuatro años de *Microbiología SEM* (1994–1997)	J. Mas-Castellà	13(4)	1997	509–516
Un año de *Microbiología SEM* en Internet	J. Garcia-Gil	13(4)	1997	517–522
European Journals on Microbiology	C. Ronda, M. Vázquez	13(4)	1997	499–508
Desarrollo de la Microbiología y pensamiento científico				
New pathogens and old resistance genes	J. Davies	10(1–2)	1994	9–12
Forty years of screening for antibiotics	S. Mochales	10(4)	1994	331–342
Microscopía en España	D. Fernández-Galiano	10(4)	1994	343–356
Maclyn McCarty: un sabio olvidado	R. López	10(4)	1994	429–432
Los paradigmas perdidos.T. S. Kuhn	C. Chica	12(4)	1996	641–646
En el centenario de Ludwik Fleck	J. J. Marcén	12(4)	1996	659–660
Midwife to the greens	J. E. Lovelock	13(1)	1997	11–22
Two generations of spore research	H. O. Halvorson	13(2)	1997	131–148
Necrólogicas: Personas y Hechos				
André Lwoff (1902–1994)	M. O. Soyer-Gobillard	10(4)	1994	433–434
Cyrii Ponnamperuma (1923–1994)	R. A. Goldsby	11(2)	1995	273–274
Maria Therezinha Martins (1936–1995)	V. Campos, G. Toranzos	11(3)	1995	409–410
Elena N. Kondratieva (1925–1995)	R. N. Ivanovsky	12(2)	1996	317–319
José Luis Cánovas (1934–1995)	G. Giménez Martín	12(3)	1996	465–468
David Vázquez (1930–1986)	J. R. Villanueva	12(4)	1996	631–640
Enrique Montoya (1928–1996)	J. M. Arias	13(1)	1997	79–82
Jacobo Cárdenas (1940–1996)	I. Núñez de Castro	13(1)	1997	83–84
Gonzalo Vidal (1943–1997)	M. Moczydlowska-Vidal	13(2)	1997	215–220
Sergei I. Kuznetsov (1900–1987)	N. N. Lyalikova	13(3)	1997	353–356

* Por razones de espacio, no se incluyen los títulos completos; éstos deben buscarse en los volúmenes y páginas correspondientes.

Distribución de ejemplares. Difusión

Son alrededor de 2400 ejemplares los que se distribuyen por correo, en España y en el extranjero. Los destinatarios son en su gran mayoría los socios de la SEM. Sin embargo, la revista se envía también a centros de investigación de España, Europa, América Latina y Estados Unidos y, en régimen de intercambio, a otras entidades (bibliotecas y centros de investigación) de esos mismos países. La relación de instituciones que la reciben se ha ido actualizando y modernizando, ya que constituye un valioso medio de difusión más allá de nuestras fronteras. En esta materia, como en otras muchas de la revista y de la SEM, es de rigor mencionar la labor que lleva a cabo con empeño y profesionalidad Isabel Perdiguero, desde la Secretaría de la Sociedad.

Cabe destacar que durante el período que nos ocupa (1994-1997) se ha implantado la publicación de *Microbiología SEM* en Internet. Si bien en una primera fase se incluyó solamente el índice, en la actualidad aparece todo el contenido. De la difusión de la revista a través de la red se ocupa de forma desinteresada el Dr. Jesús Garcia-Gil de la Universidad de Girona, a quien agradecemos su interés y las numerosas horas de trabajo que dedica. Para más detalles sobre las "visitas" a la revista y otros aspectos de interés, véase el artículo de J. Garcia-Gil "Un año de *Microbiología SEM* en Internet", en este mismo número pp. 517–522.)

Participación internacional

La revista ha contado con artículos de investigación o revisiones cuyos autores son científicos de renombre en la esfera internacional: Julian Davies, Aharon Oren, Lynn Margulis, Joan Oró, Stanley L. Miller, Antonio Lazcano, James E. Lovelock, Bruce N. Ames, Mike Sogin, Harlyn Halvorson y Ramón Margalef. Las otras secciones de la revista, incluidos los editoriales, ha contado también con firmas de prestigio, como Rita Colwell, John Maddox, Paolo Fasella, Bernard Dixon, Francisco J. Ayala, Jorge Allende y Elio Schaechter. Con ellos, junto con el resto de investigadores que nos van enviando sus artículos, *Microbiología SEM* ha ido aumentando su importancia en los últimos años.

El reconocimiento en el ámbito internacional, junto con una periodicidad regular y la utilización mayoritaria del inglés en los artículos, permitirá la inclusión de la revista en el mayor número posible de índices internacionales y, consecuentemente, que aparezca en los listados de publicaciones con índice de impacto. Actualmente está incluida en *Biosis* (BA), *Chemical Abstracts* (CA), *Medline* (ME), *Index Medicus* (IME) y *Excerpta Medica* (ES). Todo ello son elementos que pueden conferir mayor categoría a la revista y más prestigio a la SEM. Es necesario, por tanto, seguir recibiendo trabajos de calidad, y aquí llamamos nuevamente la atención sobre el papel protagonista que corresponde a los autores en este menester.

Un año de *Microbiología SEM* en Internet

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Internet como fuente de información

El "World-Wide Web" (WWW), conocido como "Red de Redes" de Internet se ha convertido en un elemento profesional o de ocio casi cotidiano. Su imparable extensión ha ido desde los centros de cálculo a los departamentos universitarios y empresariales, acabando como un componente más de millones de hogares en todo el mundo. Internet se ha convertido en parte de nuestras vidas. Para utilizar la red como una herramienta y evitar que se convierta en una finalidad imposible, debe tenerse en cuenta que se trata de un bosque extremadamente frondoso del que sólo nos interesan algunos árboles.

Internet ofrece una enorme potencialidad a todo colectivo para el que la divulgación y la comunicación sean elementos indispensables. En ocasiones, en cambio, sólo es un excelente complemento que ayuda a mantener un registro presencial. En ambos casos es imprescindible considerar las posibilidades de la Red, que combina una serie de elementos que satisfacen al más exigente: inmediatez, interactividad, comodidad y actualidad. Pero cada vez hay que ser más selectivo a causa de la ingente cantidad de información superflua que inunda la red. Es fácil indigestarse con información con sólo buscar en el WWW algunas palabras clave.

La disponibilidad de información y la aparición de un canal de transmisión con fabulosas posibilidades y perspectivas es de especial interés para la comunidad científica, cuyas fuentes de información han sido siempre muy diversas y en ocasiones de difícil acceso. Por sus numerosas posibilidades, el mundo del WWW ha calado profundamente entre la mayoría de la comunidad científica, que se ha decidido a adoptar la red como principal vehículo de comunicación ya sea a través del correo electrónico, de los grupos de discusión o de la puesta en funcionamiento de las bases de datos de interés. Para más información acerca del sentido del WWW para la Microbiología, véase "Internet para microbiólogos" de José M. Nieto publicado en esta misma revista en marzo del año 1996 y al publicado por Manuel Hernández y colaboradores en el número precedente y que lleva por título "Microbiología en Internet".

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Microbiología e Internet

Si nos centramos en la Microbiología, percibimos todo un universo de información e interactividad, aunque todavía queda un largo camino por recorrer. En la red, un microbiólogo puede hallar desde la bibliografía sobre un tema determinado hasta las información sobre la última novedad en instrumentación de laboratorio. A través de los grupos de discusión especializados de los servidores "News", podemos conocer los temas más candentes y ponernos en contacto con microbiólogos de todo el mundo en un enorme tablero electrónico de anuncios donde es fácil obtener respuestas y establecer contactos profesionales. Destacan los grupos "sci.bio.microbiology" y "bionet.microbiology". El mundo editorial también se mueve con rapidez en Microbiología. Todas las editoriales disponen de servidores con las principales novedades bibliográficas, que pueden adquirirse mediante sencillas operaciones. También clásicos de la información sobre publicaciones periódicas, como *Current Contents* del ISI se hallan ya en el WWW. Otras bases de datos como *UnCover* permiten buscar un artículo publicado en cualquiera de las revistas que se editan en la actualidad y disponer de él por fax en menos de 24 horas. Por otro lado, prácticamente todas las universidades del mundo ofrecen excelentes páginas de información y cada vez son más los departamentos de Microbiología que mantienen un buen tono divulgativo acerca de sus actividades docentes e investigadoras. Es conveniente acostumbrarse a la aparición en el WWW de servidores de información en Microbiología para mantenernos al día.

Microbiología SEM "on line"

No obstante, hay que ser conscientes de las dificultades de los momentos iniciales para mantener servidores de información. Aunque por fortuna, es cada vez más frecuente disponer de una conexión a la red desde un organismo público (universidad, centros de investigación, hospitales, etc.) es todavía difícil disponer de los dos elementos básicos para mantener un WebSite: un servidor —generalmente una máquina que opere con sistema operativo UNIX o similar— y una persona con conocimientos adecuados de informática —y tiempo extralaboral suficiente— para construir páginas en lenguaje HTML (del inglés HyperText Markup Language), instalarlas en el servidor y crear los accesos y conexiones necesarias su visualización por *navegadores* tipo Netscape o Internet Explorer desde cualquier parte del mundo.

Hasta el momento, una revista se publica íntegra y exclusivamente en el WWW: *Molecular Plant Pathology On Line* (*MPPOL*). Su calidad y rigurosidad están fuera de toda duda, manteniendo el mismo sistema de selección de los manuscritos a publicar que el resto de revistas internacionales. Las ventajas de la edición de una revista en formato electrónico son múltiples, por ejemplo, reducción de costes —papel, imprentas, distribución, etc.— que permite ofrecer subscripciones a bajo precio, la rapidez para publicar artículos en un volumen a medida que vayan siendo aceptados y finalmente la posibilidad de ofrecer sumarios más sencillos y sistemas de recuperación de información más eficientes. *MPPOL* puede ser un ejemplo para futuras publicaciones.

La publicación "on line" de una revista científica como *Microbiología SEM* no pretende, de momento, seguir los pasos de *MPPOL*. Se trata de un experimento más modesto cuya finalidad es acercar la revista oficial de la SEM a los profesionales de la Microbiología en España. La idea nació en Torremolinos en la primavera de 1996, durante la reunión de Microbiología Acuática, e inmediatamente se la propusimos al

Prof. Guerrero, actual Editor-Coordinador. Rápidamente nos pusimos manos a la obra, conun primer diseño de los contenidos del "Web-Site", tratando de elaborar la presentación del último número [vol. 12(1)]. Este primer número fue muy laborioso, puesto que disponíamos de un material Hypertext ciertamente rudimentario. La edición electrónica fue hecha enteramente en Girona aprovechando los pocos momentos libres (sobremesa, sábados, etc.). La adquisición del "software" adecuado y la colaboración inestimable de David Isamat en la Universidad de Barcelona y del propio Prof. Guerrero en la preparación y envío del material, ya en lenguaje "Hypertext", facilitó enormemente las cosas. La conversión del texto era mucho más sencilla y, al hacerse en colaboración con el propio equipo de la revista, todo el proceso se simplificó.

Posteriormente se redefinió el diseño de la página principal y se instaló un contador para un control aproximado del número de visitas a nuestras páginas. Cuando escribimos estas líneas acabamos de superar los 1500 "lectores", que, en conjunto, han producido cerca de 20.000 accesos en poco más de nueve meses, una cifra bastante aceptable, considerando el carácter científico de esta dirección "web". No obstante, mediante la consulta a los sistemas de registro disponibles en el servidor, hemos visto que, por diversos motivos, el contador deja de registrar un número de conexiones que puede llegar a ser el 30% del total. Debemos considerar finalmente que hemos detectado la conexión de al menos cuatro servidores de "Proxy" distintos, los cuales almacenan toda la información para actuar de servidores-repetidores —generalmente en universidades. De esta forma, los interesados en los contenidos de *Microbiología SEM* pueden acceder a ellos conectándose directamente a un servidor muy próximo —y por tanto, muy rápido. No es posible conocer cuantos usuarios se conectan al servidor de "Proxy".

Estadísticas del servicio

En febrero de 1997 se instaló un software de control de los accesos al directorio del servidor donde se halla almacenada la información de *Microbiología SEM* "on line". Este sistema permite conocer de forma pormenorizada aspectos del servicio que, debidamente analizados, son útiles para los editores.

Un porcentaje relativamente elevado de conexiones (34,49%) se producen desde terminales cuya procedencia no pueden resolverse debido a que su identidad aparece con el número IP de la red o subred a la que están conectados. De las conexiones con origen resuelto, aquellas cuyas direcciones de origen vienen con el punto y la extensión del país (por ejemplo ".uk") casi un 40% se producen desde nuestro país. Una cantidad apreciable (20%) se producen desde direcciones comerciales (".com"). El resto está muy repartido entre los distintos países del mundo (Tabla 1). En total son 64 los países que han accedido a nuestro servidor de los cuales 15 pertenecen a la esfera de América Latina.

La distribución temporal de los accesos ofrece también interesante información. Por ejemplo las conexiones a lo largo del día se reparten en tres grandes bloques (Fig. 1). El primero, desde las 11 de la noche a las 6 de la mañana, probablemente atribuible a lectores del otro lado del Atlántico. El segundo bloque correspondería al período comprendido entre las 9 de la mañana y la 1 del mediodía. La hora del almuerzo (2 a 3 de la tarde) queda reflejada con una disminución de los accesos,que se recuperan a partir de las 5 de la tarde, en lo que configuraría el tercer bloque.

En relación al día de la semana, el número de conexiones es similar en las jornadas laborables con un aumento en jueves difícil de explicar. Sábados y domingos las conexiones bajan a la mitad. Observando la dinámica por meses, se pueden extraer dos conclusiones. Primero que

TABLA 1. Procedencia de las conexiones

Contactos	% Bytes	Dominio
6145	34.49%	(direcciones IP no resueltas)
4689	20.29%	.es (España)
2189	12.38%	.com (Comercial)
888	6.03%	.edu (USA Educacional)
324	2.08%	.mx (Mexico)
358	2.02%	.de (Alemania)
290	1.88%	.uk (Reino Unido)
157	1.64%	.ca (Canada)
172	1.05%	.fr (Francia)
131	0.91%	.nl (Holanda)
182	0.87%	.co (Colombia)
162	0.84%	.ar (Argentina)
130	0.83%	.au (Australia)
121	0.71%	.it (Italia)
139	0.66%	.be (Bélgica)
88	0.59%	.fi (Finlandia)
56	0.45%	.il (Israel)
57	0.41%	.se (Suecia)
41	0.33%	.nz (Nueva Zelanda)
68	0.33%	.uy (Uruguay)
50	0.33%	.jp (Japón)
29	0.29%	.ch (Suiza)
29	0.26%	.gov (USA Gubernamental)
34	0.25%	.no (Noruega)
30	0.21%	.ve (Venezuela)
40	0.20%	.su (Antigua USSR)
37	0.19%	.dk (Dinamarca)
28	0.19%	.cl (Chile)
27	0.19%	.kr (Corea del Sur)
36	0.18%	.hu (Hungria)
25	0.17%	.pe (Perú)
47	0.16%	.org (Organizaciones "Non Profit")
37	0.14%	.at (Austria
25	0.13%	.pl (Polonia)
20	0.13%	.si (Eslovenia)
33	0.13%	.pa (Panamá)
32	0.13%	.do (República Dominicana)
27	0.12%	.pt (Portugal)
13	0.11%	.us (Estados Unidos)
17	0.08%	.id (Indonesia)
10	0.07%	.za (Sudáfrica)
18	0.10%	.th (Tahilandia)
13	0.06%	.cn (China)

Continúa en la página siguiente

TABLA 1.— *Continuación*

Contactos	% Bytes	Dominios
5	0.06%	.lt (Lituania)
12	0.06%	.hk (Hong Kong)
3	0.05%	.mil (USA militar)
10	0.05%	.cr (Costa Rica)
17	0.05%	.my (Malasia)
8	0.05%	.sg (Singapur)
9	0.05%	.bo (Bolivia)
8	0.05%	.gr (Grecia)
4	0.04%	.yu (Yugoslavia)
5	0.04%	.in (India)
12	0.04%	.ru (Federación rusa)
6	0.04%	.arpa (Arpanet)
9	0.03%	.py (Paraguay)
2	0.03%	.tr (Turquía)
4	0.03%	.ie (Irlanda)
10	0.03%	.ni (Nicaragua)
14	0.03%	.cz (República Checa)
3	0.02%	.ph (Filipinas)
4	0.02%	.sk (República Eslovaca)
1	0.01%	.gt (Guatemala)
1	0.01%	.kw (Kuwait)
3	0.01%	.is (Islandia)

los accesos siguen un patrón sinusoidal con máximos poco después del reparto por correo de la revista impresa (Fig. 2). Se aprecia, en lo que va de año, dos claros picos correspondientes a los dos primeros números (marzo y junio) del volumen 13. Cabe señalar también un notable incremento en la cantidad de conexiones del segundo pico en relación al primero, lo que podría ser indicador de un incremento progresivo de visitas al "web site".

Consideraciones finales

La publicación de *Microbiología SEM* en Internet ha alcanzado, con menos de un año de experiencia, un elevado grado de optimización en cuanto al tiempo de adaptación al formato electrónico. No obstante, cabe recordar una vez más que todo es el resultado de una colaboración absolutamente voluntarista. La apertura hacia nuevos horizontes podría incluir la incorporación de una base de datos, la recopilación de más información relacionada con la Microbiología y la confección de un índice de sumarios de las principales revistas científicas en el campo de la Microbiología, entre otros.

En las actuales circunstancias, las bases de datos y la información amplia sobre las publicaciones científicas, constituyen un elemento muy valioso para el investigador. Para lograrlo en nuestra revista, es necesrio el replanteamiento de una serie de factores que incluyen la mejora de la infraestructura, la adecuación de los recursos humanos y la convicción por parte de los Microbiólogos españoles que éste es un

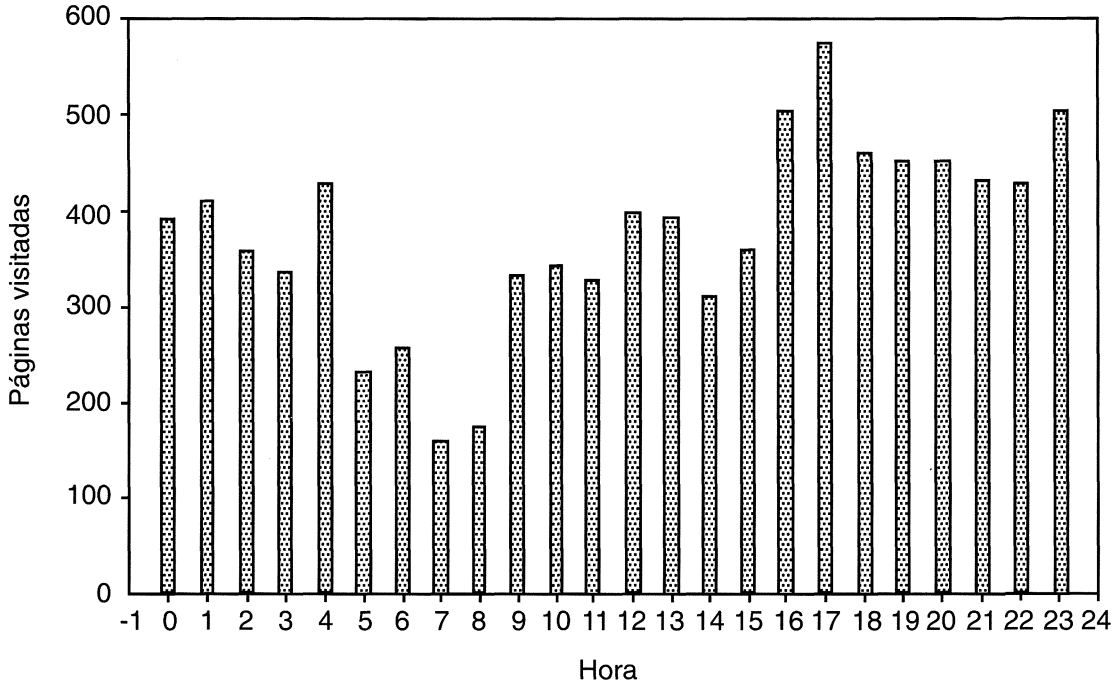

FIG. 1. Distribución horaria de los accesos a páginas de la revista entre febrero y octubre de 1997.

buen camino. Desde el Área de Microbiología de la Universidad de Girona siempre hemos creído firmemente en las enormes posibilidades que Internet en general y el WWW en particular ofrecen para la difusión de la ciencia entre la comunidad científica. Por ello se ha emprendido esta aventura con la mayor de las ilusiones, conscientes de las limitaciones humanas y materiales y teniendo como única pretensión la de abrir un camino que nos mantenga conectados a la vanguardia. Ahora sólo falta convencer a los escépticos y demostrarles que la calidad de la información no depende del soporte con el que se transmite.

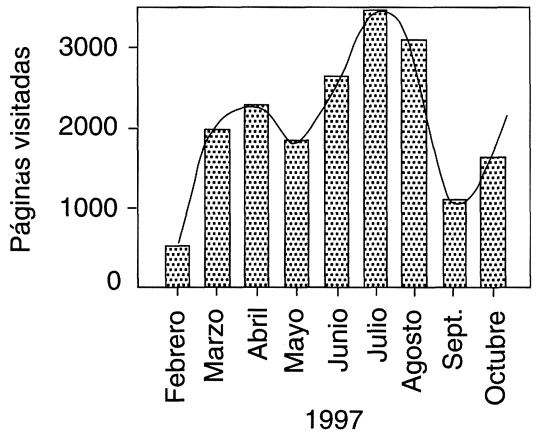

FIG. 2. Accesos por meses. Se muestran las oscilaciones coincidentes con la distribución de la revista impresa.

No es menos importante considerar las oportunidades que este medio ofrece a los investigadores de los países con menos recursos técnicos, industriales o económicos (me resisto al término tercer mundo). Los investigadores de esos países pueden suplir con un medio económico y rápido las grandes carencias de información científica y técnica a que se ven sometidos en su formación y en el desarrollo de su trabajo.

MICROBIOLOGÍA SEM **13** (1997), 523–526 **OPINION**

The importance of microbiology in improving pre-college science education

Richard L. Hinman

Pfizer Inc, Central Research Division, Groton, Connecticut, USA

It is a widely held perception that pre-college public education in science and mathematics is inadequate to prepare students for the rapidly growing high-tech work place, and for effective participation in the industrialized society of the 21st century. The call for improvement is widespread. In the U.S.A., the national movement for reform is now in its second decade. In the U.K. it has been under way for at least as long. Other countries are reexamining the effectiveness of their pre-college science and mathematics education programs. The effort has drawn the attention and support of institutions not associated with formal education per se, including businesses and universities—and many individual scientists and engineers.

As a company grounded in research and high technology manufacturing, Pfizer Inc. has been especially concerned about the mismatch between the requirements of the work place and the inadequate preparation of prospective employees. In response, Pfizer has been working actively since 1990 to help improve K–12 (i.e., from the kindergarten to grade 12 [16–18-year old students]) science education. Pfizer's programs are designed to engage and sustain student interest in science and mathematics with the ultimate goal of helping raise standards of scientific literacy for all students.

A fundamental tenet of the Pfizer Educational Initiative (PEI) is that non-educational institutions can be most effective through personal interaction with schools, teachers, and students. Pfizer establishes partnerships with local schools in communities where Pfizer has its facilities. Pfizer employee volunteers—who mentor teachers and students, guide tours of Pfizer facilities, demonstrate science in schools, judge science fairs, etc.,—are the key to the success of these partnerships. We now maintain partnerships with some forty schools at fourteen Pfizer locations in the U.S.A., Puerto Rico, and England, reaching 500 science and mathematics teachers and through them some

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21,000 students (Fig. 1). Over 750 Pfizer employees serve as volunteers. This was initially an entirely U.S.A. program. Its success, as measured by the enthusiastic response of teachers and students, and the willingness of our volunteers to dedicate their time and energy, has prompted us to expand it to other countries. School partnerships are now under way in France and Ireland, and planning is going on to include similar activities in other European countries where Pfizer has a major presence.

At the core of the Pfizer approach is the belief that hands-on experiments provide the base from which the inquiry process and content of science can be most effectively learned. These activities engage student interest much more effectively than traditional classroom methods. Further, "hands-on" engages interest broadly, helping offset perceived gender and racial learning differences. Our belief in this concept is illustrated by the fact that Pfizer supports educational experiments in which all science texts in elementary schools are replaced by hands-on kits of instructional materials. For schools that lack laboratory facilities, Pfizer constructs and equips laboratories with the motto "you can't do hands-on science if you have nothing to put your hands on". To date, we have built 10 laboratories in elementary, middle, and high schools in the U.S.A. and Puerto Rico.

In our experience with hands-on activities in the pre-college classroom, nothing beats microbiology! Experiments are rapid, easy to comprehend if they are presented effectively, and they are appealing to students because they reveal an unknown world. Introduction of microbiology at an early age is important for another reason—it helps overcome microbe-phobia, the uneasiness, even dread, with which many people perceive the microbial world. Discussion of microbiology and biotechnology leads into a broad range of essential topics, including ethical issues, which are important for enhancing scientific literacy. Further, the skills attained in microbiological

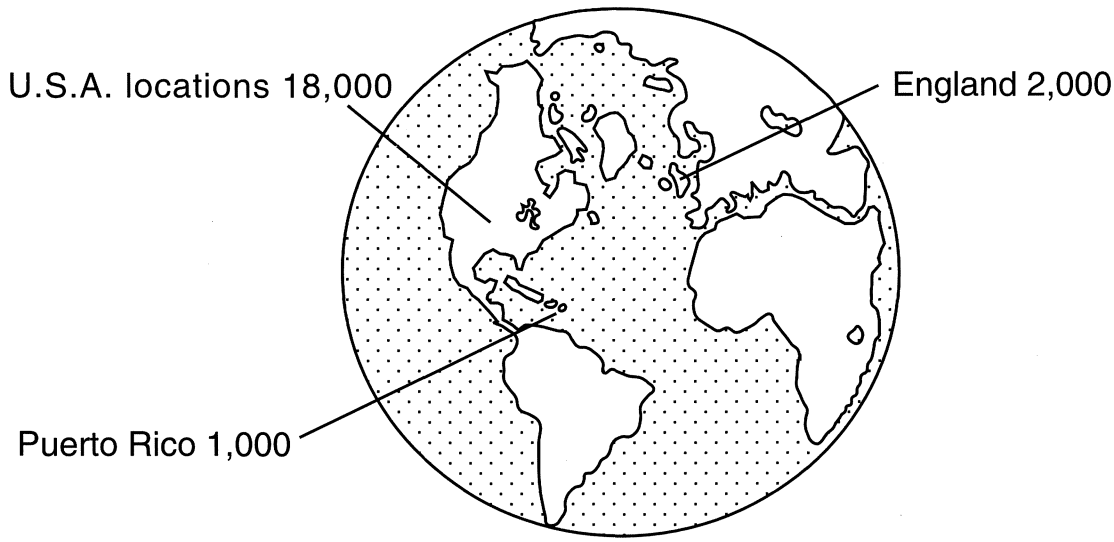

FIG. 1. Pfizer education initiative (PEI) site locations. Pfizer has formed PEI partnerships with more than 40 schools in 17 communities where it operates in the United States, Puerto Rico and Europe.

laboratories are valuable in preparing for many careers in industry, especially the rapidly growing sector of biotechnology.

Despite these benefits, microbiology, as a teaching concept, is relatively underemployed. Interest is growing, prompted especially by the American Society for Microbiology. Some projects have been developed for school use. The Microcosmos series, developed at Boston University, is well-known; experiments on biotechnology and food, published by the Institute of Food Technologists in the U.S.A., is another. Closer to home, Pfizer has developed for school use a series of enzyme experiments which use a recombinant calf rennin to clot milk. It is easy to demonstrate enzyme reaction rates as a function of temperature, pH, and fat content, for example. And since recombinant chymosin was the first food additive approved by the U.S. Food and Drug Administration, it offers a ready introduction to the broader issues of biotechnology. Pfizer has also produced a short video tracing the development of recombinant chymosin from the idea to actual production. This video, featuring local high school students, has won several awards. It is titled "Biotechnology–Careers for the 21st Century", and is available from the National Association of Biology Teachers, Reston, VA, U.S.A.

All-in-all, experiments with microorganisms constitute a very effective hands-on teaching tool. It simply needs more emphasis and more widespread use. And, of course, teachers must be trained in its use. In our experience, teacher workshops, sponsored by universities and industry, have proved very effective and popular. Wesleyan University and three Connecticut-based pharmaceutical companies—Bayer, Boehringer-Ingelheim and Pfizer—jointly sponsor three teacher workshops each year in biotechnology. Topics range from plating on agar to DNA fingerprinting. Opportunities to observe larger scale operations are especially popular and rewarding. Most pre-college teachers think of microbiology in terms of Petri dishes and test tube slants. Observing fermentation at the 10-liter level affords an entirely new dimension, and seeing operations at pilot plant or manufacturing scale is truly an eye-opening experience.

The Pfizer program is a dynamic one, which changes as we learn. Of all the lessons we have learned in the eight years since the inception of this program, the need to provide support for teachers, especially at the elementary and middle school levels, is the most important. Teachers at this level are often inadequately prepared to teach science. Their uncertainty and discomfort is easily communicated to students. Programs to overcome this discomfort are especially beneficial. Pfizer offers summer internships in research and manufacturing laboratories for these underprepared teachers. By working side-by-side with professional scientists and engineers, teachers gain the self-confidence and professional esteem that offsets the lack of adequate scholastic preparation. Another clear benefit of working through teachers is that it greatly amplifies any individual scientist's efforts. Teachers contact many students each day, and different students each year, with an influence that no individual scientist can achieve in an occasional classroom relationship.

Another major point is that the classroom is not the only center for aiding science education reform. In science museums and science centers, participants create their own hands-on experiences and—a significant difference from the classroom—they do so at their own pace. The significance of these centers for science education is shown by the fact that annual attendance at U.S.A. science museums is now approaching the one hundred million mark—roughly the same as the attendance at all professional U.S.A. sport-

ing events! Science centers offer exceptional opportunities for scientists and engineers to intercede in science education. Here too, microbiology, however, is an underdeveloped resource. More and better exhibits and demonstrations on this theme would be an important extension of classroom work. In recognition of this opportunity, Pfizer has sponsored the construction of an extensive hands-on exhibit on "Microbes; Invisible Invaders; Amazing Allies", which will travel to a number of major science centers in North America, beginning with the Ontario Science Center in Toronto, Canada, in the fall of 1997, and the Liberty Science Center in New Jersey, in early 1998.

In sum, there is a role to play in improving science education for every member of the scientific community—government, academia, and industry—whether raising money to enhance local school science programs, mentoring teachers, or aiding the local science museum. It is a wonderfully rewarding experience. We are part of a critical movement. And microbiologists have at their command the tools with which to really make a difference in improving pre-college science education.

MICROBIOLOGÍA SEM **13** (1997), 527–528 **OPINIÓN**

Tres reuniones sobre publicaciones científicas

Carmen Chica

Redacción de *Microbiología SEM*

6th General Assembly and Conference of EASE (European Association of Scientific Editors). Helsinki, mayo 25–28, 1997

Bajo el lema "Quality in communication: the editor role" se trataron, entre otros: El auge y potencial de la publicación electrónica. El siempre candente tema de la revisión paritaria y su contribución (o no) a la mejora del artículo. El papel del editor. La problemática de las diferentes publicaciones en lengua no inglesa. La producción de los países en vías de desarrollo y las dificultades de su incorporación a las redes de comunicación científica. Las bases de datos y los índices de impacto. Las diversas formas del fraude en ciencia.

EASE cuenta con 900 miembros de 47 países y más de 450 revistas representadas. Lleva a cabo una meritoria labor en el marco de la edición científica sobre temas que interesan a la mayoría. Publica tres veces al año y distribuye entre sus socios el boletín "European Science Editing".

The International Congress on Biomedical Peer Review and Global Communication. Praga, septiembre 17–21, 1997

Este congreso reúne cada cuatro años a un potente grupo de publicaciones compuesto por los diferentes Archives y Journals de la *American Medical Association*, y el grupo editor del *British Medical Journal*. En esta ocasión, se trataron las tendencias en la autoría, los conflictos de intereses, medidas para mejorar el sistema de evaluación (enmascarando o no la identidad de los autores), los sesgos y prejuicios en las publicaciones (transculturales, geográficos, sexistas, no convencionales y de otros tipos). El sistema de revisión "peer review" está recibiendo muchas críticas. Las discusiones no buscan su eliminación, sino su mejora, quitar los factores indeseados como posibilidad de plagio, ejercicio de prepotencia, juicios contradictorios, subjetividad, etc. La principal crítica hacia el congreso es que en la práctica se hayan frivolizado los contenidos, rayando en ocasiones en auténtico ejercicio de fruslería. Sorprende que, puestos a analizar sesgos y prejuicios (se supone que para evitarlos), se incurra gravemente en ellos. Sucedió, cuando un trabajo con una metodología seria y resultados objetivos se incluyó en la sección de pósters debido a su procedencia. Casualmente, otro trabajo similar, pero incompleto en todos sus apartados, fue expuesto oralmente por su autor, eso sí, miembro de la organización y editor de una revista de prestigio.

La edición científica es un mundo amplio, diverso y complejo. Pero esa complejidad aumenta en la medida en que los editores crean distancias. Se está produciendo la que antaño existía entre científicos, con su aureola de sabios, y el público. Hoy es el editor (por

supuesto, el de las revistas de primera línea, altamente profesionalizado) quien establece su superioridad sobre los autores. Pero ¿con qué criterio? En Praga ha saltado a la vista que bastantes de esos editores han olvidado qué significa investigar y cómo se lleva a cabo. Si es una postura para crear una distancia saludable en aras de la objetividad, corre el riesgo de convertirse en abismo insalvable. Pero no sería justo que una opinión subjetiva abarcara todo el colectivo de editores científicos. Hay diferencias entre los de diversas revistas, según los temas y especialidades.

II Taller sobre Publicaciones Científicas Latinoamericanas. Guadalajara (México), noviembre 26–29, 1997

Las dificultades de las revistas científicas latinoamericanas para lograr difusión a escala internacional son similares a las de otros países, como España, que no pertenecen al ámbito anglosajón. Para hacer frente a esta situación se organizó en 1994 la primera edición del Taller cuya iniciativa y continuación se deben a la labor de la Dra. Ana María Cetto y un grupo de colaboradores y asesores de primera línea. El Taller recibe el apoyo de instituciones locales, Universidad de Guadalajara, Universidad Nacional Autónoma de México, Consejo Nacional de Ciencia y Tecnología de México, y de ICSU y Unesco, organizaciones interesadas en que la ciencia en estos países reciba la consideración necesaria para continuar su desarrollo.

A lo largo de tres días de trabajo intensivo se analizó la labor de mejora de las publicaciones y del proceso editorial, los pasos para la elaboración de un censo de revistas que evite duplicaciones, permita conocer el potencial de lectores y las posibilidades de autofinanciación, todo lo cual viene a responder a las conclusiones emanadas del I Taller. A este respecto se ha puesto en marcha el proyecto Latindex como instrumento de información de las publicaciones científicas latinoamericanas. Latindex ha elaborado un directorio de publicaciones periódicas y continúa la labor que, junto con otras iniciativas integradoras permitirá disponer de instrumentos de información, evaluación y difusión de la producción científica.

Los trabajos presentados discurrieron sobre los cuatro temas propuestos : (1) La edición electrónica frente a la edición en papel. (2) Sostenibilidad de las publicaciones científicas seriadas. (3) Presencia y visibilidad de las publicaciones latinoamericanas. (4) Normalización y criterios para la evaluación de las publicaciones.

Entre los temas discutidos y las conclusiones destacaron la importancia de las nuevas tecnologías, la necesidad de evaluar los costos del formato electrónico y la formación del personal necesario; establecimiento de criterios diferenciadores de evaluación en materia de ciencias sociales; utilidad y necesidad de identificar al público usuario de las publicaciones; dimensiones del problema de la lengua en relación a la cultura y tradiciones locales; consecuencias de la comercialización de las revistas, etc. Un aspecto largamente discutido fue la utilización que algunas instituciones hacen de los diferentes índices de ISI para evaluar individuos. No siendo ésa la finalidad para la que tales índices fueron creados, esta práctica provoca distorsión y debe ser rechazada. El Taller estaba concebido como punto de interacción de los responsables de la publicación científica para (a) analizar el estado y perspectivas de las revistas científicas de la región (b) detectar las necesidades y obstáculos a la expansión de las revistas, (c) búsqueda de estrategias para superar las dificultades y (c) evaluar el impacto de las nuevas tecnologías. La clausura se efectuó en el marco de la XI Feria del Libro de Guadalajara, uno de los acontecimientos culturales de mayor importancia de América Latina con repercusión mundial.

MICROBIOLOGÍA SEM **13** (1997), 529–532 **OPINIÓN**

La Red Latinoamericana de Ciencias Biológicas (RELAB)

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Breve reseña histórica

La Red Latinoamericana de Ciencias Biológicas (RELAB) nació en 1975 como Programa Regional de Entrenamiento de Postgrado en Ciencias Biológicas, patrocinado por el Programa de Naciones Unidas para el Desarrollo (PNUD) y por la UNESCO. Se inició con cinco países (Bolivia, Chile, Colombia, Ecuador y Perú), y terminó diez años después con diez países incorporados (Argentina, Bolivia, Brasil, Chile, Colombia, Ecuador, Paraguay, Perú, Uruguay y Venezuela).

El objetivo del programa es la integración de las ciencias biológicas de la región a través de la colaboración en la formación de jóvenes científicos de los países participantes. Fue tanto lo que se logró con pocos recursos (tres millones de dólares para los diez años), que los gobiernos y científicos de estos países decidieron continuar la tarea al finalizar, en 1985, el proyecto financiado por el PNUD. Así surgió la RELAB.

Ya en 1981, había servido como modelo de iniciativas para International Council of Scientific Unions (ICSU), UNESCO y el International Biosciences Networks (IBN). Esta iniciativa generó redes biológicas en África, Países Árabes y Asia, vinculadas a la comunidad científica internacional mediante las organizaciones de ICSU.

Organización

La estructura RELAB ha evolucionado desde su creación. Sus Miembros Nacionales, han aumentado a catorce con la agregación de México, Honduras, Cuba, Costa Rica, y Panamá. Hay seis Miembros Regionales y dos Miembros Asociados. Los Miembros Nacio-nales son países latinoamericanos cuyos gobiernos designan un Comité Nacional, formado por científicos que representan las principales instituciones de investigación y docencia superior en ciencias biológicas en ese país. Los Miembros Regionales son: Asociación Panamericana de Sociedades de Bioquímica y Biología Molecular (PABMB), Sociedad Iberoamericana de Biología Celular (SIABC), y las Asociaciones Latinoamericanas de Botánica (ALB), Farmacología (ALF), Ciencias Fisiológicas (ALACF) y Genética (ALAG). Estas sociedades regionales agrupan a los biólogos latinoamericanos en grandes áreas de las ciencias

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biológicas, y constituyen una organización representativa con una actividad continuada en el ámbito latinoamericano. Los Miembros Asociados son instituciones que comparten los objetivos de la RELAB en la promoción de la integración científica de América Latina en el campo de la biología. Con esta composición de miembros, se puede asegurar que RELAB es una organización altamente representativa de las ciencias biológicas de la región.

El órgano máximo es el Consejo Directivo Regional (CDR), compuesto por: (i) un delegado de cada uno de los gobiernos de los países participantes, (ii) un delegado científico designado por cada uno de los Comités Nacionales de RELAB, (iii) un delegado de cada uno de los Miembros Regionales y Asociados, (iv) el Presidente y el Coordinador Técnico de RELAB, y (v) observadores permanentes de la UNESCO, la OEA y el ICSU, instituciones que copatrocinan la RELAB.

El CDR se reúne cada dieciocho meses para escoger los nuevos miembros, definir las políticas generales y el plan de acción de RELAB para el siguiente bienio.

En el XVIII CDR, celebrado en octubre de 1997 en Asunción, se creó el cargo de Presidente de la RELAB, para el cual se nombró al Dr. Jorge E. Allende, y al Dr. Oscar Grau como Coordinador Regional en substitución del Dr. Allende, quien había ejercido ese cargo durante veintidós años, desde la fundación de la Red.

Durante los primeros diez años (1975–1985), en los que RELAB funcionó en el marco de un proyecto PNUD/UNESCO, apoyó 68 becas de postgrado y 46 cursos de entrenamiento intensivo, generó y apoyó 88 proyectos binacionales y trinacionales de colaboración en investigación, y financió parcialmente numerosas actividades organizadas por los Comités Nacionales.

En su segunda etapa (1985–1994), la RELAB ha contado con un financiamiento más limitado, que ha sido generosamente otorgado por UNESCO, ICSU, y ocasionalmente por la OEA y el PNUD. En esta etapa, la mayor parte de las actividades se concentró en cursos intensivos, talleres y simposios.

En 1997 la Junta de Gobernadores del International Centre for Genetic Engineering and Biotechnology (ICGEB) aprobó oficialmente la incorporación de la RELAB como "red regional afiliada" a dicha entidad internacional. Esto permitirá a RELAB presentar solicitudes de cursos y reuniones científicas para ser realizadas en un país miembro.

Actividades recientes

Un tercer período comenzó en 1994, cuando RELAB pasó a formar parte de la gestión de COSTED/IBN para América Latina. COSTED (Comité de Ciencia y Tecnología para Países en Desarrollo) e IBN (International Biosciences Networks) se fusionaron, en una iniciativa conjunta de ICSU y UNESCO, para impulsar el desarrollo científico y tecnológico de las regiones del Tercer Mundo. Actualmente, COSTED/IBN ha organizado, en América Latina, redes con objetivos similares en: Química (RELACQ), Física (RELAFI), Matemáticas (RELAMA), Ciencias de la Tierra (RELACT) y Astronomía (RELAA), en cuya organización participa también la Academia de Ciencias de América Latina (ACAL). La primera reunión regional latinoamericana de representantes de estas seis ramas de la ciencia tuvo lugar el 6 y 7 de junio de 1994 en la sede de la Academia Chilena de Ciencias, en Santiago. Posteriormente se celebraron reuniones del Comité Coordinador de Redes Científicas Latinoamericanas (CCRCLA) en México D. F. (agosto, 1995) y Rio de Janeiro (enero, 1997). Ese mismo año se celebró en el mes de septiembre una segunda

reunión en Rio de Janeiro, junto con la reunión de la Third World Academy of Sciences (TWAS).

La XVIII reunión del CDR de la RELAB tuvo lugar en Asunción, Paraguay, en octubre de 1997. En esta ocasión se realizó el Simposio RELAB N° 6 que abordaba los estudios de postgrado en Ciencias Biológicas con vistas al siglo XXI. Presentaron resúmenes de sus tesis once jóvenes doctorados en los últimos dos años. El contenido de este Simposio dará lugar al sexto libro editado por RELAB. Los volúmenes publicados se indican en la Tabla 1.

La Corporación RELAB

La Corporación RELAB, o Corporación de Apoyo a la Red Latinoamericana de Ciencias Biológicas, es una organización privada, sin fines de lucro, destinada a actividades científicas en los países miembros de RELAB.

En su mayoría los fondos para RELAB han provenido de organismos internacionales. Los principales han sido UNESCO (directamente y a través de su Oficina Regional de Ciencia y Tecnología [ORCYT]) e ICSU (a través de las IBN), con aportes ocasionales de la OEA y del PNUD.

El Consejo Directivo Regional, reunido en México en 1991, discutió sobre las limitaciones del financiamiento de RELAB por parte de las instituciones internacionales, que es muy reducido. Se aprobó por unanimidad que los países miembros contribuyeran con una cuota anual de US $5000. Este acuerdo, sin embargo, aún no ha podido consolidarse de forma regular, debido a la difícil situación económica en que se encuentran algunos de los países miembros.

Actividades apoyadas por RELAB en 1996

- III Curso Latinoamericano sobre Control Microbiano de Insectos. Castelar (Argentina). Noviembre.
- Curso: Recientes Avances en Histocompatibilidad e Inmunogenética. Buenos Aires (Argentina). Agosto.
- Curso: Biophysical and Chemical Approaches to the Study of Excitable Cells. Córdoba (Argentina). Agosto.

Tabla 1. Volúmenes publicados de los Simposios RELAB

Simposio No.	Título	Editorial	Año
1	La Biología como instrumento de desarrollo para América Latina	Universitaria	1990
2	La formación, retención y recuperación de recursos humanos en ciencias biológicas para América Latina: una estrategia para enfrentar la fuga de cerebros	Universitaria	1992
3	El financiamiento de la investigación en ciencias biológicas en América latina	Universitaria	1993
4	Los grandes temas actuales y futuros de las investigaciones biológicas en América Latina	En publicación	1994
5	La sociedad latinoamericana y la ciencia: un encuentro necesario	En publicación	1995
6	El postgrado en Ciencias Biológicas para la América Latina del siglo XXI	En publicación	1997

• Curso: Caracterización, estructura y función de macromoléculas de parásitos. Buenos Aires (Argentina). Octubre.

• VIII Conferencia Internacional sobre la Na^+/K^+–ATPasa. Mar de Plata (Argentina). Agosto.

• Curso Internacional: Avances en Virología y Bioquímica Molecular. La Paz (Bolivia). Septiembre.

• Curso Internacional de Postgrado: Aspectos Matemáticos y Estadísticos de la Genética Molecular de Poblaciones Humanas. La Paz (Bolivia).Octubre–Noviembre.

• 4° Simposio Brasileiro sobre Matrix Extracelular. Rio de Janeiro (Brasil). Septiembre.

• Santiago Southern Summer Symposium 1996–Genes y Genomas: Estructura y Mapeamiento. Santiago (Chile). Enero.

• PABMB–Congreso Panamericano de Bioquímica y Biología Molecular. Pucón (Chile). Noviembre.

• Simposio Molecular Mechanisms of Viral Pathogenesis (en el marco del Congreso PABMB). Noviembre.

• Curso Internacional de Conservación de Germoplasma Vegetal. San José (Costa Rica). Junio.

• Curso de Introducción a la Biología Molecular. Quito (Ecuador). Octubre.

• Simposio RELAB N° 5: La Sociedad Latinoamericana y la Ciencia: Un encuentro necesario, y XVIII reunión del Consejo Directivo Regional de RELAB. San José (Costa Rica). Mayo.

Actividades apoyadas por RELAB en 1997

• Curso ICRO: Molecular Techniques of Genome Mapping and Screening. Santiago (Chile). Enero.

• Curso Internacional Tópicos en Citogenética Molecular. La Paz (Bolivia). Noviembre.

• 26ª Reunião Anual da Sociedade Brasileira de Bioquimica e Biologia Molecular. Caxambú (Brasil). Mayo.

• Taller sobre Aumento de la Eficiencia Reproductiva y Conservación del Genoma Mediante el Uso de la Criopreservación. Santiago (Chile). Noviembre.

• Taller y Curso de Biotecnología. Heredia (Costa Rica). Junio.

• Simposio: "El enfoque darwiniano aplicado al estudio de la diversidad biológica" (en el marco de la Conferencia Anual de la Asociación de Biólogos Tropicales). San José (Costa Rica). Junio.

• IV Simposio de Zoologia. La Habana (Cuba). Noviembre.

• Curso de marcadores moleculares para estudio de diversidad de plantas silvestres y cultivadas en bancos de germoplasma. Quito (Ecuador). Noviembre.

• Curso Patología de peces. Guyaquil (Ecuador). Noviembre.

• Simposio: Human Genome Research: Implications for Health in Latin America. San Juan del Rio, México. Marzo.

• Fourth South-North Human Genome Conference. Guadalajara (México). Marzo.

• Curso de Postgrado Microscopía Electrónica. Lima (Perú). Junio.

• XIX Congreso Latinoamericano de Ciencias Fisiológicas: Integración de metabolismo. Caracas (Venezuela). Septiembre.

• Curso "Dendrocronología: principios, metodología y aplicaciones de las ciencias ambientales". Mendoza (Argentina). Noviembre.

• Simposio RELAB N° 6: La Enseñanza de Postgrado en Ciencias Biológicas para la América Latina del Siglo XXI, y XVIII reunión del Consejo Directivo Regional de RELAB. Asunción (Paraguay). Octubre.

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Revisión de libros

Introducción a la Inmunología Humana

Miguel Sánchez-Pérez (ed.)

Editorial Síntesis, Madrid, 1997. 383 pp. Precio: 3930 PTA. ISBN 84-7738-534-3

El editor Miguel Sánchez-Pérez comienza el prólogo con una frase de E. S. Golub que resume la increíble evolución de la inmunología en este siglo. Esta ciencia en efecto ha experimentado un empuje considerable en tiempos recientes. El impacto social de la inmunología no solamente se debe al hecho de haberse ampliado considerablemente las fronteras de la ciencia, sino también a que ha permitido que algunas de las enfermedades que siempre han aterrorizado a la humanidad sean parcial o en algunos casos totalmente controlables en la actualidad.

Con la participación de numerosos autores —investigadores y docentes de prestigio en el campo de la inmunología—, Miguel Sánchez-Pérez ha diseñado un libro de texto extremadamente útil y clarificador sobre los conocimientos actualizados de esta ciencia, a menudo menoscabada, que es la inmunología. El libro va dirigido fundamentalmente a alumnos de las ciencias de la salud (medicina, farmacia), aunque puede ser de gran utilidad también para estudiantes de otras disciplinas como la biología, bioquímica, veterinaria, etc.

Este volumen, de contenido eminentemente básico, se complementa con un segundo volumen de la misma colección que lleva por título *Conceptos Básicos de Inmunología Aplicada y Técnicas Inmunológicas.*

El formato del libro y del mismo texto hacen de él una obra atractiva y amena para la lectura y el estudio. La infografía utilizada profusamente en las figuras a lo largo del libro es de extrema calidad y con una uniformidad de símbolos, tipos y estilos —lo cual es siempre de agradecer. Unos recuadros explicativos en cada capítulo destacan conceptos especialmente importantes, e incluso dan informaciones que no suelen ser habituales en los libros de texto: premios Nobel en inmunología, trasplantes de piel, medicina legal, etc.

El libro consta de 14 capítulos, cada uno de los cuales se acompaña de una relación de citas bibliográficas muy recientes y, en la mayoría de los casos, de revistas internacionales de primer orden. Cada capítulo está dividido en partes y subpartes que hacen que cualquier estudiante pueda leer comprendiendo y asimilando los conceptos que van sucediéndose a lo largo de aquél. Las últimas páginas del libro están dedicadas a un útil diccionario de términos.

El primer capítulo, dedicado a la historia de la inmunología, constituye un apartado que todo estudiante de la materia debería conocer, y que es una introducción adecuada.Los hitos conceptuales que se han ido sucediendoen el desarrollo de esta ciencia se relatan con todo lujo de detalles y con imágenes curiosas (por ejemplo, recortes de periódico de 1917 sobre

muertes por rabia). Las teorías evolutivas se relacionan también en este capítulo con el desarrollo del sistema inmunitario.

Los siguientes capítulos tratan tanto de la organización como de la filogenia y la ontogenia del sistema inmunitario. Otros desarrollan aspectos del funcionamiento de la acción inmunitaria con sus agentes protagonistas (células, macrófagos, linfocitos, complemento, complejo principal, citocinas, etc.). Los demás capítulos se centran en otros aspectos que resultan igualmente imprescindibles en el conocimiento de esta ciencia: la biología molecular de la presentación antigénica, la genética y la generación de diversidad en la respuesta adaptativa del sistema inmunitario.

Se trata en definitiva de un libro de texto de carácter básico muy recomendable en el estudio de la inmunología en el contexto universitario, preparado por especialistas y adecuado a la predominante periodicidad cuatrimestral de las asignaturas en nuestras universidades.

Como recomendación al alumnado, al que principalmente va dirigido el libro, se sugiere en el prólogo que se siga la estrategia del sistema inmunitario, que basa su increíble potencial no sólo en la memoria, sino también en la creatividad y la versatilidad. La memoria es importante, pero sólo si demuestra ser útil.

El título de la obra, sin embargo, no resulta del todo acertado a mi parecer, puesto que una inmunología básica no puede circunscribirse únicamente a la especie humana. Incluso el mismo autor afirma en el prólogo que el libro aborda con detalle la pregunta de cómo es posible que un metazoo sea capaz de contrarrestar el potencial de variación de los microorganismos, teniendo en cuenta sus posibilidades de transferencia génica horizontal.

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Environmental Applications of Nucleic Acid Amplification Techniques

Gary A. Toranzos (ed.)

Technomic Publishing Company, Inc., Lancaster, 1997. 266 pp. Precio: $ 125,00. ISBN 1-56676-408-4

Gary A. Toranzos, investigador y profesor de microbiología ambiental en la Universidad de Puerto Rico, ha desarrollado y aplicado técnicas avanzadas de biología molecular al estudio de problemas ambientales. El conocimiento y experiencia acumulada en muchos años de investigación le ha llevado coordinar la preparación y edición del libro que ahora se comenta en todos su capítulos, para el que ha contado con la colaboración de un buen número de especialistas, diecisiete en total.

El primer capítulo, que es a la vez introducción, ha estado a cargo de A. K. Bej y G. A. Toranzos y recoge aspectos generales sobre la recuperación y purificación de los ácidos nucleicos de muestras medioambientales; detección de patógenos bacterianos y algunos indicadores transmitidos por vía hídrica; una introducción a la técnica de PCR en fase sólida y su aplicación a la detección de patógenos; aspectos sobre la técnica de "PCR-gene probe hybridization" y su aplicación en estudios de microorganismos manipulados genéticamente; así como la aplicación de PCR a estudios de biorremediación y otros aspectos medioambientales.

El capítulo 2, *Basic Methodology for DNA and RNA Amplification*, es obra de A. J. Alvarez y G. A. Toranzos, y se centra en las principales técnicas de amplificación de ácidos nucleicos (DNA y RNA). Lo más sobresaliente es la cla-

ridad de la exposición y el planteamiento de las técnicas a través de cuyas explicaciones el lector pueda desarrollarlas en su laboratorio.

El capítulo 3 (*Assessing Genetic Diversity of Microbes using Repetitive Sequence-based PCR*), de F. J. Louws, M. Schneider y F. J. DeBruin, aborda un aspecto muy importante en la microbiología medioambiental como es el de la biodiversidad microbiana, y los métodos quimiotaxonómicos y genéticos que se están aplicando para estos fines. Los autores ponen especial énfasis en la importancia de la técnica de rep-PCR para llevar a cabo el "fingerprinting" de los aislados y establecer las similitudes o diferencias inter- e intraespecíficas, es decir, en cuanto a especies o a cepas. El capítulo concluye con una detallada descripción de la técnica de rep-PCR y de los análisis de rep-PCR "fingerprints".

I. L. Pepper, T. M. Straub y C. P. Gerba son los autores del capítulo 4, *Detection of Microorganisms in Soils and Sludges*. Se ofrecen detalles especifícos acerca de la introducción de la técnica de PCR en la detección de bacterias y virus de suelos y lodos. Un aspecto interesante del capítulo es la aplicación de estas técnicas moleculares a la detección de bacterias viables pero no cultivables.

En el capítulo 5 (*Detection of Viruses in Water Samples by Nucleic Acid Amplification*), a cargo de M. Abbaszadegan y R. DeLeon, se describen los principales virus que pueden transmitirse por las aguas y se comparan los métodos de detección tradicionales, como cultivos celulares, con los que se basan en la técnica de PCR. En el capítulo 6 (*Detection of* Giardia *Cysts and* Cryptosporidium *Oocysts in Water Samples by PCR*) de M. Abbaszadegan, se propone una interesante metodología de detección de estos protozoos entéricos por PCR. La descripción de las técnicas es muy completa y accesible al lector.

PCR Detection of Airborne Microorganisms, (cap. 7) ha sido escrito por M. P. Buttner, L. D. Stetzenbach, A. J. Alvarez y G. A. Toranzos. Con la aparición de la legionelosis causada por *Legionella pneumophila* los microbiólogos hemos puesto mucha atención en los microorganismos transmitidos por vía aérea o por aerosoles, proponiéndose el término de Bioaerosol. La detección de microorganismos patógenos en los bioaerosoles es muy compleja debido a la falta de una metodología fiable y adecuada. En este capítulo se propone la detección de estos patógenos por medio de técnicas de PCR, que puede, sin lugar a dudas, suponer una importante contribución científico-tecnológica. El capítulo 8 (*Detection of* Legionella *and* Legionella pneumophila *DNA from Natural Water Samples by PCR*), conecta con el capítulo anterior y ha sido escrito por las Dras. T. Picone, T. Young y E. Fricker.

El último capítulo (*DNA Amplification Techniches in Fossilized Samples*), está a cargo de un autor bien conocido y con amplia experiencia en el tema, R. J. Cano. Se estudia y discute la posible aplicación de PCR y de otras técnicas moleculares que requieren amplificación del DNA para estudios cronobiológicos. Es una parte totalmente novedosa que puede suponer una aportación muy significativa para especialistas.

El interés de la obra radica principalmente en el tratamiento y aplicación específica de las técnicas moleculares y de PCR a la microbiología ambiental, donde hay tantos problemas por resolver. Aunque se han editado numerosos textos sobre las enormes posiblidades de las técnicas en biología y genética molecular y de PCR, no se había abordado sin embargo su aplicación en los estudios de microbiología ambiental

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Microbiología médica de Divo, 5th ed.

Oswaldo Carmona, Ma. Josefina Gómez, Franklin Mariño, Tibaire Montes, Carmen Marcano

McGraw-Hill Interamericana, 1997. 444 pp. Precio: 8935 PTA. ISBN 970–10–1160–0

Nos encontramos ante un libro pensado y diseñado para ser utilizado principalmente por estudiantes de medicina que ha constituido un texto popular en las facultades de medicina, venezolanas principalmente, pero también en el resto de universidades de la región. De su popularidad en este medio dice mucho el que continúen apareciendo ediciones, máxime en una época de abundancia de textos universitarios. Mucho más todavía, si se considera que no se trata de una traducción, sino de una obra escrita originalmente en español. El nombre de Alejandro Divo, autor de las cuatro primeras ediciones ha quedado incorporado al título a partir de esta quinta, como homenaje a su larga dedicación docente.

Los autores dejan claro lo que persigue la obra: ofrecer un resumen conciso de los conceptos fundamentales de la microbiología médica. Y lo que no es ni pretende ser: libro de consulta para profesionales especialistas. Con esta idea en mente, y sin perder la perspectiva de los destinatarios de la obra, estudiantes de medicina y resto de carreras relacionadas con las ciencias de la salud, se organizan y desarrollan las materias. De éstas, la mayor actualización corresponde a las aportaciones de la microbiología e inmunología que han podido ser incorporadas a la práctica clínica. El conocimiento de los mecanismos de la respuesta inmunitaria ha permitido avances indudables en el tratamiento de muchas enfermedades, aunque es cierto que queda mucho camino por delante.

El enfoque didáctico queda de manifiesto por la procedencia de los autores, profesores de la cátedra de microbiología de la Universidad Central de Venezuela. El contenido, distribuido en cinco grandes apartados, comprende: (1) bacteriología general; (2) inmunología; (3) bacteriología especial (una muy extensa parte en la que cada uno de los géneros susceptibles de ocasionar enfermedades, es descrito de acuerdo a un patrón que cubre prácticamente todas sus características: clasificación, caracteres generales, de cultivo, actividad bioquímica, propiedades metabólicas, resistencia, composición antigénica, etc. para acabar con profilaxia y tratamiento); siguen el apartado (4) dedicado a virología, y el (5) a la micología, y un apéndice final de procedimientos microbiológicos. En esta última parte se exponen de manera esquemática diversas técnicas de diagnóstico. Su propósito es facilitar la consulta del estudiante cuando ha integrado los conocimientos teóricos de la materia. Hay que destacar en la obra la claridad y sencillez de la exposición, tendente a hacer más dinámico el aprendizaje y comprensión de los procesos infecciosos y las respuestas inmunitarias que desencadenan.

El libro es muy sencillo en su presentación, no incorpora el color en ninguna de las gráficas y el papel es de baja calidad. Posiblemente para ofrecer un precio asequible, que suponemos más reducido en el país de origen, Venezuela, que el que tiene en España. Se echa también en falta una mínima bibliografía complementaria sobre los temas tratados, que podría ir al final de cada capítulo o al final de la obra. No hay que olvidar que la información bibliográfica es un elemento tan importante como la propia materia en cuestiones docentes.

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Life: An Unauthorised Biography

Richard Fortey

Harper Collins Publishers, London, 1997. 340 pp. Precio: £ 20,00. ISBN 000-255560-3

Prior to the now-cliched NASA image, it occured neither to Fortey nor to anyone else that the entire planet is alive. The early world (4,000 to 1,500 million years ago) writes Fortey, "was growing into an interconnected web of life, chemistry, oceans and geology—even the most sceptical reader will be able to accept that this much of Professor Lovelock's *Gaia* hypothesis must be true. Life made the surface of the Earth what it is, even while it was Earth's tenant." NASA's photo showed us the mobile facial expressions of the planet. Eureka! This giant blue marble is at once both a body, of which we people are a part, and a home. Seeing the Earth rise from a vantage point on the moon made millions of us face the planetary being to realize that since it is alive it does have a personal history. Now for Fortey the totality of planet-bound Earth-life is a subject ripe for biographical inquiry. The fact that its biography is "unauthorized" is clearly a major aspect of its charm. Presumably if "authorized", the book would read like any Historical Geology text and all the personal spunk would have been purged.

The order is chronological, beginning with life's first appearance. In its thirteen chapters, illustrated with 88 plates of black and white photographs, microbes originate, plants come ashore and animals, including human apes, appear, evolve and extinguish. Fortey ends with the statement that "Life will probably cope". Replete with small errors, my main misgiving is the extent of Fortey's biological misunderstandings. No longer is plant all that is not animal: much of the Ediacara biota, hardly a "fauna", is protoctist. Fortey, unclear about just what animals, plants, fungi, protoctists and bacteria are, fails to tell us when they first appear in the record. So too he is unaware that cyanobacterial atmospheric oxygen comes entirely from water (from which its hydrogens are greedily extracted) and not at all from the air's carbon dioxide. Atmospheric carbon dioxide is used by cyanobacteria, sulfur, methanogenic and other bacteria which he mentions to make cell material.

Although extraordinarily well read and self consciously international the failings of this ebullient author will disappear as he limits himself less to British zoology. We more than forgive him: what makes this text wonderful is its lively language and personal narrative. He leads us in a dance through time that is enthralling; we begin to know personally the trilobite-ridden Cambrian sea flooding over Australia. ("Trilobites are *my* family", he writes, "they probably adopted me long before I went to Spitzbergen, when, as a lad armed with optimism and a coal hammer, I tapped along Welsh cliffs and river banks in their pursuit."). We meet Ordovician nautiloids through the smoke filled eyes of his one-night room mate, the quintessential eccentric Rousseau H. Flower. From his youthful field trip in the severe landscape of Spitzbergen to his recent forays into the world of cladistics, Fortey is highly specific about people and places all over the world. He seems to have been everywhere and met everyone actively participating in the paleontological search to reconstruct the history of life on Earth: Dolf Seilacher, Stephen J. Gould, Chris Stringer, Bob Bakker and all sorts of other hard working paleontologists come in and out of these pages. His personal comments even extend to lumunaries now dead such as the three Charleses: Lyell, Darwin and Lapworth.

The Ordovices were a Welsh hill tribe at the time of the Roman empire known to Lapworth, the practical and artistic geologist, who began as a school teacher. Lapworth, who ended his career as Professor at Birmingham, gave the name "to the meat in the sandwich between Cambrian and Silurian." In his famous 1879 compromise (between Cantebridgian Professor Adam Sedgewick´s Cambrian claims and Sir Roderick Murchison´s Silurian) Lapworth assigned the older Welsh rocks to the Cambrian and the younger to the Silurian while he carved out a middle time period; now from 505 until 438 million years ago, a time-rock division of the lower Paleozoic Era. Our author is "deeply fond of this Ordovician country, for all its concealed bogs and incomprehensible gates." He notes that in a few places (Snowdon, Cadair Idris) where they are "reinforced by flows of lava and pumice which once erupted angrily over a sea swarming with brachipods, trilobites and conodont animals, over which lazy graptolites drifted in their incessant trawl for plankton" such Ordovian rocks make mountains.

Throughout geological time, the rocks tell us, at given landscapes still before our eyes lush green continents, their shelves, slopes and offshore depths have been frozen, scoured and desertified by enormous, inexorably expanding, rocky glacial ice masses.

How do we know for sure? Who has the audacity to claim that 438 million years ago the Ordovician seascape, icy and barren, closed never to reopen? With extraordinary skill for scientific narration of the field experience, Fortey, at his best, describes the ice age suffering of the end of the Ordovician world. On what does this paleointerpreter base his evidence? Suffice it to say that the author reads the landscape in Canada, Norway, Switzerland, Wisconsin, Spitzbergen, Greenland, Australia, the antiAtlas mountains of Morocco as part of the reconstruction of history from clues he takes to be representational. Most pertinently is his own description of the relentless sunbeat sands of the Arabian peninsula at Oman. He tells us of a huge valley scored by great deepening grooves, of rock gauges and scratches that looked as if they had been "scraped by the fingernails of some Titan clawing the ground in rage."

Fortey convinces us that polished rock, deep scratches and unsorted boulders on the valley walls were only some of the remarkable remnants of the huge ice blocks that used to occupy today's burning valleys of Oman. He leads us to see the unmistakable clues of advancing ice sheets that stop for no one. We realize the unity of the single ancient Earth system as he reconstructs for us the massive ice blanket, once contiguous, on Africa, South America, India, the Arabian peninsula, Antarctica and Australia. Moving down from the shifted south pole our mind's eye sees the huge Permian impertinence of perpetual ice on a planetwide continental mass. We discover, that is, not today's *Gaia* (= Geae) but the ancient world's "*Pan-geae*". So many times in the past the Earth's surface has suffered a long-term chill that left its record in the rocks.

Whether or not we deny it. the geological facts summarized by Fortey make it clear that all of us are embedded in this single continuous story of the Earth's surface. Independence from the planet is a dangerously ingenuous political delusion. Even if you never read again about this living Earth's body and newly envisaged face, of which we are all a part, I recommend you enjoy Fortey's version of our biography.

Lynn Margulis
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A more extensive version of this review was published in The Times *Higher Education Suppl., Oct. 31, 1997.*

Los Primeros Pasos de la Ecología en España

Santos Casado de Otaola

Publicaciones de la Residencia de Estudiantes, Madrid, 1997. 529 pp. Precio: 2200 PTA. ISBN 84-491-0222-7

El inicio y desarrollo de una disciplina científica en un país depende directamente de unas personas que lo hacen posible. Sin embargo, además de su espíritu emprendedor y dedicación personal, el resultado está sujeto a los condicionantes de tipo social, político, ideológico e institucional de cada época. El libro muestra cómo los naturalistas españoles de la segunda mitad del siglo pasado desarrollaron una nueva visión científica de los sistemas naturales y de los organismos que los componen a tenor del impacto de las teorías evolucionistas. Con todo lujo de detalles y basándose en una profunda investigación de esa etapa, el autor muestra cómo los naturalistas de la primera mitad del siglo XX asumieron nuevos enfoques ecológicos, surgidos en las ciencias naturales del cambio de siglo, y se esforzaron por incorporar la ecología a nuestro panorama científico. Entre los que dieron, quizá sin proponérselo, los primeros pasos de la ecología en España estaban Celso Arévalo, Odón de Buen, Tomás Andrés, Joaquín M. de Castellarnau, Luis Pardo, Emilio H. del Villar y José Cuatrecasas.

El autor aporta argumentos que sostienen que el desarrollo de la historia natural en España tiene que ver con proyectos colectivos de carácter nacional y concepción nacionalista, con los que guarda un amplio paralelismo. Esos proyectos proporcionaron un conocimiento histórico-natural de la Península, hasta entonces muy imperfecto. En 1871 se fundó la Sociedad Española de Historia Natural con un cierto componente de motivación patriótica. Al año siguiente se creó la revista *Anales de la Sociedad Española de Historia Natural*, para dotar a la comunidad de naturalistas españoles de un medio de publicación que canalizase su incipiente reactivación científica y difundiera los resultados de sus investigaciones. Aún más notorio fue el componente nacionalista que tuvo la Institució Catalana d'Història Natural fundada en Barcelona en 1899 para el estudio y propagación de las ciencias naturales en el ámbito catalán.

En contraposición a la proyección inicial y mayoritaria de las ciencias naturales en España en el sentido de conocer y catalogar todos los seres vivos de la península, algunos pocos científicos —en el campo de la hidrobiología y la botánica sobre todo— abogaron por un cambio de rumbo hacia la sinecología. El libro permite entender por qué se mantuvo el interés en ampliar el conocimiento de la flora, por poner un ejemplo, de la Península y, sin embargo, no desarrollar su estudio sinecológico. Tomando una cita de Celso Arévalo (*La vida en las aguas dulces,* 1929), reflejada en el último capítulo del libro: "Los naturalistas formados en la escuela clásica (...) habituados a no concebir el estudio de los seres de la Naturaleza más que partido en grupos taxonómicos, se les antoja una ciencia de carácter enciclopédico, sin pensar que es mucho más enciclopédico estudiar una familia, lo mismo de Europa, que Malasia o de Patagonia..."

La obra ofrece un riguroso análisis histórico que mezcla muy acertadamente la vertiente científica, social, política e institucional. Trabajos de esta magnitud en otros campos de la ciencia permitirían a los "gestores de la ciencia" conocer la evolución del desarrollo científico en España y evitar, en la medida de lo posible, errores ya cometidos en el pasado.

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Avances en Climatología Histórica en España

Javier Martín-Vide (ed.)

Editorial Oikos-tau, Barcelona, 1997, 224 pp. Precio: 2500 PTA. ISBN 84-281-0905-2

La importancia que el IV Programa Marco (1994–1998) de la Unión Europea concede al estudio del clima y del cambio climático es uno de los elementos que contribuyó a la inclusión de los estudios científicos sobre el clima en España en el III Plan Nacional de Investigación y Desarrollo Tecnológico (1996–1999). La presentación en 1994 de dos tesis sobre climatología histórica, indicaba la necesidad ya inminente de incorporar los estudios sobre el clima en la política científica de nuestro país.

Avances en Climatología Histórica en España forma parte de dos proyectos, europeo y nacional, cuyo objetivo general es obtener una plena caracterización del clima en Europa a fin de cuantificar el cambio climático producido como respuesta al efecto invernadero. Posiblemente se irán publicando otros volúmenes a medida que se avance en la investigación y en la localización y consulta de la documentación. En este volumen, con los datos referidos exclusivamente a España, se explora la rica documentación existente, y seguramente ignorada, lo cual aumenta su utilidad.

La reconstrucción del clima en el llamado período preinstrumental (anterior a 1780), se vale de los registros de las ceremonias rogativas que, una vez conocidos los procedimientos institucionales que regían su celebración, permiten su utilización como indicador climático. Según datos de los autores, eran actos religiosos en respuesta a una tensión medioambiental real. Se desarrollaban de forma homogénea en toda la península debido al control de la Santa Sede sobre su realización. La información que proporcionan es cuantificable, homogénea y válida. La climatología histórica dispone principalmente de informaciones documentales susceptibles de cuantificación. España presenta unas particularidades climáticas cuyo conocimiento presenta un indudable interés. A esta peculiaridad contribuye una orografía compleja, latitudes subtropicales, riqueza y variedad de fenómenos y acusado gradiente de temperatura entre el Atlántico y el Mediterráneo.

Este primer volumen se centra en: (i) Comprobación de la calidad de la información documental mediante la distribución de Poisson. (ii) Riesgos meteorológicos en Barcelona a través de registros históricos con datos de los siglos XIV–XIX. (iii) Series meteorológicas instrumentales antiguas de Madrid y Barcelona. (iv) Aplicación de las cadenas de Markov a secuencias de días lluviosos, Barcelona 1780–1860 y 1951–1990. (v) Incidencia de la actividad volcánica en las temperaturas. Y (vi) Evolución reciente de las temperaturas medias anuales en España.

Los estudio sistemáticos sobre el clima van a ser beneficiosos para muchas áreas del conocimiento científico. Cada vez va a resultar más evidente su estrecha relación con numerosos campos de investigación como geografía, antropología física y cultural y ciencias de la tierra. Mención especial merece su relación con la ecología microbiana, disciplina integradora, donde las haya, en la que todos los factores del medio y de los seres vivos que lo habitan, ejercen una constante y recíproca influencia. A efectos prácticos, el conocimiento que aporte resultará esencial para una correcta planificación de los recursos, gestión de espacios naturales, predicción de fenómenos climáticos y actuaciones adecuadas ante su aparición.

Carmen Chica
Redacción de Microbiología SEM

Laboratory Earth

The Planetary Gamble We Can't Afford to Lose

Stephen H. Schneider

Weidenfeld & Nicolson, London, 1996. 184 pp. Precio: £ 11.99. ISBN 0-297-81644-6

"It's a matter of scale", así introduce el autor Stephen H. Schneider la necesidad de observaciones del Sistema Tierra que permitan percibir la enorme variedad de la naturaleza. Reconocido como uno de los expertos más destacados del mundo en investigación atmosférica y cambio global, S. H. Schneider es profesor del Departamento de Ciencias Biológicas de la Universidad de Stanford y Director del *Advanced Study Project* en el National Center for Atmospheric Research en Boulder, Colorado .

En162 páginas distribuidas en los siguientes capítulos: (i) *The Organic and Nonliving Earth: A Dynamic Cohesion*, (ii) *The Coevolution of Climate and Life*, (iii) *What Causes Climate Change?*, (iv) *Modeling Human-Induced Global Climate Change*, (v) *Biodiversity and the Battle of the Birds*, y (vi) *Integrated Assessments of Policy Options*, se explica en qué consiste el cambio climático y sus consecuencias ecológicas y sociales.

El autor se plantea una serie de interrogantes: ¿Cuánto tardaron en evolucionar el clima y la vida? ¿Cómo interaccionan las porciones orgánicas e inorgánicas de la Tierra? ¿Cómo modifica la especie humana el planeta? ¿Cabe la posibilidad de reconciliación entre protección ambiental y desarrollo económico? La revolución industrial, el agotamiento de recursos y aumento de residuos, junto con la explosión demográfica y la degradación del suelo, han producido un cambio global muy rápido en el ambiente. Por ello es necesario conocer la forma en que alteramos el clima global y las consecuencias de la destrucción de la naturaleza. ¿La respuesta será debida a *Gaia* o a la coevolución del planeta con la vida?

¿Nos precipitamos al declarar *Gaia* real?, o, por el contrario, ¿las nuevas ideas de apoyo al influjo biológico en el cambio climático quedan confirmadas por el ajuste entre los sistemas? En 1969, James E. Lovelock formuló la hipótesis de *Gaia*. En sus propias palabras: "las condiciones físicas y químicas de la superficie de la Tierra, de la atmosfera y de los océanos se han hecho adecuadas y confortables para la vida debido a las actividades de los seres vivos. Esto contrasta con la visión anterior, que mantenía que la vida se había adaptado a las condiciones existentes en la Tierra, y que ésta y la vida habían evolucionado separadamente". Según *Gaia*, la Tierra se regula como un organismo vivo que controla automáticamente su ambiente para hacerlo óptimo para los organismos. Para el autor, el clima y la vida han coevolucionado.

Es un título atractivo para un excelente libro de alta divulgación científica, con sólida base interdisciplinaria. Contribuye a la propagación de la nueva ciencia del Sistema Tierra y, tal vez, ojalá, a la construcción de un mundo menos contaminado. El autor sería un candidato idóneo a representante en la "Asamblea de las Culturas", propuesta por Yehudi Menuhin. Éste, en el pregón de las Fiestas de la Merced de Barcelona (1997), proclamó que "hay que reconocer que existe una economía mucho más amplia que la financiera de un país industrializado, y que incluye la educación, la salud, el equilibrio mental, la creatividad de cada individuo y, finalmente, el bienestar de la naturaleza, de los bosques, los animales, el agua y la atmosfera que hemos explotado, despreciado y contaminado hasta el día de hoy".

Asunción Peral Socías
I. B. Torras i Bages, Barcelona

Diccionario Enciclopédico Ilustrado de Medicina Dorland, 28a. ed.

McGraw-Hill Interamericana, Madrid, 1997. 2 volúmenes. 2118+xxxviii pp. Precio: 19.900 PTA. ISBN (obra completa) 84–486–01777–7

El *Dorland's Illustrated Medical Dictionary* ha sido una obra de consulta tradicional, que cuenta en su haber con casi un siglo de existencia. Tradicional ha sido también la colaboración en las sucesivas ediciones de un numeroso grupo de especialistas de todas las áreas de la medicina, algunas de las cuales son más modernas que el propio diccionario. En esta edición, el número de colaboradores se eleva a 39, procedentes de facultades de medicina y hospitales universitarios de Estados Unidos.

Es una obra extensa, como suelen serlo todas las de este tipo si se quiere abarcar la enorme cantidad de términos para constituirse en referencia autorizada en terminología médica y de otros campos de las ciencias de la salud y la atención sanitaria. Obedeciendo a esta necesidad, parece haberse llevado a cabo una exhaustiva revisión con las necesarias modificaciones que han afectado a todas y cada una de las especialidades. Se ofrece así un vocabulario actualizado con una práctica presentación de la información. Otras novedades son la comodidad de la búsqueda por el simple hecho de darle una nueva línea a cada entrada y la incorporación de dos nuevos anexos al índice. La intención es proporcionar al lector una guía actualizada y autorizada sobre el vocabulario médico.

En la edición original inglesa los términos empleados utilizan la nomenclatura proporcionada por obras de referencia o acuerdos internacionales (en aquellas especialidades que cuentan con ellos) que han venido a constituirse en norma de aceptación prácticamente universal. Así, los términos anatómicos aparecen definidos de acuerdo con la *Nomina Anatomica*, procedente de la XII Conferencia Internacional de Anatomistas (Londres 1985). La nomenclatura enzimática se rige esencialmente por las Recomendaciones del Comité de Nomenclatura de la Unión Internacional de Bioquímica y Biología Molecular. La terminología bacteriológica toma como referencia los volúmenes 1 a 4 del Manual de Bergey de 1989 y así se procede en las especialidades farmacológicas y psiquiátricas.

Además del vocabulario, que compone el bloque general de la obra, con más de 2000 páginas, hay otros apartados de interés como el dedicado a los fundamentos de etimología médica, equivalencias de temperatura en Celsius y Fahrenheit, tablas de dosis métricas con sus equivalentes farmacéuticos y valores de referencia para la interpretación de pruebas de laboratorio. El índice de tablas y el de figuras permite su fácil localización en cualquiera de los dos volúmenes.

El Diccionario ha sido traducido al español por un grupo de especialistas en las distintas materias y sometido a una revisión técnica. Es de esperar por tanto que se haya mantenido la máxima fidelidad en la equivalencia de los términos, junto a la adecuada corrección y estilo lingüístico. En cualquier caso, la terminología científica en español es todavía una asignatura pendiente, tanto por parte de la Real Academia de la Lengua como del resto de Academias científicas. Sabemos que hay proyectos en marcha para subsanar esta laguna, por lo que es de esperar que sean pronto una realidad, pues no cabe duda de que una instancia de referencia en terminología científica supondrá una gran ayuda en todos los sentidos.

Ricard Guerrero
Redacción de Microbiología SEM

MICROBIOLOGÍA SEM **13** (1997), 543–546

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Frances Luttikhuizen
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Javier Martín-Vide (ed.)
Editorial Oikos-tau, Barcelona, 1997, 224 pp. Precio: 2500 PTA. ISBN 84-281-0905-2
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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
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Genes y nuevas terapias
Alberto Muñoz
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D. Balasubramanian, C. F. A. Bryce, K. Dharmalingam, J. Green, K. Jayaraman (ed.)
Universities Press, India, 1996. 425 pp. Precio: £ 19,95. ISBN 0-86311-667-1
Revisado en 13(1), 107

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Revisado en 13(4), *544*

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Una exploració sobre la informació electrònica i el futur de l'edició
Lluís Codina
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La ciencia como una luz en la oscuridad
Carl Sagan
Editorial Planeta, Barcelona, 1997. 493 pp. Precio: 4300 PTA. ISBN 84-08-02043-9
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Un desafío pendiente

Luis Carrasco
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Environmental Applications of Nucleic Acid Amplification Techniques

Gary A. Toranzos (ed.)
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In the company of mushrooms.

A Biologist Tale

Elio Schaechter
Harvard University Press. Cambridge, Massachusetts. London, England, 1997. 280 pp. Precio: $ 24.95. ISBN 0-674-44554-6
Revisado en 13(2), 247

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Ediciones UNESCO, Madrid, 1996. 372 pp.
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ISBN 84-294-5128-5 (Santillana)
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Miguel Sánchez-Pérez (ed.)
Editorial Síntesis, Madrid, 1997. 383 pp.
Precio: 3930 PTA. ISBN 84-7738-534-3
Revisado en 13(4), *535*

Immunology: The Making of a Modern Science

Richard B. Gallagher, Jean Gilder, G. J. V. Nossal, Gaetano Salvatore (ed.)
Academic Press, London, 1995. 246 pp.
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Revisado en 13(3), 375-376

Laboratory Earth.

The Planetary Gamble We Can't Afford to Lose

Stephen H. Schneider
Weidenfeld & Nicolson, London, 1996. 184 pp.
Precio: £ 11.99. ISBN 0-297-81644-6
Revisado en 13(4), *543*

Life: An Unauthorised Biography

Richard Fortey
Harper Collins Publishers, London, 1997. 340 pp. Precio: £ 20,00. ISBN 000-255560-3
Revisado en 13(4), *539–540*

Los primeros pasos de la ecología en España

Santos Casado de Otaola
Publicaciones de la Residencia de Estudiantes, Madrid, 1997. 529 pp. Precio: 2200 PTA.
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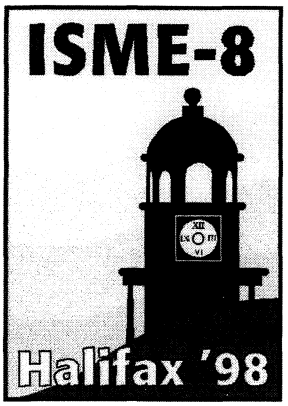

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