Volumen 10, nº 3
Septiembre 1994
ISSN 02 13-4101

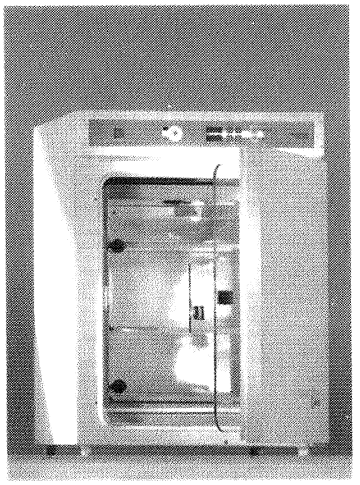
PUBLICACION DE LA SOCIEDAD
ESPAÑOLA DE MICROBIOLOGIA

Microbiología

SEM

Heraeus
INSTRUMENTS

Incubador de CO2 BB-16
Hemos incubado el huevo de Colón .....



En esta nueva generación de aparatos nos hemos concentrado especialmente en las caracteristicas de funcionamiento esenciales. Naturalmente no hemos hecho ninguna concesión en lo referente a la calidad de nuestros aparatos.

Sencillo y funcional
Las características técnicas de la BB 16 han sido estudiadas para facilitar el trabajo al usuario para las aplicaciones de rutina. Este aparato no representa complicaciones en su manejo, y está equipado con los dispositivos de seguridad necesarios.

Pequeño y compacto
La BB16 necesita poco sitio. Especialmente diseñado para espacios reducidos, el montaje especial de su puerta permite su colocación prácticamente a ras de los muebles de laboratorio.

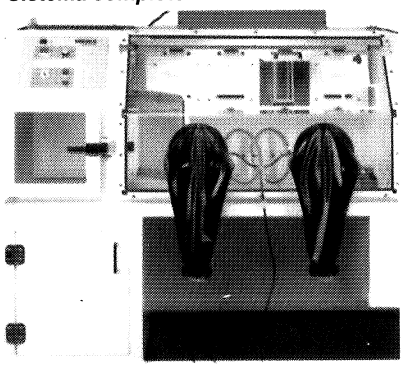
Next to in vivo
Un sistema de medición de CO2 de nueva creación, basado en la medición de la termoconductividad, asegura un valor pH estable de larga duración, incluso en el rango inferior de CO2.

Equipamiento orientado a la aplicación
Para condiciones especialmente estables se puede instalar, sobre demanda, una puerta de vidrio subdividida dotada de 3 pequeñas puertas, que permite un acceso segmentado.

Técnicas para el futuro
La BB-16 Function Line posee la acreditada calidad de Heraeus: Es un incubador con el que Ud. podrá contar también en el próximo milenio.

Camara para el cultivo de Anaerobios
Mod. WA 6200

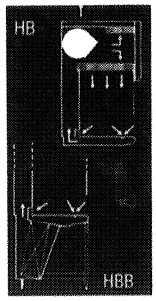
- ***Trabajo sin guantes***
- ***Manejo simple***
- ***Visión óptima***
- ***Control completamente automático***
- ***Incubador integrado hasta 70º C***
- ***Exento de condesación***
- ***Conexión para dos tipos de gas***
- ***Sistema completo***



Para trabajos en atmósfera exenta de oxigeno.
Cabina de flujo laminar para aplicaciones en el campo de la microbiologia, genética, farmacia microbiología médica en una atmósfera exenta de oxígeno. Apropiada para sobreinocuación de microorganismos sensibles al contacto con el oxigeno. Perfectamente adecuada para su empleo en laboratorios para la comprobación de la calidad de medicamentos y productos químicos que exigen atmósferas controlables, así como para la producción de componentes electrónicos en atmósfera de gas protector.

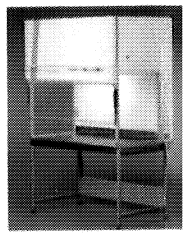
Construcción
Construcción en acero inoxidable barnizada con cristal frontal de plexiglás y dos aperturas con cierre para trabajar sin guantes. Incubador de aerobios hasta 70º C integrado y controlado por microprocesador, así como disyuntor de sobre-temperatura ajustable. Esclusa de anaerobios integrada, con carro móvil para la introducción de las muestras. Control de vacío completamente automático para el ga[illegible]iento rápido de la esclusa. Sis[illegible] automático de regulación de la humedad del aire. Dispositivo de seguridad manométrico de la camara.

Probablemente la mejor cabina de fluj laminar del mundo



HB/HBB Cabinas de seguridad clase

Seguridad clase II con 2 juegos de filtros HEPA para máxima protección producto, operario y medio ambiente. Verificador y construida segun todas normas internacionales.

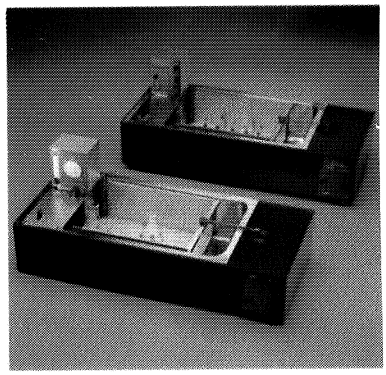


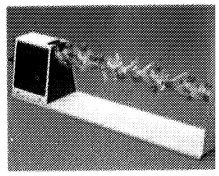
- Diseño insuperable y confortable para el usuario
- Bajo nivel de ruido
- Fácil mantenimiento, filtros facilmente intercambiables
- Alarma de seguridad
- Mesa de acero inoxidable
- Laterales en cristal para mejor iluminación
- Para usarse en laboratorios P-3

El modelo HBB tiene un sistema de filtros HEPA debajo de la mesa, proporcionando un filtrado extra de aire.
Corta distancia entre la parte superior de la mesa y el 1" filtro HEPA lo que proporciona una mínima area contaminada.

Heto es mucho más!

Adicionalmente al ámplio rango de baños presentados en este catalogo HETO ofrece más equipos para control de muestras. Aquí introducimos baños de agitación, baños termostatizados y mezcladores de muestras.





Mezcladores de muestr

Un amplio rango de mezcladores rotación y tareas de agitación. Capacid hasta 150 tubos simultaneamen adaptable a casi todo tipo de muestras.

- ***Tubos Eppendorf***
- ***Tubos conicos***
- ***Cajas Petri***
- ***Tubos de recogida de sangre***
- ***Soporte de protamuestras***

Con lectura digital de velocidad y tie velocidades extra 1-45 RPM.Amplio ran anillos para muestras opcionales.

(Preguntar por catalogo adicional).

Hetomix

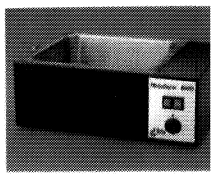
Heto ofrece un amplio rango de baños de agitación y completo rango de accesorios para el equipamiento completo del baño.
El baño de agitación Heto combinado con termostato Heto proporciona un sistema de control de temperatura preciso entre - 25°C y + 110° C.

Preguntar por catalogo adicional.

Baños de agua termostatizados

Para altas temperaturas o tareas básicas de regulación Heto ofrece un rango de baños de agua termostatizados con rasgos desde ambiente hasta + 100° C. Con compensación de agua evaporada. Existe un amplio rango de tapas y accesorios.

Preguntar por catalogo adicional.



Heraeus, S. A.
C/ Manuel Tovar, 24
28034 - Madrid

Para mayor información, recortar este cupón y remitir a:

Nombre.....
Empresa.....
Dirección .....Dpto. .....
C. P. .....Provincia .....
Teléfono de contacto .....

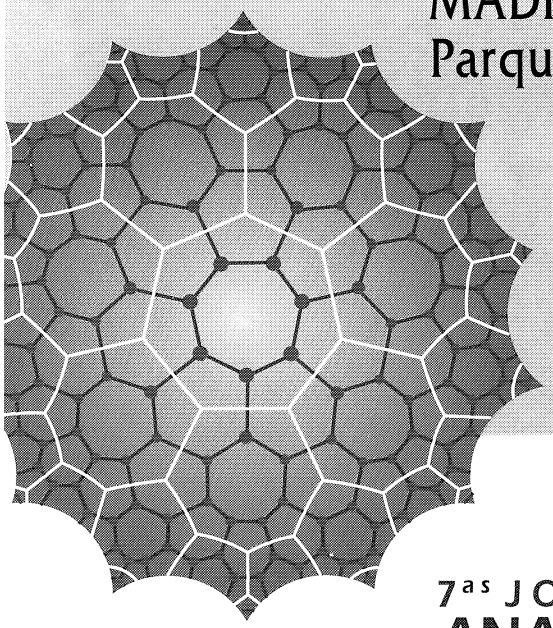
❑ Incubador de CO2 BB-16 ❑ Cabina Flujo Laminar ❑ Camara de Anaerobios ❑ Termostatos
❑ Baños con Agitación ❑ Agitadores de Tubos ❑ Baños de Agua

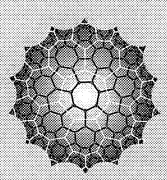
Ref. Revista: MICROBIOLOGIA

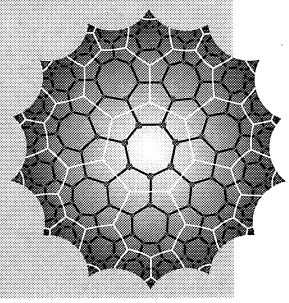
EXPOANALITICA + BIOCIENCIA

MADRID 4-7 ABRIL 1995
Parque Ferial Juan Carlos I

Feria de Madrid







7as JORNADAS DE ANALISIS INSTRUMENTAL

JAI

3-6 ABRIL 1995

1as JORNADAS DE BIOCIENCIA

JBC

4-6 ABRIL 1995

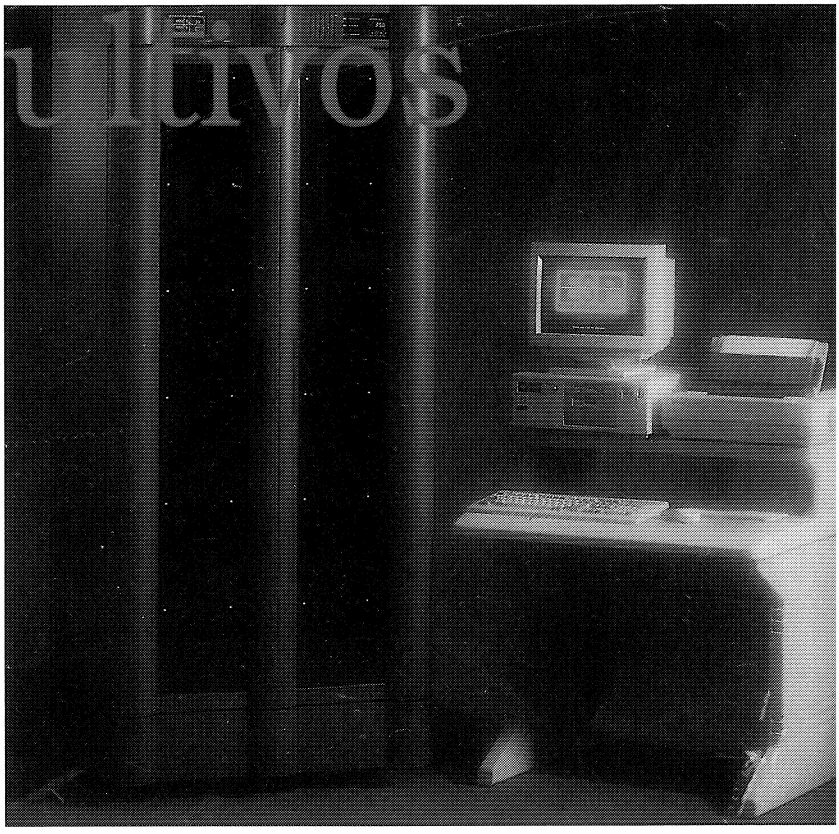
Organizado por:
EXPOQUIMIA

SECRETARIA: Avda. Reina Mª Cristina, Palacio nº 1 - 08004 Barcelona (España)
Tel. (34-3) 423 31 01 - Fax (34-3) 423 86 51

Hemocultivos ESP

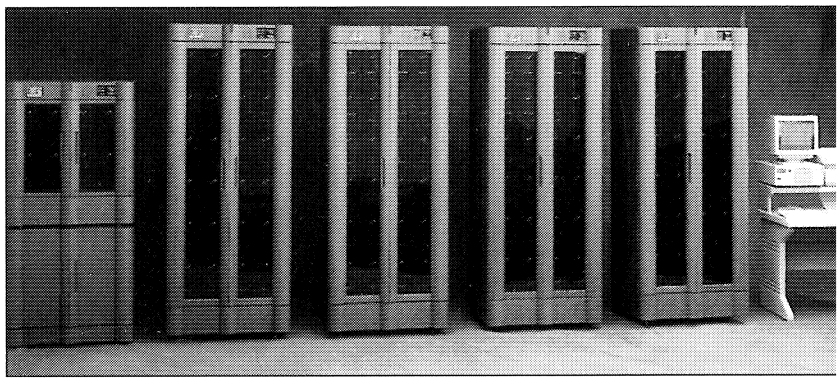
LA NUEVA TECNOLOGIA CONDUCE A UNA MAYOR RAPIDEZ

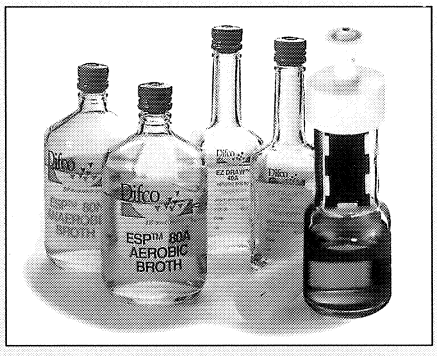
ESP ofrece lectura contínua, no invasiva, de los hemocultivos con automatización total y monitorización tanto de consumo como de producción de cualquier gas.



Configuraciones

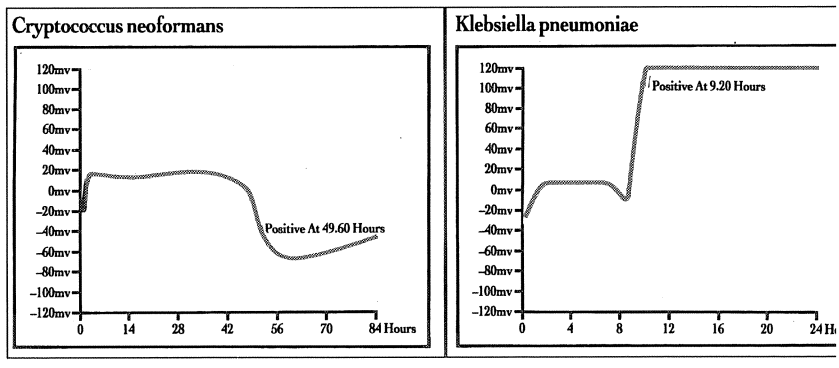
El sistema de Hemocultivos ESP, se ajusta con precisión a su volúmen de trabajo. Los instrumentos están disponibles en dos tamaños, 128 y 384 botellas. El ordenador controla hasta 5 instrumentos de cualquier capacidad.





Formatos de botellas

Los medios de nuevo desarrollo se ofrecen en una selección de tamaños. La botella de 30 ml. acepta muestras entre 0,1 y 10 ml. La botella pediátrica acepta hasta 5 ml. en extracción directa.
La botella bifásica contiene además una lengueta con Agar Chocolate y Sabouraud Dextrose.



Mayor recuperación. Mayor rapidez

Los sensores están contínuamente monitorizando tanto el consumo como la producció cualquier gas de forma que los positivos son detectados antes que con sistemas que monitorizan la producción de CO_2.

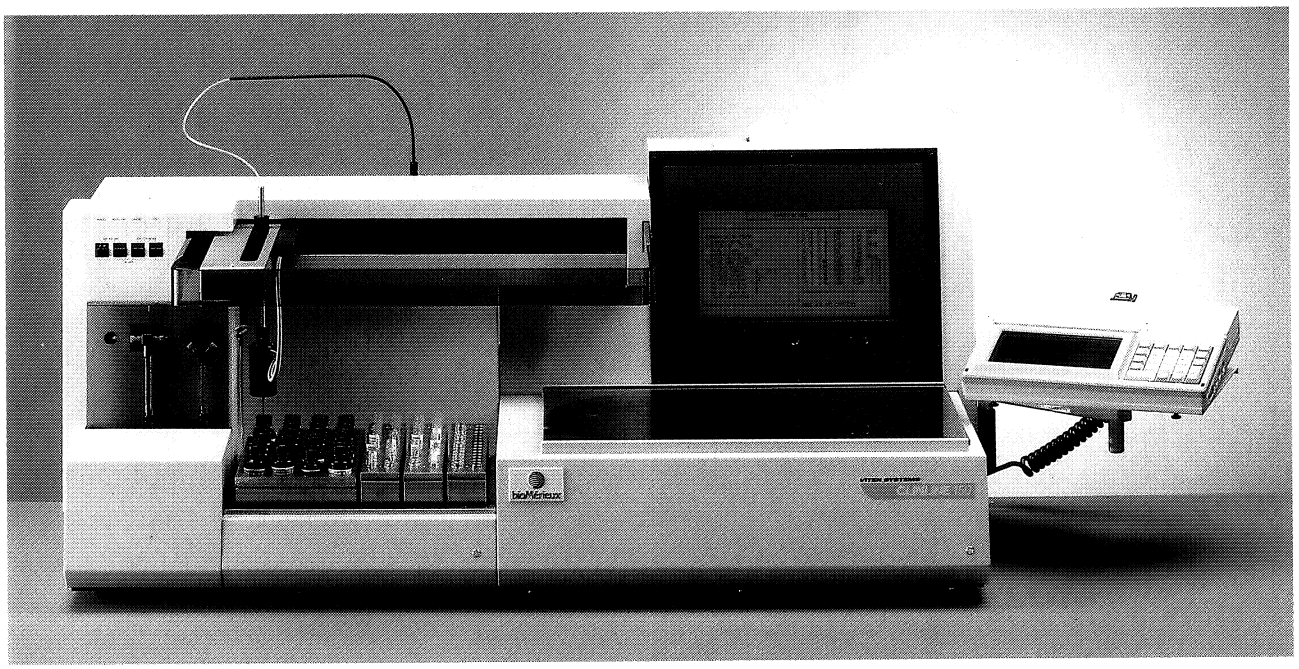
DISTRIBUIDOR PARA ESPAÑA:

FRANCISCO SORIA MELGUIZO, S.
Caramuel, 38 - 28011 MADRID
Telf.: 464 94 50 - 464 36 00 • Fax: 464

BIOMÉRIEUX: LOS SISTEMAS...

CLINLINE 150

La inteligencia al servicio del análisis

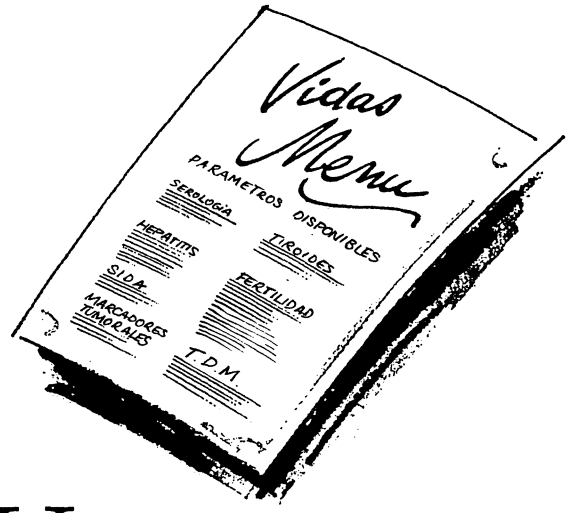


Aparato multiparamétrico selectivo de Química clínica

- Velocidad: 150 test/hora.
- 28 posiciones de reactivos.
- 2 menús de 24 tests.
- 60 posiciones de muestras (cubeta y tubo primario).
- Urgencias: pipeteo inmediato.
- Calibración: lineal, no lineal, factor fijo (hasta 10 calibradores).
- Visualización de curvas de reacción, calibración, densidades ópticas.
- Diluciones: prediluciones automáticas de muestras, dilución de los puntos de una gama de calibración.
- Termostatización: 30°C, 37°C, temperatura ambiente.
- Cubetas lavables en POLIMETILMETACRILATO.
- Software sencillo y traducido al castellano.
- Posibilidad de conexión bidireccional.

bioMérieux

69280 Marcy-l'Étoile / France / Tél. 78 87 20 00 / Fax 78 87 20 90
España: Manuel Tovar, 36 / 28034 Madrid / Tel. (91) 358 11 42 / Telex 46620
Otras filiales en Alemania, Australia, Austria, Bélgica, Brasil, Canadá, Chile, Hong Kong-China, Italia, Japón, México, Países Bajos, Polonia, Portugal, Reino Unido, Suiza, U.S.A.



UN MENU CAPAZ DE SATISFACER LOS GUSTOS MAS EXIGENTES

¡Pídanos la carta!

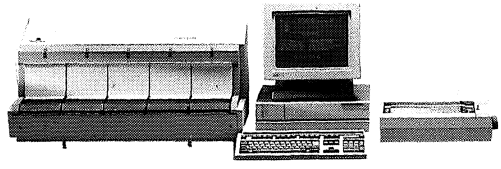
Un menú de reactivos de inmunoanálisis no se puede improvisar
30 años de experiencia han hecho posible para bioMérieux desarrollar un auténtico menú de "gourmet": Serología, Hepatitis, SIDA, Tiroides, Fertilidad, Marcadores tumorales, TDM, Detección de antígenos bacterianos y virales...

En nuestra "carta" encontrará más de 30 parámetros y lugar para muchos más.

VIDAS

La solución adaptada a cada laboratorio.

mini VIDAS



VIDAS

bioMérieux

bioMérieux España s.a. Manuel Tovar, 36 - 28034 MADRID
Tel. (91) 358 11 42 - Fax (91) 358 06 29

MICROBIOLOGÍA SEM

Publicación de la Sociedad Española de Microbiología

* See addresses in pp. 323–324.

[MICROBIOLOGÍA SEM 10 (3): 211–324 (1994)]

Dirección: Sociedad Española de Microbiología. Hortaleza, 104
28004 Madrid (España). Tel. (91) 308 23 22. Ext. 211.
Aparecen cuatro números al año (1994), que se integran en un volumen.
Precio de suscripción anual. Año 1994: España 8.000 ptas. (IVA incluido);
Europa 88 US $. Resto países 99 US $

IMPRIME: Graesal, Madrid.
DEPOSITO LEGAL: M-30455-1985.

EDITORIAL GARSI, S.A.
GRUPO MASSON

Edita: EDITORIAL GARSI, S. A. Juan Bravo, 46. 28006 Madrid.
Teléfono (91) 402 12 12. Avda. Príncipe de Asturias, 20.
08012 Barcelona. Teléfono (93) 415 45 44.

APP
ASOCIACION
DE PRENSA
PROFESIONAL

Editorial Office/Dirección editorial:
Microbiología SEM
Apartado 16009
08080 Barcelona
Tel . +34-3-4482373. Fax +34-3-3341079
E-mail: guerrero@porthos.bio.ub.es

Preparación y composición de originales:

Josep Julve
Eulàlia Massana

La revista *Microbiología SEM* y la Sociedad Española de Microbiología agradecen la ayuda recibida de distintas personas y centros de la **Universidad de Barcelona**.

[MICROBIOLOGÍA SEM 10 (3): 211–324 (1994)]

***Guidelines for Authors*:**
Information about the Journal, including guidelines on the preparation and submission of manuscripts, is published on pp. 321–322 of this issue, and may also be obtained from the Editorial Office.

ÍNDICE

Página

Editorial 215

Enzyme diversity in halophilic archaea. *Oren, A.* 217

Invasion and intracellular proliferation of *Salmonella* within non-phagocytic cells. *García-del Portillo, F., Finlay, B. B.* 229

Chitin synthetases in *Candida albicans*: a review on their subcellular distribution and biological function. *Martínez, J. P., Gozalbo, D.* 239

Virulence factors and O groups of *Escherichia coli* strains isolated from cultures of blood specimens from urosepsis and non-urosepsis patients. *Blanco, M., Blanco, J. E., Alonso, M. P., Blanco, J.* 249

Incidence of motile *Aeromonas* spp. in foods. *Pin, C. Marín, M. L., García, M. L., Tormo, J., Selgas, M. D., Casas, C.* 257

Different responses of the marine diatom *Phaeodactylum tricornutum* to copper toxicity. *Reiriz, S., Cid, A., Torres, E., Abalde, J., Herrero, C.* 263

Utilization of some phenolic compounds by *Azotobacter chroococcum* and their effect on growth and nitrogenase activity. *Abd-Alla, M. H.* 273

Otitis externas infecciosas: etiología en el área de Terrassa, métodos de cultivo y consideraciones sobre la otomicosis. *Bayó, M., Agut, M., Calvo, M. A.* 279

A direct membrane filter method for enumerating somatic coliphages in drinking water. *Alonso, M. C., Sánchez, J. M., Moriñigo, M. A., Borrego, J. J.* 285

Aplicación de métodos inmunológicos al análisis de bacterias biolixiviadoras. *Coto, O., Fernández, A. I., León, T., Rodríguez, D.* 297

Conservación de bacterias acidófilas. *Velasco, A., Lorenzo, P., de Silóniz, M. I., Perera, J.* 305

The Spanish Type Culture Collection of microorganisms (CECT). *Uruburu, F.* 311

Revisión de libros 315

Normas para los autores/Guidelines for authors 321

Direcciones de los miembros del Consejo Editorial/Editorial Board addresses 323

CONTENTS

Page

Editorial [In Spanish] 215

Enzyme diversity in halophilic archaea. *Oren, A.* 217

Invasion and intracellular proliferation of *Salmonella* within non-phagocytic cells. *García-del Portillo, F., Finlay, B. B.* 229

Chitin synthetases in *Candida albicans*: a review on their subcellular distribution and biological function. *Martínez, J. P., Gozalbo, D.* 239

Virulence factors and O groups of *Escherichia coli* strains isolated from cultures of blood specimens from urosepsis and non-urosepsis patients. *Blanco, M., Blanco, J. E., Alonso, M. P., Blanco, J.* 249

Incidence of motile *Aeromonas* spp. in foods. *Pin, C., Marín, M. L., García, M. L., Tormo, J., Selgas, M. D., Casas, C.* 257

Different responses of the marine diatom *Phaeodactylum tricornutum* to copper toxicity. *Reiriz, S., Cid, A., Torres, E., Abalde, J., Herrero, C.* 263

Utilization of some phenolic compounds by *Azotobacter chroococcum* and their effect on growth and nitrogenase activity. *Abd-Alla, M. H.* 273

Infectious otitis of the outer ear: etiology in the Terrassa area, culture methods and observations about otomycosis. *Bayó, M., Agut, M., Calvo, M. A.* [In Spanish] 279

A direct membrane filter method for enumerating somatic coliphages in drinking water. *Alonso, M. C., Sánchez, J. M., Moriñigo, M. A., Borrego, J. J.* 285

Use of immunogical methods in the analysis of bioleaching bacteria. *Coto, O., Fernández, A. I., León, T., Rodríguez, D.* [In Spanish] 297

Long-term storage of acidophilic bacteria. *Velasco, A., Lorenzo, P., de Silóniz, M. I., Perera, J.* [In Spanish] . 305

The Spanish Type Culture Collection of microorganisms (CECT). *Uruburu, F.* 311

Other sections [In Spanish] 315

MICROBIOLOGÍA SEM **10** (1994), 215–216

Editorial

El sistema de evaluación de trabajos científicos denominado "revisión paritaria" (en inglés "peer review", es decir, entre iguales), se aplica a la revisión de artículos (enviados para su publicación a revistas científicas) o a la evaluación de proyectos de investigación (entregados a los organismos correspondientes para su posible financiación). De gran tradición en países anglosajones, en España se viene aplicando desde hace pocos años y, tanto aquí como en el extranjero, el tema suscita a la vez interés y polémica. La experiencia española con la Agencia Nacional de Evaluación y Prospectiva (ANEP), en su aspecto de evaluación de proyectos, es considerada en conjunto muy positiva por una mayoría de investigadores. En lo referente a la evaluación de artículos en revistas nacionales la tradición es aún limitada. Es deseo de *Microbiología SEM* aplicar el sistema sin excepciones y, al mismo tiempo que se utiliza, tratar de evaluarlo y mejorarlo.

El Second International Congress on Peer Review in Biomedial Publication se celebró en Chicago del 9 al 11 de septiembre de 1993. Un número reciente de la revista *The Journal of the American Medical Association* (JAMA, vol. 272, núm. 2, 13 de julio de 1994) publica 24 de las contribuciones presentadas, más las dos conferencias invitadas (la de Jerome P. Kassirer y Edward W. Campion, editores del *New England Journal of Medicine*, y la de Horace L. Judson, autor, entre otros libros, de "El octavo día de la Creación"). A este congreso asistieron 275 editores, investigadores y otras personas interesadas en la publicación biomédica, procedentes de 20 países distintos. Este congreso tenía el objetivo genérico de impulsar la investigación en los aspectos que más preocupan sobre el proceso de la revisión paritaria, a la par que promover la investigación sobre lo que constituye uno de los más importantes mecanismos de control de la ciencia y establecer una base de datos para la continuación de esos estudios. Como objetivo último explícito se invocaba uno tan concreto, y ambiguo a la vez, como es el de "mejorar el proceso".

Este proceso de revisión paritaria suscita discusiones entre los investigadores, pero también los consejos editoriales de las publicaciones se plantean numerosos interrogantes. Las discusiones e interrogantes van generalmente dirigidos al perfeccionamiento del sistema, no a cuestionar su existencia. Por ejemplo, de cuántos revisores habría que disponer en cada evaluación, cuál sería el mejor modo de seleccionarlos, si hay que seguir manteniendo el anonimato ante los autores. Al margen de la celebración de los congresos indicados, estas cuestiones surgen en las reuniones de los consejos editoriales de todas las publicaciones científicas del mundo.

De todas las preguntas anteriores, la del anonimato de los revisores sigue siendo una de las más discutidas. Gracias a los estudios que se están desarrollando se puede concluir que el anonimato permite una mejor realización del trabajo en los ámbitos que por especializados son necesariamente reducidos, pero los datos son todavía insuficientes para poder llegar a una conclusión definitiva.

Lo que es innegable es que la revisión paritaria de artículos y proyectos es un proceso caro, que consume una gran cantidad de tiempo y que suele verse sometido a críticas si el resultado es desfavorable para los investigadores evaluados. Pero es un proceso necesario que tiene una incidencia directa para garantizar un mínimo de calidad en una época de recursos económicos (o espacio, en el caso de las revistas) limitados.

Parafraseando el título de uno de los trabajos presentados en el congreso citado, la revisión paritaria es un proceso "rudo, poco estudiado... pero indispensable". Y, por consiguiente, seguiremos preocupados y ocupados con él, en estas mismas páginas.

Ricard Guerrero
Director-Coordinador

MICROBIOLOGÍA SEM **10** (1994), 217–228 **MINIREVIEW**

Enzyme diversity in halophilic archaea

Aharon Oren

Division of Microbial and Molecular Ecology and the Moshe Shilo Center for Marine Biogeochemistry, The Institute of Life Sciences, The Hebrew University of Jerusalem, Israel

Received 15 June 1994/Accepted 15 July 1994

Summary

The halophilic archaea display a considerable extent of enzyme diversity. The presence or absence of certain enzymatic activities is closely linked with the taxonomic status of the strains investigated. Thus, *Halobacterium* species such as *Hb. salinarium*, *Hb. halobium*, and *Hb. cutirubrum* differ from most other Halobacteriaceae tested by the possession of an NAD^+-dependent glycerol dehydrogenase, by the absence of methylglyoxal synthase activity, and the ability of fermentative growth on arginine. Species such as *Hb. saccharovorum* and *Hb. sodomense*, which are still classified within the genus *Halobacterium*, have an enzymatic machinery greatly different from that of the *Hb. salinarium-Hb. halobium* group, confirming the need for a taxonomic reappraisal of these species. The presence of NAD^+-dependent D-lactate dehydrogenase is characteristic of representatives of the genus *Haloarcula*, which possess only low activities of NAD^+-independent L- and D-lactate dehydrogenases, if at all. Other enzymes which show considerable diversity are fructose 1,6-bisphosphate aldolase, of which two classes exist, and ribulose 1,6-bisphosphate carboxylase, which is present in a limited number of species.

Key words: halophilic archaea, *Halobacterium*, *Haloferax*, *Haloarcula*, enzyme diversity

Resumen

Las arqueas halófilas presentan una gran diversidad enzimática. La presencia o ausencia de ciertas actividades enzimáticas aparece estrechamente ligada a la posición taxonómica de las cepas investigadas. Así, especies de *Halobacterium* tales como *Hb. salinarium*, *Hb. halobium* y *Hb. cutirubrum* difieren

* *Correspondence to*: Aharon Oren. Division of Microbial and Molecular Ecology and the Moshe Shilo Center for Marine Biogeochemistry. The Institute of Life Sciences. The Hebrew University of Jerusalem. 91904 Jerusalem. Israel. Telephone: +972-2-584951. Fax: +972-2-528008

de la mayoría de las otras Halobacteriaceae estudiadas por presentar una glicerol deshidrogenasa dependiente de NAD^+, por carecer de una actividad metilglioxal sintasa, y por poder fermentar la arginina. Especies tales como *Hb. saccharovorum* y *Hb. sodomense*, que aún están clasificadas dentro del género *Halobacterium*, tienen una maquinaria enzimática muy diferente de la que presentan las bacterias del grupo *Hb. salinarium-Hb. halobium*, lo cual confirma la necesidad de un reajuste taxonómico de estas especies. La presencia de D-lactato deshidrogenasa dependiente de NAD^+ es característica de los representantes del género *Haloarcula*, que presenta una baja (o nula) actividad de las L- y D-lactato deshidrogenasas independientes de NAD^+. Otras enzimas que muestran una considerable diversidad son la fructosa 1,6-bisfosfato aldolasa, de las cuales existen dos clases, y la ribulosa 1,6-bisfosfato carboxilasa, que se encuentra en un número limitado de especies.

Introduction

The halophilic archaea (family Halobacteriaceae) are found worldwide in hypersaline environments with salt concentrations exceeding 200-250 g/l. Until recently, the halophilic archaea were considered a group of microorganisms with little metabolic versatility, growing mainly at the expense of amino acids. In recent years the number of recognized species within the Halobacteriaceae has increased considerably. The following genera are recognized at present: the neutrophilic *Halobacterium, Haloarcula, Haloferax,* and *Halococcus*, and the alkaliphilic *Natronobacterium* and *Natronococcus* (42). The classification into genera and species is based in part on such properties as salt and specific ionic requirements, and the nature of the glycolipids in the cell membrane (42, 44).

With the increase in the number of genera and species, the metabolic diversity known to occur within the group has increased considerably. The present classification reflects this metabolic diversity. Thus, properties such as the ability to grow on carbohydrates as the sole or principal carbon source, with or without acid production, the hydrolysis of starch, the production of indole from tryptophan, etc., are used in numerical taxonomic studies of the group, and form an important part of the species descriptions.

While the differences in properties of the species of the Halobacteriaceae are necessarily based on differences in their enzymatic machinery, little attention has been paid to the comparative enzymology of the halophilic archaea. On the other hand, comparative studies involving other archaeal groups as well have shown a considerable enzyme diversity within the archaeal domain. The properties of the archaeal enzymes and the metabolic pathways composed by them often differ greatly from their (eu)bacterial counterparts (9, 10).

The present paper reviews the current knowledge of the extent of enzyme diversity within the halophilic archaea, and the relationship between the taxonomic status of the isolates of the Halobacteriaceae and their enzymatic machinery.

Carbohydrate degradation and gluconeogenesis

While *Hb. salinarium* and related species (*Hb. halobium, Hb. cutirubrum*) use amino acids as their principal carbon and energy source, many other species display a well-developed ability to use

carbohydrates. Carbohydrates are used as the sole or principal carbon source by all members of the genera *Haloferax* and *Haloarcula*, and by a few *Halobacterium* species (e.g., *Hb. saccharovorum*).

Those halophilic archaea that utilize glucose, degrade the sugar via a modification of the Entner-Doudoroff pathway, in which oxidation precedes phosphorylation (glucose → gluconate → 2-keto-3-deoxygluconate → 2-keto-3-deoxygluconate-6-phosphate → pyruvate + glyceraldehyde 3-phosphate) (9, 10, 36, 43). The glucose dehydrogenase (EC 1.1.1.47) of *Hb. saccharovorum* was reported to have a dual cofactor specificity: $NADP^+$ was found to be the most efficient electron acceptor, but cell extracts also reduced NAD^+ in the presence of glucose at a rate of 8% of that observed with $NADP^+$ (43).

Nicotinamide nucleotide-dependent dehydrogenases of (eu)bacteria and eukarya are characteristically specific for either NAD^+ or $NADP^+$, but additional cases of dual cofactor specificity have been reported within the archaeal domain (9).

The Embden-Meyerhof glycolytic pathway is probably not operative in halophilic archaea growing on glucose, as 6-phosphofructokinase (EC 2.7.1.11) activity is lacking, though ^{13}C-NMR experiments with *Hb. halobium* indicated the existence of the glycolytic pathway in addition to the modified Entner-Doudoroff pathway (38). A modified Embden-Meyerhof pathway was shown to operate in *Ha. vallismortis* during growth on fructose. In this case, fructose does not enter the pathway by the usual phosphoenolpyruvate-dependent fructose phosphotransferase system, but is phosphorylated to fructose 1-phosphate in an ATP-dependent reaction, followed by conversion to fructose 1,6-bisphosphate by the action of ketohexokinase (EC 2.7.1.3) (3).

All enzymes required for a functional reversal of glycolysis (gluconeogenesis) were detected in *Hb. halobium*. Fructose 1,6-bisphosphate aldolase (EC 4.1.2.13), which participates in this pathway, is of special interest. The enzyme acts in gluconeogenesis, but may work in the opposite direction during growth of *Ha. vallismortis* on fructose (2). Two classes of fructose 1,6-bisphosphate aldolase are known. Class I enzymes, which are characteristically found in animals, higher plants, protozoa, and green algae, form a Schiff-base intermediate with the substrate. Borohydride treatment reduces the Schiff- base, resulting in the irreversible inactivation of the enzyme. Class II aldolases, found in yeast, other fungi, and in most bacteria, require divalent cations for activity, and are inhibited by EDTA. However, a few (eu)bacteria are known that have a class I aldolase.

Both class I and class II enzymes occur within the halophilic archaea, but only one class of fructose 1,6-bisphosphate aldolase was found in any one species. Presence of a class I enzyme was reported in *Halobacterium* strain R-113 (11, 13). Class I aldolases were further found in *Hb. saccharovorum* and in *Ha. vallismortis* (12, 23). Representatives of the *Hb. salinarium-Hb. halobium-Hb. cutirubrum* group possess a class II enzyme, and so do the *Haloferax* species tested (*Hf. volcanii, Hf. mediterranei*), which display a particularly high fructose 1,6-bisphosphate aldolase activity (Table 1).

Glycerol metabolism

Glycerol is produced in large quantities as an osmotic solute by algae of the genus *Dunaliella*, the main or only primary producers present in salt lakes in which halophilic archaea thrive. Glycerol is readily used as a carbon and energy source by most or all Halobacteriaceae. Many halophilic archaea, notably those belonging to the genera *Haloferax* and *Haloarcula*, produce acids when glycerol is added

TABLE 1
DISTRIBUTION OF ENZYMATIC ACTIVITIES[a] AMONG THE SPECIES OF THE HALOBACTERIACEAE

Strain	FPA	GDH	LDH	L-LDH	D-LDH	LR	MGS
Halobacterium							
Hb. salinarium	II	+	–	±	–	–	–
*Hb. halobium**	II	+					
*Hb. cutirubrum**	II	+	–	±	–		–
*Hb. trapanicum**							
Halobacterium							
Hb. saccharovorum	I	–	–	+	+	–	+
Hb. sodomense		–	–	+	+		
Hb. lacusprofundi							
Hb. distributum							
Haloferax							
Hf. volcanii	II	–	–	+	+	+	+
Hf. denitrificans		–	–	+	+		+
Hf. mediterranei	II	–	±	+	+	–	+
Hf. gibbonsii							
Haloarcula							
Ha. vallismortis	I	–	+	–	±	+	+
Ha. marismortui		–	+	±	±	+	+
Ha. hispanica							
Ha. japonica							
*Ha. californiae**							
*Ha. sinaiiensis**							
Halococcus							
Hc. morrhuae							
Hc. turkmenicus							
Hc. saccharolyticus							
Natronobacterium							
Nb. pharaonis							
Nb. magadii							
Nb. gregoryi							
Natronococcus							
Nc. occultus							

[a] Initials at the headings of columns: FPA, type of fructose 1, 6-bisphosphate aldolase (Class I or Class II, see text). GDH, glycerol dehydrogenase. LDH, NAD^+-dependent D-lactate dehydrogenase. L-LDH, NAD^+-independent L-lactate dehydrogenase. D-LDH, NAD^+-independent D-lactate dehydrogenase. LR, lactate racemase. MGS, methylglyoxal synthase.

*Species incertae sedis (42); ±, weak activity only.

to the growth media. Analysis by high performance liquid chromatography and enzymatic analyses of supernatants of *Haloferax* cultures grown in the presence of glycerol showed that all converted part of the glycerol to D-lactate and acetate, while cultures of two *Haloarcula* species tested produced pyruvate and acetate from glycerol (31).

Two pathways of glycerol metabolism are known in the prokaryotic world. One starts with the phosphorylation of glycerol by glycerol kinase (ATP:glycerol 3- phosphotransferase, EC 2.7.1.30), followed by dehydrogenation of the glycerol 3- phosphate. In the second pathway, glycerol is first oxidized to dihydroxyacetone by glycerol dehydrogenase (glycerol:NAD^+ 2-oxidoreductase, EC 1.1.1.6), whereafter phosphorylation occurs. Bacteria able to utilize glycerol as carbon and energy source may possess either or both pathways.

Activity of an NAD^+-dependent glycerol dehydrogenase was demonstrated in *Hb. salinarium* already 40 years ago (4). Comparative studies in which a variety of halophilic archaea was tested showed that glycerol dehydrogenase activity is restricted to the *Hb. salinarium - Hb. halobium - Hb. cutirubrum* group (32, 36). The activity was found to be constitutive, and addition of glycerol to the growth medium did not lead to increased glycerol dehydrogenase activities. No glycerol dehydrogenase could be detected in any of the *Haloferax* and *Haloarcula* species tested, nor in *Halobacterium* species such as *Hb. sodomense* and *Hb. saccharovorum*, which do not show a close relationship with the other members of the genus *Halobacterium*, and deserve a taxonomic reappraisal (42). Glycerol kinase activity was demonstrated in all strains tested, including those *Halobacterium* species that also have glycerol dehydrogenase (32, 36); it may be noted here that the levels of glycerol kinase activity in *Hf. mediterranei* and *Ha. vallismortis* reported by Oren and Gurevich (32) are about an order of magnitude higher than those measured by Rawal et al. (36).

Lactate metabolism

The presence of at least three different lactate dehydrogenases has been documented in the halophilic archaea, and also in this case the presence or absence of certain classes is correlated with the taxonomic position of the species examined (Table 1). An NAD^+-linked D-lactate dehydrogenase (EC 1.1.1.28) has been isolated from *Ha. marismortui* and characterized (19). A high rate of NADH-dependent pyruvate reduction was further shown in *Ha. vallismortis*, while *Hf. mediterranei* showed a much lower activity. Activities were found constitutive, and were not greatly altered by addition of lactate or glycerol, which gives rise to the formation of lactate in certain species (31). Other species tested did not show any NADH-dependent pyruvate reduction (Oren and Gurevich, Can. J. Microbiol., submitted for publication). A study of Rawal et al. (36) confirmed the presence of NAD^+-linked lactate dehydrogenase in *Ha. vallismortis* and in *Hf. mediterranei*. In addition, a low activity was detected also in *Hb. salinarium*. NAD^+-dependent activity in *Hb. salinarium* strain M1 was reported to be somewhat increased when the cells were grown in the presence of glucose (7). However, as no quantitative data were given, comparison with activities reported in other isolates is not possible.

Whereas the presence of NAD^+-linked lactate dehydrogenase is restricted mainly to *Haloarcula* species, NAD^+-independent lactate dehydrogenases, which in vitro reduce electron acceptors such as ferricyanide or dichlorophenol indophenol, are widespread in the Halobacteriaceae. The occurrence of

an NAD^+-independent lactate dehydrogenase in *Hb. salinarium* was first reported almost forty years ago (5). Tests for NAD^+- independent L-lactate dehydrogenase (EC 1.1.2.3) and D-lactate dehydrogenase (EC 1.1.2.4) showed that both activities were present in extracts of all nine species tested (Oren and Gurevich, Can. J. Microbiol., submitted for publication). However, L-lactate dehydrogenase activity in *Hb. salinarium* and *Hb. cutirubrum* was very low, and *Haloarcula* species, which possess a high activity of NAD^+-linked D-lactate dehydrogenase (19), showed very low activities, if at all, of both NAD^+-independent L- and D-lactate dehydrogenase. Addition of lactate to the growth medium did not greatly alter the activities.

Little is known on the function of the three lactate dehydrogenases in halophilic archaea. The equilibrium of the NAD^+-linked reaction greatly favors formation of lactate from pyruvate and NADH. However, *Ha. marismortui* and *Ha. vallismortis*, being the only species which displayed a high activity of the NAD^+-linked enzyme, produced pyruvate rather than lactate when grown in the presence of glycerol or sugars (31). Moreover, in spite of the constitutive presence of an NAD^+-independent L-lactate dehydrogenase in most strains tested, L-lactate added to the medium was not used by most species, unless lactate racemase was present as well.

An inducible lactate racemase (EC 5.1.2.1) with an unusually high pH optimum was detected in *Hf. volcanii*. Both *Haloarcula* species tested also showed lactate racemase activity, whether or not lactate was present in the growth medium. No activity could be demonstrated in *Hb. salinarium, Hb. saccharovorum,* and *Hf. mediterranei* (Oren and Gurevich, Can. J. Microbiol., submitted for publication). An additional pathway that may contribute to the formation of D-lactate in halophilic archaea is the methylglyoxal bypass. In this pathway, methylglyoxal is formed from dihydroxyacetone phosphate by methylglyoxal synthase (EC 4.2.99.11), whereafter glyoxalase I (EC 4.4.1.5) produces the D-lactoyl derivative of glutathione, or in the case of the halophilic archaea, γ-glutamylcysteine. While activity of glyoxalase I was found in all halophilic archaea tested, the distribution of methylglyoxal synthase was species-dependent: no activity was found in *Hb. salinarium* and *Hb. cutirubrum*, while all other strains tested, including *Hb. saccharovorum*, contained the enzyme (Oren and Gurevich, submitted). The activity of *Hf. volcanii* methylglyoxal synthase was highest in the absence of salt, and the presence of 3 M NaCl or KCl caused about 50% inhibition. Such a behavior is highly unusual for enzymes of halophilic archaea, most of which function optimally in the presence of 3–4 M salt.

Ribulose 1,6-bisphosphate carboxylase

Halophilic archaea grow heterotrophically at the expense of amino acids, carbohydrates, and other organic compounds. Therefore the discovery of ribulose 1,6- bisphosphate carboxylase/oxygenase (RuBisCo, EC 4.1.1.39) activity in part of the members of the Halobacteriaceae came as a surprise (37). Not all halophilic archaea possess the enzyme, and those that do have activities which are at best only a few percent of those typically found in autotrophic bacteria.

Studies on the distribution of RuBisCo in halophilic archaea showed that the activity is restricted to a few carbohydrate utilizing species. Relatively high activities were detected in *Ha. mediterranei* and *Hf. volcanii*, while *Ha. marismortui* extracts displayed a much lower activity. NADH, and not NADPH, was the reductant required. No activity of RuBisCo could be demonstrated in *Ha. vallismortis, Hb. halobium, Hb. salinarium, Hb. cutirubrum* and *Hb. saccharovorum* (1, 35, 36, 37). A possible correlation

has been suggested between the RuBisCo content of halophilic archaea and their ability to accumulate poly-ß-hydroxybutyrate (PHB) as storage material (1). Thus, *Hf. mediterranei* may have an extremely high PHB content under certain growth conditions (17), and the presence of PHB has been documented in *Ha. marismortui* (22). Only low quantities of PHB were found in *Ha. vallismortis*, and those strains that did not show RuBisCo activity also did not accumulate PHB (1). No explanation for the apparent correlation between RuBisCo activity and PHB accumulation has been brought forward as yet.

Though an organism such as *Hf. mediterranei* possesses not only a relatively high RuBisCo activity, but also all other enzymes required for the operation of the reductive pentose phosphate cycle, autotrophic growth has never yet been demonstrated in the halophilic archaea. The combination of the presence of the light-driven proton pump bacteriorhodopsin with the presence of the enzymes of the Calvin cycle may theoretically enable photoautotrophic growth (10), but RuBisCo has not yet been found in strains containing bacteriorhodopsin. Thus, the physiological role of RuBisCo activity in halophilic archaea is still unknown.

Hydrocarbon degradation

Until recently, halophilic archaea were known to grow only on amino acids, carbohydrates, and related compounds as carbon and energy source. However, two studies showed that certain isolates may also be able to degrade different long-chain hydrocarbons. From a saltern in the south of France a pleomorphic *Halobacterium* or related organism was isolated that grows on saturated hydrocarbons such as tetradecane, hexadecane, eicosane, heneicosane, and pristane, certain unsaturated hydrocarbons, and even aromatic hydrocarbons such as phenantrene and anthracene (6). Screening of other isolates revealed six strains, resembling *Hb. salinarium*, *Hf. volcanii*, and *Hb. distributum*, that emulsified petroleum and grew on petroleum hydrocarbons. Hardly or no petroleum degradation was detected in *Hb. halobium*, *Hb. saccharovorum*, *Hf. mediterranei*, and *Halococcus turkmenicus* (24). A more extensive ability to degrade aromatic compounds was found in a recently isolated *Haloferax* strain (16).

The elucidation of the enzymatic basis of hydrocarbon and aromatic compound degradation, and a systematic survey of the presence of the enzymes involved among the Halobacteriaceae, may add another interesting aspect to enzyme diversity in the halophilic archaea.

Alternative electron acceptors in respiration

While the halophilic archaea basically lead an aerobic way of life, at least four alternative electron acceptors have been shown to replace oxygen in the respiration of different representatives of the group: nitrate, dimethylsulfoxide (DMSO), trimethylamine N-oxide (TMAO), and fumarate (Table 2).

Anaerobic growth with nitrate as electron acceptor, with the formation of the gaseous products dinitrogen and/or nitrous oxide (denitrification), has been demonstrated in all *Haloarcula* species tested: *Ha. vallismortis*, *Ha. marismortui*, *Ha. hispanica*, and *Ha. japonica* (42), and in two *Haloferax* species: *Hf. denitrificans* and *Hf. mediterranei* (27, 42). Additional species, e.g. *Hb. sodomense*, *Hb. distributum*, *Hf. volcanii*, and the alkaliphile *Natronobacterium pharaonis*, slowly reduce nitrate to nitrite without gas formation (42), while others, such as *Hf. gibbonsii*, do not reduce nitrate. The differences in behavior

TABLE 2
DISTRIBUTION OF MODES OF ANAEROBIC METABOLISM[a] AND RETINAL PIGMENTS[b] AMONG THE SPECIES OF THE HALOBACTERIACEAE

Strain	N_2	NO_2^-	DMSO	TMAO	Fum.	Arg.	Brhod.	Hrhod.
Halobacterium								
Hb. salinarium	–	–	+	±	–	+	+	
*Hb. halobium**	–	–	+	+	+	+	+	+
*Hb. cutirubrum**	–	–	+	+	+	+	+	
*Hb. trapanicum**								
Halobacterium								
Hb. saccharovorum	–	+	–	–		–	–	
Hb. sodomense	–	+				–	+	
Hb. lacusprofundi	–	+					–	
Hb. distributum	–	+						
Haloferax								
Hf. volcanii	–	+	+	±	+	–	–	
Hf. denitrificans	+	+	+	–	+	–	–	
Hf. mediterranei	+	+	+	+	–	–	–	
Hf. gibbonsii	–	–	–	–	–	–	–	
Haloarcula								
Ha. vallismortis	+	+	+	+	–	–	–	
Ha. marismortui	+	+	+	+	–	–	–	
Ha. hispanica	+	+						
Ha. japonica	+	+						
*Ha. californiae**		+					±	±
*Ha. sinaiiensis**							±	±
Halococcus								
Hc. morrhuae	–	+						
Hc. turkmenicus	–	+						
Hc. saccharolyticus	–	+						
Natronobacterium								
Nb. pharaonis	–	±	–	±		+		+
Nb. magadii	–	–						
Nb. gregoryi	–	–						
Natronococcus								
Nc. occultus	–	+						

[a] Initials at the headings of columns: N_2 nitrate to gas. NO_2^- reduction of nitrate to nitrite. DMSO, dimethylsulfoxide. TMAO, trimethylamine N-oxide. Fum., fumarate. Arg., arginine fermentation. (See text.)
[b] Pigments: Brhod., bacteriorhodopsin. Hrhod., halorhodopsin.
*Species incertae sedis (42); ±, weak activity only.

towards nitrate reflect the presence or absence of dissimilatory nitrate reductase and nitrite reductase, thus adding to the metabolic and enzyme diversity within the Halobacteriaceae.

DMSO, TMAO, and fumarate can be used as alternative electron acceptors by many halophilic archaea, and they are reduced to dimethylsulfide, trimethylamine, and succinate, respectively (30, 33). In most cases DMSO, TMAO or fumarate reduction enabled growth in the absence of molecular oxygen. No correlation was found between the ability to reduce nitrate to nitrite or to gaseous products, and the ability to reduce DMSO or TMAO (33).

Arginine fermentation

Hb. halobium, Hb. salinarium, and *Hb. cutirubrum* were reported to grow anaerobically in the dark when the growth medium was supplemented with L-arginine. Under these conditions energy is generated by fermentation of arginine via citrulline to ornithine and carbamoylphosphate, which is subsequently degraded to carbon dioxide and ammonia with the phosphorylation of ADP to ATP (18). The key enzyme ornithine carbomoyltransferase (EC 2.1.3.3) has been isolated from *Hb. salinarium* (14). Tests for anaerobic growth on arginine performed with a variety of Halobacteriaceae (Oren and Gurevich, unpublished results, see Table 1) confirmed the ability of arginine fermentation in above strains. The alkaliphilic *Na. pharaonis* was also found able to grow anaerobically when arginine was added to the medium, but no anaerobic growth with arginine was found in any of the other neutrophilic species tested.

Bacteriorhodopsin and other retinal pigments

The great majority of studies performed with halophilic archaea since the early 1970's dealt with the properties of bacteriorhodopsin and other retinal pigments (halorhodopsin, sensory rhodopsins) of *Hb. halobium*. These pigments enable *Hb. halobium* to use light energy for the maintenance of proton and chloride gradients over the cell membrane, and to direct the cells to optimal light conditions. Cells containing bacteriorhodopsin were even shown to grow photoheterotrophically under anaerobic conditions (18). However, it is often not realized that the possession of these retinal pigments is not a universal feature of the Halobacteriaceae, but may be the exception rather than the rule (Table 2). Most species are colored red-orange due to the presence of carotenoids (α-bacterioruberin and derivatives), and bacteriorhodopsin has not been detected in most recognized species of halophilic archaea.

The occurrence of purple membrane containing bacteriorhodopsin has thus far been documented only in *Hb. halobium*, the closely related *Hb. cutirubrum* (25), and in *Hb. sodomense* (29). In a number of additional *Halobacterium* isolates, whose taxonomic position was not elucidated, retinal-based proton pumps similar to the bacteriorhodopsin of *Hb. halobium* were found, but differing in the primary structure of the protein moiety (28, 39). The bacteriorhodopsin-like pigment from such a *Halobacterium* isolate from Australia has been designated archaerhodopsin (28, 41). In a study of novel isolates from Guerrero Negro, Mexico, and Shark Bay, Australia, 5 strains were found that contain bacteriorhodopsin genes which differ from those of *Hb. halobium*, as ascertained by DNA hybridization studies (34). The *Haloarcula* strains *Ha. californiae* and *Ha. sinaiiensis* (both species incertae sedis) (42) were reported to contain both bacteriorhodopsin and halorhodopsin (21, 40). The bacteriorhodopsin content of *Ha.*

californiae was only 2–5% of that of *Hb. halobium* grown under similar conditions (21). In most other species of the Halobacteriaceae bacteriorhodopsin seems to be absent, but systematic studies of the occurrence of the pigment are lacking.

Little information is available on the distribution of halorhodopsin and sensory rhodopsins. Halorhodopsin-like molecules were detected only in part of those strains from Guerrero Negro and Shark Bay that contained bacteriorhodopsin (34). Sensory rhodopsins are not expected to occur in those species that lack motility. Except in *Hb. halobium*, the presence of a phoborhodopsin has been ascertained in certain haloalkaliphilic bacteria (8), and the phoborhodopsin of *Na. pharaonis* has been studied in depth, both with respect to structure (26) and function (15, 20). A sensory rhodopsin was found also in *Ha. sinaiiensis* (40), and in *Halobacterium* SG1, a strain whose taxonomic affiliation has not yet been ascertained (39).

Conclusions

Comparative enzymatic studies show a considerable extent of enzyme diversity within the Halobacteriaceae (Table 1, 2). Not all species have yet been tested for all enzymes mentioned, and particularly few biochemical data are available on the halococci and the alkaliphiles. Furthermore, not all strains used in biochemical studies have been characterized taxonomically, and in addition considerable confusion exists on the identity of certain strains which have been assigned to a species, but may differ greatly from the type strain of that species (42). However, sufficient data have been collected to support a number of conclusions.

A close correlation can be observed between the enzymatic data and the taxonomic position of the species, as determined by polar lipid composition and numerical taxonomy (42, 44). Thus, *Halobacterium* species such as *Hb. salinarium*, *Hb. halobium*, and *Hb. cutirubrum*, differ from the other members of the Halobacteriaceae by the presence of an NAD^+-dependent glycerol dehydrogenase, the absence of methylglyoxal synthase, and the ability to grow anaerobically on arginine. Species such as *Hb. saccharovorum* and *Hb. sodomense*, which at present are still classified within the genus *Halobacterium*, have an enzymatic machinery greatly different from that of the *Hb. halobium-Hb. salinarium* group, confirming the need for the removal of these species from the genus *Halobacterium* (42). The presence of NAD^+-linked D-lactate dehydrogenase is characteristic of representatives of the genus *Haloarcula*, which in their turn show very low activities of NAD^+-independent D- and L-lactate dehydrogenases, if at all. Another enzyme displaying considerable diversity is fructose 1,6-bisphosphate aldolase, with any species investigated containing either the class I or the class II enzyme. The correlation of the taxonomic position of the species with the presence or absence of retinal pigments, RuBisCo, and the ability to use different alternative electron acceptors, is less obvious. Comparative studies on other enzymes may supplement our picture on enzyme diversity in the halophilic archaea, and these may also aid in the elucidation of the taxonomic and evolutionary relationships within the group.

Acknowledgments

My recent research on enzyme diversity in halophilic archaea was supported by grants from the Ministry of Science and Culture, Land Niedersachsen, and the Israel Science Foundation administered by the Israel Academy of Sciences and Humanities.

References

1. Altekar, W., Rajagopalan, R. (1990). Ribulose bisphosphate carboxylase activity in halophilic Archaebacteria. Arch. Microbiol. **153**, 169–174.
2. Altekar, W., Rangaswamy, V. (1990). Indication of a modified EMP pathway for fructose breakdown in a halophilic archaebacterium. FEMS Microbiol. Lett. **69**, 139–144.
3. Altekar, W., Rangaswamy, V. (1991). Ketokinase (ATP:D-fructose 1-phosphotransferase) initiates fructose breakdown via a modified EMP pathway in halophilic archaebacteria. FEMS Microbiol. Lett. **83**, 241–246.
4. Baxter, R. M., Gibbons, N. E. (1954). The glycerol dehydrogenases of *Pseudomonas salinaria*, *Vibrio costicola*, and *Escherichia coli* in relation to bacterial halophilism. Can. J. Biochem. Physiol. **32**, 206–217.
5. Baxter, R. M., Gibbons, N. E. (1956). Effects of sodium and potassium chloride on certain enzymes of *Micrococcus halodenitrificans* and *Pseudomonas salinaria*. Can. J. Microbiol. **2**, 599–606.
6. Bertrand, J. C., Almallah, M., Acquaviva, M., Mille, G. (1990). Biodegradation of hydrocarbons by an extremely halophilic archaebacterium. Lett. Appl. Microbiol. **11**, 260–263.
7. Bhaumik, S. R., Sonawat, H. M. (1994). Pyruvate metabolism in *Halobacterium salinarium* studied by intracellular ^{13}C nuclear magnetic spectroscopy. J. Bacteriol. **176**, 2172–2176.
8. Bivin, D. B., Stoeckenius, W. (1986). Photoactive retinal pigments in the haloalkaliphilic bacteria. J. Gen. Microbiol. **132**, 2167–2177.
9. Danson, M. J. (1988). Archaebacteria: the comparative enzymology of their central metabolic pathways. Adv. Microb. Physiol. **29**, 165–231.
10. Danson, M. J., Hough, D. W. (1992). The enzymology of archaebacterial pathways of central metabolism. *In* Danson, M. J., Hough, D. W., Lunt, G. G. (ed.), The Archaebacteria: Biochemistry and Biotechnology, pp. 7–21. Portland Press, London.
11. Dhar, N. M., Altekar, W. (1986). A class I (Schiff base) fructose-1,6-bisphosphate aldolase of halophilic archaebacterial origin. FEBS Lett. **199**, 151–154.
12. Dhar, N. M., Altekar, W. (1986). Distribution of class I and class II fructose bisphosphate aldolases in halophilic archaebacteria. FEMS Microbiol. Lett. **35**, 177–181.
13. D'Souza, S. E., Altekar, W. (1983). A halophilic fructose 1,6-biphosphate aldolase from *Halobacterium halobium*. Indian J. Biochem. Biophys. **19**, 135–138.
14. Dundas, I. E. D. (1970). Purification of ornithine carbamoyltransferase from *Halobacterium salinarium*. Eur. J. Biochem. **16**, 393–398.
15. Duschl, A., Lanyi, J. K., Zimanyi, L. (1990). Properties and photochemistry of a rhodopsin from the haloalkalophile, *Natronobacterium pharaonis*. J. Biol. Chem. **265**, 1261–1267.
16. Emerson, D., Chauhan, S., Oriel, P., Breznak, J. A. (1994). *Haloferax* sp. D1227, a halophilic archaeon capable of growth on aromatic compounds. Arch. Microbiol. **161**, 445–452.
17. Fernandez-Castillo, R., Rodriguez-Valera, F., Gonzalez-Ramos, J., Ruiz-Berraquero, F. (1986). Accumulation of poly(ß-hydroxybutyrate) by halobacteria. Appl. Environ. Microbiol. **51**, 214–216.
18. Hartmann, R., Sickinger, H.-D., Oesterhelt, D. (1980). Anaerobic growth of halobacteria. Proc. Natl. Acad. Sci. USA **77**, 3821–3825.
19. Hecht, K., Langer, T., Wrba, A., Jaenicke, R. (1990). Lactate dehydrogenase from the extreme halophilic archaebacterium *Halobacterium marismortui*. Biol. Chem. Hoppe-Seyler **371**, 515–519.
20. Hirayama. J., Imamoto, Y., Shichida, Y., Kamo, N., Tomioka, H., Yoshizawa, T. (1992). Photocycle of phoborhodopsin from haloalkaliphilic bacterium (*Natronobacterium pharaonis*) studied by low-temperature spectroscopy. Biochemistry **31**, 2093–2098.
21. Javor, B., Requadt, C., Stoeckenius, W. (1982). Box-shaped halophilic bacteria. J. Bacteriol. **151**, 1532–1542.
22. Kirk, R. G., Ginzburg, M. (1972). Ultrastructure of two species of *Halobacterium*. J. Ultrastr. Res. **41**, 80–94.
23. Krishnan, G., Altekar, W. (1991). An unusual class I (Schiff base) fructose-1,6-bisphosphate aldolase from the halophilic archaebacterium *Haloarcula vallismortis*. Eur. J. Biochem. **195**, 343–350.

24. Kulichevskaya, I. S., Milekhina, E. I., Borzenkov, I. A., Zvyagintseva, I. S., Belyaev, S. S. (1992). Oxidation of petroleum hydrocarbons by extremely halophilic archaebacteria. Microbiologiya (English translation) **60**, 596–601.
25. Kushwaha, S. C., Kates, M., Stoeckenius, W. (1976). Comparison of purple membrane from *Halobacterium cutirubrum* and *Halobacterium halobium*. Biochim. Biophys. Acta **426**, 703–710.
26. Lanyi, J. K., Duschl, A., Hatfield, G. W., May, K., Oesterhelt, D. (1990). The primary structure of a halorhodopsin from *Natronobacterium pharaonis*. J. Biol. Chem. **265**, 1253–1260.
27. Mancinelli, R. L., Hochstein, L. I. (1986). The occurrence of denitrification in extremely halophilic bacteria. FEMS Microbiol. Lett. **35**, 55–58.
28. Mukohata, Y., Sugiyama, Y., Ihara, K., Yoshida, M. (1988). An Australian halobacterium contains a novel proton pump retinal protein: archaerhodopsin. Biochem. Biophys. Res. Commun. **151**, 1339–1345.
29. Oren, A. (1983). *Halobacterium sodomense* sp. nov., a Dead Sea halobacterium with an extremely high magnesium requirement. Int. J. Syst. Bacteriol. **33**, 381–386.
30. Oren, A. (1991). Anaerobic growth of halophilic archaeobacteria by reduction of fumarate. J. Gen. Microbiol. **137**, 1387–1390.
31. Oren, A., Gurevich, P. (1994). Production of D-lactate, acetate, and pyruvate from glycerol in communities of halophilic archaea in the Dead Sea and in saltern crystallizer ponds. FEMS Microbiol. Ecol. **14**, 147–156.
32. Oren, A., Gurevich, P. (1994). Distribution of glycerol dehydrogenase and glycerol kinase activity in halophilic archaea. FEMS Microbiol. Lett. **118**, 311–316.
33. Oren, A., Trüper, H. G. (1990). Anaerobic growth of halophilic archaeobacteria by reduction of dimethylsulfoxide and trimethylamine N-oxide. FEMS Microbiol. Lett.**70**, 33–36.
34. Otomo, J., Tomioka, H., Sasabe, H. (1992). Bacterial rhodopsins of newly isolated halobacteria. J. Gen. Microbiol. **138**, 1027–1037.
35. Rajagopalan, R., Altekar, W. (1991). Products of non-reductive CO_2 assimilation in the halophilic archaebacterium *Haloferax mediterranei*. Indian J. Biochem. Biophys. **28**, 65–67.
36. Rawal, N., Kelkar, S. M., Altekar, W. (1988). Alternative routes of carbohydrate metabolism in halophilic archaebacteria. Indian J. Biochem. Biophys. **25**, 674–686.
37. Rawal, N., Kelkar, S. M., Altekar, W. (1988). Ribulose 1,5-bisphosphate dependent CO_2 fixation in the halophilic archaebacterium, *Halobacterium mediterranei*. Biochem. Biophys. Res. Commun. **156**, 451–456.
38. Sonawat, H. M., Srivastava, S., Swaminathan, S., Govil, G. (1990). Glycolysis and Entner-Doudoroff pathways in *Halobacterium halobium*: some new observations based on ^{13}C NMR spectroscopy. Biochem. Biophys. Res. Commun. **173**, 358–362.
39. Soppa, J., Duschl, J., Oesterhelt, D. (1993). Bacterioopsin, haloopsin, and sensory opsin I of the halobacterial isolate *Halobacterium* sp. strain SG1: three new members of a growing family. J. Bacteriol. **175**, 2720–2726.
40. Stoeckenius, W., Bivin, D., McGinnis, C. (1982). Photoactive pigments in halobacteria from the Gavish sabkha. *In* Friedman, G. M., Krumbein, W. E. (ed.), Hypersaline Ecosystems. The Gavish Sabka, pp. 288–295. Springer-Verlag, Berlin.
41. Sugiyama, Y., Maeda, M., Futai, M., Mukohata, Y. (1989). Isolation of a gene that encodes a new retinal protein, archaerhodopsin, from *Halobacterium* sp. aus-1. J.Biol. Chem. **264**, 20859–20862.
42. Tindall, B. J. (1992). The family Halobacteriaceae. *In* Balows, A., Trüper, H. G., Dworkin, M., Harder, W., Schleifer, K.-H. (ed.), The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications, pp. 768–808. Springer-Verlag, New York.
43. Tomlinson, G. A., Koch, T. K., Hochstein, L. I. (1974). The metabolism of carbohydrates by extremely halophilic bacteria: glucose metabolism via a modified Entner-Doudoroff pathway. Can. J. Microbiol. **20**, 1085–1091.
44. Torreblanca, M., Rodriguez-Valera, F., Juez, G., Ventosa, A., Kamekura, M., Kates, M. (1976). Classification of non-alkaliphilic halobacteria based on numerical taxonomy and polar lipid composition, and description of *Haloarcula* gen. nov., and *Haloferax* gen. nov. Syst. Appl. Microbiol. **8**, 89–99.

MICROBIOLOGÍA SEM **10** (1994), 229–238 **MINIREVIEW**

Invasion and intracellular proliferation of *Salmonella* within non-phagocytic cells

Francisco García-del Portillo,* B. Brett Finlay

Biotechnology Laboratory and the Departments of Microbiology and Biochemistry, University of British Columbia, Canada

Received 18 January 1994/Accepted 16 May 1994

Summary

Salmonella species penetrate (invade) and proliferate within non-phagocytic cells such as epithelial cells. These two processes are essential for *Salmonella* virulence and have been shown to occur in the intestinal epithelium and cultured epithelial cells. In recent years the signals that *Salmonella* transmits to the epithelial cell have begun to be elucidated. In this review, we summarize the most recent findings about the molecular nature of the interactions between *Salmonella* and epithelial cells. These studies reveal that *Salmonella* causes dramatic changes in the morphology of the host plasma membrane during bacterial invasion, and that this pathogen exploits other host structures such as actin filaments and lysosomes to trigger internalization and intracellular proliferation within non-phagocytic cells.

Key words: *Salmonella*, intracellular bacteria, cell invasion, vacuole, epithelial cell

Resumen

Las especies del género *Salmonella* invaden células no fagocíticas, tales como las células epiteliales, y proliferan en el interior. Estos dos procesos son necesarios para desarrollar virulencia y se ha demostrado que tienen lugar en el epitelio intestinal y en células epiteliales crecidas en cultivo. En los últimos años se han empezado a conocer las señales que *Salmonella* transmite a la célula epitelial. En esta revisión se resumen los datos más recientes relativos a la biología molecular de la interacción de *Salmonella* con células epiteliales. Estos datos han demostrado que *Salmonella* induce cambios notables

* *Correspondence to*: Francisco García-del Portillo. Centro de Biología Molecular. Universidad Autónoma de Madrid. 28049 Cantoblanco (Madrid). Tel.: 91-3978083. Fax: 91-3978344.

en la morfología de la membrana citoplásmica de la célula huésped durante la invasión bacteriana. Este patógeno utiliza también otras estructuras de la célula huésped, como filamentos de actina y lisosomas, para inducir la internalización y proliferación intracelular en células no fagocíticas.

Introduction

Salmonellosis includes several clinical manifestations such as gastroenteritis (food poisoning), enteric fever, bacteremia, and a carrier state (24). Although all serotypes of *Salmonella* can cause these different types of salmonellosis, certain clinical symptoms are related to particular *Salmonella* species or serotypes (24). Gastroenteritis is predominantly caused by the *S. enteriditis* serotypes *enteriditis* (known as *S. enteriditis*, with higher incidence in Europe) and *typhimurium* (known as *S. typhimurium*, with more cases in North America). Enteric fever is usually associated with *S. typhi* and bacteremia with *S. choleraesuis*.

Nearly all *Salmonella* infections occur from oral ingestion of bacteria, followed by penetration of the mucosa at the distal ileum of the small intestine and the proximal large bowel. At this stage, bacteria penetrate the intestinal mucosa and reach the mesenteric lymph follicles, where they proliferate. Most infections do not proceed beyond the local lymph nodes. More invasive species such as *S. typhi* and *S. choleraesuis* spread to deeper tissues, causing bacteremia which leads to infection and intracellular proliferation within cells of the liver and spleen. Therefore, in at least two stages during the course of infection, *Salmonella* interacts with non-phagocytic cells: (i) during penetration of the intestinal epithelium and (ii) during intracellular proliferation within cells of the liver and spleen. It is conceivable that *Salmonella* may proliferate intracellularly in the cells of the intestinal epithelium. Although not discussed in this review, *Salmonella* interacts with phagocytic cells such as macrophages underlying the intestinal mucosa. The survival of *Salmonella* within phagocytic cells is also essential for virulence. This aspect of the *Salmonella* pathogenesis has been recently reviewed (6).

The molecular biology of the interaction between *Salmonella* and epithelial cells has been characterized using several methods, including infection of cultured epithelial cells and specific genetic selection procedures (9). It is generally accepted that the interaction of *Salmonella* with non-phagocytic cells involves bacteria internalization into the host cell, a process triggered by the infecting organism and formally known as *invasion*, which is followed by the intracellular bacterial proliferation process (9). Intracellular bacterial growth takes place in an expanding vacuole that occupies the entire pre-existing cytoplasm (9, 10, 11). Invasion of the epithelial cell is an active process that requires bacterial protein synthesis and an intact actin filament network supplied by the host cell (7, 38). Mutants belonging to different *Salmonella* serotypes with defects in invasion of epithelial cells have been isolated, and the so-called "invasion genes" characterized (reviewed in 6). Host plasma membrane receptors are elements of the host system used by *Salmonella* to trigger bacterial uptake (3, 6), and the current data suggest that the invasion machinery used by *Salmonella* to invade epithelial cells could be part of a more common mechanism used by other invasive bacteria (25).

The invasion process

Morphological description. In 1967, Takeuchi (42) provided a detailed morphological description of *S. typhimurium* interacting with and invading guinea pig ileal intestinal epithelial cells. The alterations in the morphology of the host cell were summarized as follows: when bacteria come into close proximity to the epithelial

cell surface, microvilli in the immediate vicinity begin to degenerate. Evident protrusion or swellings containing large amounts of cytoplasm are observed in this area, and bacteria are internalized in membrane bound vacuoles, often surrounded by the cytoplasmic protrusion. A localized and prominent endocytic activity is also observed in this area. A characteristic of *Salmonella* invasion is that initially each organism is internalized within an individual vacuole, although at later times these vacuoles may fuse. *Salmonella* reside within vacuoles during the entire intracellular stage, in contrast to other invasive bacteria such as *Shigella* spp. and *Listeria monocytogenes* which lyse the bacteria-containing vacuole to gain access to the cytoplasm of the host cell. The description made by Takeuchi was considered representative of the *Salmonella* infection of non-phagocytic cells and has been confirmed by several other groups (reviewed in 6).

In recent years, diverse in vitro models have been used to study the internalization of invasive bacteria into non-phagocytic cells. Certain cell lines (of canine kidney and of intestinal human origin) form polarized epithelial monolayers, in which cells have well developed microvilli, tight junctions and defined apical and basolateral domains, mimicking a columnar epithelial cell barrier. When *S. typhimurium* and *S. choleraesuis* were used to infect these cells as a model for intestinal penetration, identical morphological alterations were observed as those reported by Takeuchi in the infected intact intestinal epithelium (10, 11). Therefore, this model is considered extremely valid to examine the molecular biology of the invasion process of *Salmonella*. The same alterations in the host cell were described with adherent, non-polarized epithelial cells (15, 43), showing the efficiency of the entire invasion process induced by *Salmonella*, which occurs within minutes (15).

Bacterial factors required for invasion. Two main genetic strategies have been used to select and identify bacterial loci involved in invasiveness. They include the screening for an invasive phenotype in a normally non-invasive bacterium (i.e., *Escherichia coli* K 12) after transferring DNA from an invasive bacterium. This method is easy to test, but requires that the invasive phenotype be encoded by a single gene or localized genes. This selection procedure permitted the identification and characterization of the *inv* gene from *Yersinia pseudotuberculosis* (encoding the invasin protein) (26), and DNA fragments related to invasion from *Mycobacterium tuberculosis* and *S. typhi* (2, 4). In these two later cases the genes are still uncharacterized.

A second strategy, transposon mutagenesis, has been used with diverse *Salmonella* species to select for mutants unable to invade cultured epithelial cells. Non-invasive mutants have been isolated in *S. typhimurium*, *S. typhi*, *S. enteriditis* and *S. choleraesuis* (13, 16, 35, 41). These mutants have been classified into different classes, and as a result, it is known that mutations in lipopolysaccharide biosynthesis decrease invasiveness in *S. choleraesuis* and *S. typhi*, but not in *S. typhimurium* (13, 35). Motility seems to be a bacterial factor required for invasion of *S. typhi* and *S. typhimurium* (29, 33). It has been suggested that *S. typhimurium* invasion is affected by the direction of the flagellar rotation and the physical orientation of flagella around the bacteria (27). This requirement of motility in invasion can be avoided by centrifuging bacteria onto the monolayer of epithelial cells.

All these genetic studies emphasize the complexity of the genetic machinery used by *Salmonella* to invade non-phagocytic cells. Nevertheless, an extensive and detailed characterization of an invasion locus has been reported for *S. typhimurium* (1, 16, 18). In a complementation study of an invasion mutant of *S. typhimurium*, Galán and Curtiss (16) isolated a DNA fragment that restored the invasive phenotype

of the wild type strain and mapped at 59 minutes on the chromosome. In this study they reported the existence of at least three genes, called *invA-C*. Lesions in these genes result in a slight decrease in virulence when bacteria were delivered orally in the mouse, the animal model used for *S. typhimurium* virulence. Further studies showed the existence of additional genes in the upstream region, named *invE*, required for triggering the uptake of *S. typhimurium* into epithelial cells (23), *invF*, and *invG* (1). Upstream of *invF*, and transcribed in the opposite direction, is *invH*, a locus involved in adherence and strongly conserved between *Salmonella* species and serotypes (1). Recently, Groisman and Ochman (25) have also reported the existence of additional *S. typhimurium* genes located downstream of the *inv* locus and involved in invasion of epithelial cells.

Different physiological studies have shown that S. *typhimurium* invasiveness is regulated by growth state (5, 30, 40). Increased invasion rates are observed when bacteria are grown in low oxygen tension conditions (5, 30, 40). The *inv* locus of *S. typhimurium* is also regulated by changes in DNA supercoiling affected by osmolarity (17). Additionally, a "hyperinvasive" locus was identified, and mutations in this locus uncouple invasion from its regulators, yielding constitutive invasive bacteria (31). Unlike *Shigella* or *Yersinia*, *Salmonella* invasion does not depend on temperature. Inhibitors of bacterial protein or RNA synthesis block *Salmonella* adherence and internalization (7), although it has been reported that bacteria can invade epithelial cells in the presence of chloramphenicol (30). Prolonged inhibition of bacterial protein synthesis block bacterial invasion (34), suggesting that the bacterial products involved in invasion may be rapidly turned over.

Host factors required for invasion. The clear morphological alterations induced in the epithelial cell during *Salmonella* uptake indicate that bacteria transmit localized signals at the host cell surface. The final target of these signals is the host actin containing microfilaments. Treatment of cultured epithelial cells with cytochalasins, which disrupts actin filaments, blocks *Salmonella* uptake in different systems (12, 14), although drugs that block microtubule polymerization, such as nocodazole, have no effect on bacterial invasion. During invasion of epithelial cells *Salmonella* induces a localized rearrangement of cytoskeletal components, including actin, α-actinin, tropomyosin, talin and ezrin, in the area surrounding invading bacteria (12). These cytoskeletal rearrangements are closely correlated with the morphological alterations associated with *Salmonella* invasion, such as swelling and membrane ruffling. Both the cytoskeleton and the host plasma membrane return to their normal distribution and morphology once bacteria are internalized (12).

Several signals appear to be transduced in the host cell to trigger bacterial internalization (3, 38). It is known that *S. typhimurium* induces increases in the concentration of intracellular Ca^{2+}, which correlates with the changes in the distribution of cytoskeletal components (23). A role of *invE* in this process has been suggested by the incapacity of an *invE* mutant to trigger the increase in Ca^{2+} intracellular and the cytoskeletal rearrangement (23). Release of intracellular Ca^{2+} is often mediated by fluxes of inositol phosphate IP_3. *S. typhimurium* triggers fluxes of inositol phosphate upon infection of epithelial cells (39), a process that correlates with the internalization of the bacteria. It has also been shown that intracellular, but not extracellular, Ca^{2+} chelators block *S. typhimurium* invasion (39).

It has been proposed that the epidermal growth factor receptor (EGF-R) is involved in the invasion of cultured epithelial cells by *S. typhimurium* (19). In this study it was shown that wild type bacteria induce tyrosine phosphorylation of the EGF-R, an effect that was not observed with an *invA* mutant. The

addition of EGF could restore the capacity of this mutant to penetrate epithelial cells. In a more recent work, the same group proposed a model of how *S. typhimurium* transduces the signal to the epithelial cells to trigger bacterial internalization (36). Bacteria activate EGF-R, which activates MAP kinase. Active MAP kinase induce the activation of phospholipase A_2 (PLA_2) which generates arachidonic acid. This component is converted to leukotriene LTD_4 by 5-lipoxygenase and finally LDT_4 open Ca^{2+} channels, with the subsequent influx of extracellular calcium, causing membrane ruffling, cytoskeletal rearrangements, and bacteria internalization. However, there are several contradictions between this model and different effects known to be induced by *S. typhimurium*. For example, it does not include any role for sources of intracellular calcium, although chelators of intracellular calcium affect *S. typhimurium* invasion (39). Nor does it explain the involvement of phospholipase C, which presumably generates the inositol phosphates seen during *S. typhimurium* invasion. In addition, it is known that cell lines that do not express EGF-R are efficiently infected by *S. typhimurium* (19), and that drugs that inhibit specifically tyrosine protein kinases, including EGF-R, have no effect on *S. typhimurium* invasion (37). Moreover, two more recent reports have described that inhibition of rac and rho, two host proteins essential for the EGF mediated cytoskeletal ruffling, are not involved in *S. typhimurium* invasion (14, 28). The same group also showed that ruffling and bacterial internalization induced by *S. typhimurium* can occur in cell lines that do not possess EGF-R (14). In conclusion, the current evidence suggests that *S. typhimurium* invasion of epithelial cells may not require activation of the EGF-R.

Intracellular bacterial proliferation

In contrast to the large number of reports describing *Salmonella* invasion, less is known about intracellular bacteria enclosed in a vacuole, and how they proliferate actively in this intracellular environment. Two studies have described the isolation of *S. typhimurium* and *S. choleraesuis* mutants which are impaired for growth inside epithelial cells but can grow extracellularly (so-called prototrophic *rep*$^-$ mutants) (8, 32). These mutants were avirulent in the mouse typhoid model, and support the concept that specific genes are needed for the intracellular proliferation process and that they are essential for *Salmonella* virulence.

Interestingly, the capacity of *Salmonella* to proliferate within vacuoles of the epithelial cell begins approximately 4 h after the initial internalization (9, 13). This fact indicates that there is a defined lag period in which bacteria may adjust to the intracellular environment of the vacuole, probably modifying diverse host parameters to trigger the bacterial growth process. The microenvironment of *S. typhimurium*-containing vacuoles has been partially characterized in epithelial cells using transcriptional *lacZ* fusions to several regulated bacterial genes (20). Measurement of ß-galactosidase activity from such intracellular bacteria suggested that *S. typhimurium*-containing vacuoles should have low concentrations of cations such as Fe^{2+}, Mg^{2+}, mild-acidic pH, lysine and oxygen (20).

In another recent study, host membrane markers present in the *S. typhimurium*-containing vacuoles have been identified. Cell surface molecules such as class I MHC heavy chain, $ß_2$-microglobulin, fibronectin receptor ($\alpha_5ß_1$ integrin), and CD44 (hyalorunate receptor) are all aggregated by *S. typhimurium* in the host plasma membrane during bacterial internalization (22). Of these molecules, only class I MHC heavy chain is predominantly incorporated into the membrane of bacteria-containing vacuoles during the initial stages of infection (22). These data showed that *S. typhimurium* is able to induce selective aggregation

and internalization of host cell surface molecules during bacterial uptake. These processes are probably linked to the actin rearrangement induced by *S. typhimurium* (12), and are not induced by other invasive bacteria which reside in vacuoles, such as *Y. enterocolitica* or a *S. typhimurium* invasion mutant that invade epithelial cells using the *Yersinia*-mediated internalization pathway (22). Differences in the composition of the vacuolar membrane may support the capacity of *Salmonella* to proliferate intracellularly within vacuoles. A certain vacuolar membrane composition may directly target the bacteria-containing vacuoles to specific locations within the epithelial cell. Indeed, recent observations suggest that *S. typhimurium* reside in vacuoles fusogenic with lysosomes yet by-pass the late endosome compartment, an obligate stage in the endocytic route (García-del Portillo and Finlay, submitted).

Another interesting effect produced by *Salmonella* species upon invasion of epithelial cells is the intimate interaction with the lysosomal system of the host cell (21). *Salmonella* is targeted to vacuoles containing lysosomal membrane markers at approximately 2 h after infection. At 4 h after infection, and coincident with the initiation of intracellular bacterial proliferation, long filamentous structures containing lysosomal markers and connected to the bacteria-containing vacuoles are also observed (Fig. 1) (21). These filamentous structures appeared in diverse human epithelial cells infected with all the *Salmonella* species and serotypes tested, including *S. typhimurium*, *S. enteriditis*, *S. choleraesuis*, and *S. dublin*. The linkage of the redistribution of lysosomes to the intracellular bacterial proliferation is supported by several lines of evidence: (i) they are not induced by certain avirulent *S. typhimurium* and *S. choleraesuis* mutants which are unable to proliferate inside the vacuoles (Fig. 1), (ii) they do not appear when *S. typhimurium* is killed with antibiotics, and (iii) they are not induced by invasive bacteria that reside in vacuoles but not proliferate intracellularly such as *Y. enterocolitica* (Fig. 1). The filamentous lysosomes triggered by *Salmonella* require an acidic intravacuolar pH and an intact microtubule network provided by the host cell (21). These results confirm the capacity of *Salmonella* to establish specific interactions with host organelles such as lysosomes, inducing dramatic alterations in their morphology and functionality to trigger intracellular bacterial proliferation.

Concluding remarks

The most recent data relative to the molecular biology of the interaction of *Salmonella* with non-phagocytic cells are summarized in Table 1. The current data suggest that this bacterium uses a very efficient system to induce internalization by epithelial cells, although the specific receptor(s) remains unidentified (3, 6). The extensive changes in the morphology of the host plasma membrane during bacterial uptake, including membrane ruffling and increased vacuolization (10, 14, 23), are natural processes which can be triggered by other cellular factors. Binding of natural ligands to plasma membrane receptors activates a signal cascade in which proteins such as rac, rho, and ras are involved. It happens that *Salmonella* invasion does not depend on the activity of any of these host proteins (28), although activation of MAP kinase, which is located downstream in the activation cascade, has been observed (36). One controversial aspect in the field is the requirement of host protein tyrosine phosphorylation for *Salmonella* invasion. Further work is required, including characterization of the different invasion mutants isolated in diverse *Salmonella* species and serotypes. Another note of interest are the significant homologies that have recently been found between different virulence genes

TABLE 1
EXPLOITATION OF HOST FUNCTIONS BY *Salmonella* DURING INVASION AND INTRACELLULAR PROLIFERATION WITHIN EPITHELIAL CELLS

Process	Bacterial factors required	Host factors required	Induced host signal activity[c]	References
Invasion	Protein synthesis, motility[a] low oxygen growth, lipopolysaccharide[b]	Actin filaments	Phospholipase C, $[Ca^{2+}]_i$, EGF-R?, MAP-kinase	5, 12, 19, 29, 30, 33–36, 38–40
Intracellular bacterial proliferation	Factors involved in filamentous lysosome formation	Mild acidic intravacuolar pH, microtubules	??	21, 32

[a] Reported for S. *typhimurium* and *S. typhi*, but not for *S. choleraesuis*.
[b] Required by S. *typhi* and *S. choleraesuis*, but not by *S. typhimurium*.
[c] Only characterized with *S. typhimurium*.

in several bacterial pathogens (25). It is now known that the InvA protein from *S. typhimurium* shares homology with LcrD from *Yersinia* and MxiA from *Shigella flexneri*. LcrD is a membrane bound calcium regulator involved in *Yersinia* pathogenesis, and MxiA is involved in exporting *Shigella* invasion antigens to the bacterial surface. Furthermore, several plant pathogens have similar secretory systems, including HrpO from *Pseudonomas solanacearum* and HrpC2 from *Xanthomonas campestris*. The homology between *Salmonella* and *Shigella* has been extended to several *Salmonella* genes of the *inv* locus and *the spa* genes of *S. flexneri*, which, like *mxiA*, are needed for secretion of invasion antigens to the bacterial surface (25). These results indicate that the complex machinery needed to transport virulence proteins is conserved in *Yersinia*, *Shigella*, *Salmonella* and various plant pathogens.

The intracellular bacterial proliferation, a process essential for *Salmonella* virulence, emphasizes the capacity of the bacteria to interact with specific organelles of the host cell (6, 21). The formation of filamentous lysosomes that occur in epithelial cells infected with *Salmonella* provides a clear example of exploitation of host organelles. This process is probably mediated early in the infection, since differences in the membrane composition of the initial bacteria-containing vacuoles have been observed for *Salmonella* and *Yersinia* species (22). Future work needs to establish in detail the intracellular trafficking of *Salmonella*-containing vacuoles, the bacterial genes involved in this process, and the nutrient sources within the host cell that permit *Salmonella* to proliferate yet remain enclosed within a vacuole. Moreover, the unconventional targeting of *Salmonella* to lysosomes within the epithelial cell, which basically maintain the bacteria separated from the endocytic route, evidence this pathogen as a useful biological tool to understand the process of lysosome biogenesis in eucaryotic cells.

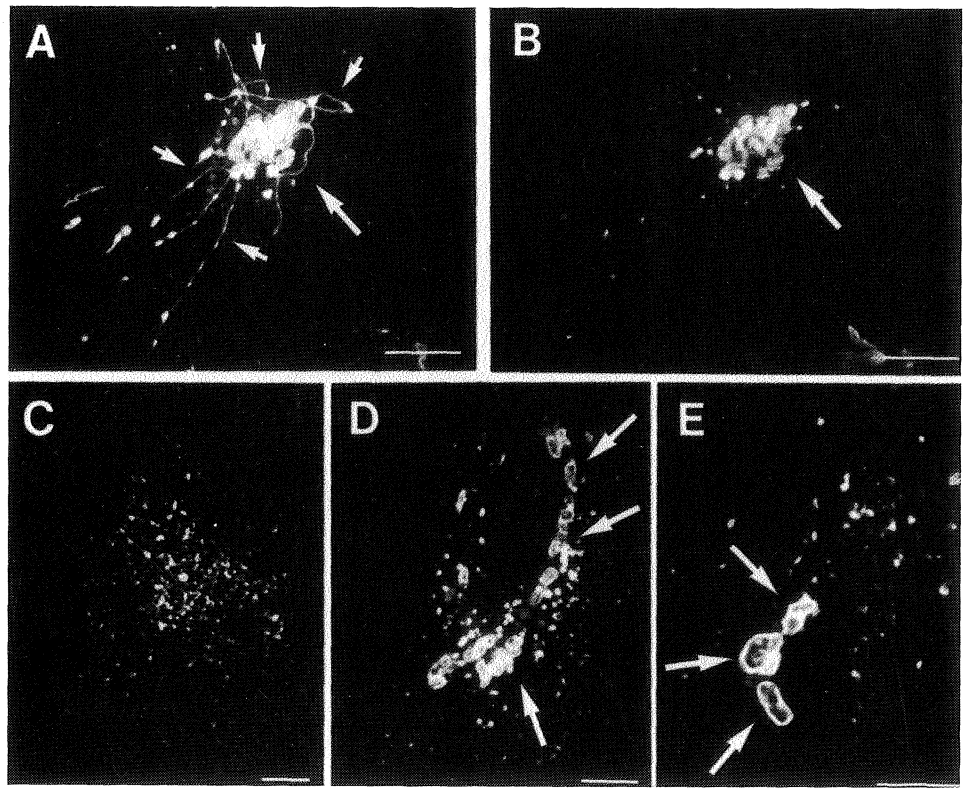


FIG. 1. Formation of filamentous lysosomes in *S. typhimurium*-infected HeLa epithelial cells. Indirect immunofluorescence images show distribution of the human lysosomal membrane glycoprotein hlamp-2 (A, C, D, E) and bacterial lipopolysaccharide (B). (A, B) *S. typhimurium* SL1344 (wild type strain)-infected cell. (C) Uninfected cell. (D) *Y. enterocolitica*-infected cell. And (E) *S. typhimurium* strain 3-11 (*rep*⁻ mutant)-infected cell. All infected cells were fixed and processed for immunofluorescence at 6 h after infection. Large arrows show location of intracellular bacteria. Small arrows indicate filamentous lysosomes connected to bacteria-containing vacuoles. Bar: 10 μm. Modified from (21).

In conclusion, genetics, biochemistry and cell biology of the interaction of *Salmonella* with non-phagocytic cells are starting to be defined. The complementary and often surprising results will provide the necessary information to combat salmonellosis, a disease which continues to be a significant health problem worldwide.

Acknowledgments

F.G.P. was a post-doctoral fellow in B.B.F.'s laboratory supported by long-term fellowships from the European Molecular Biology Organization (EMBO) (1991/92) and the Medical Research Council (MRC) of Canada (1993/94). The work in B.B.F.'s laboratory is supported by operating grants from the MRC of Canada, the Canadian Bacterial Diseases Network, and a Howard Hughes International Scholarship.

References

1. Altmeyer, R. M., McNern, J. K., Bossio, J. C., Rosenshine, I., Finlay, B. B., Galán, J. E. (1993). Cloning and molecular characterization of a gene involved in *Salmonella* adherence and invasion of cultured epithelial cells. Mol. Microbiol. **7**, 89–98.
2. Arruda, S., Bomfim, G., Knights, R., Huima-Byron, T., Riley, L. W. (1993). Cloning of a *M. tuberculosis* DNA fragment associated with entry and survival inside cells. Science **261**, 1454–1457.
3. Bliska, J. B., Galán, J. E., Falkow, S. (1993). Signal transduction in the mammalian cell during bacterial attachment and entry. Cell **73**, 903–920.
4. Elsinghorst, E. A., Baron, L. S., Kopecko, D. J. (1989). Penetration of human intestinal epithelial cells by *Salmonella*: molecular cloning and expression of *Salmonella typhi* invasion determinants in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86**, 5173–5177.
5. Ernst, R. K., Dombroski, D. M., Merrick, J. M. (1990). Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella typhimurium*. Infect. Immun. **58**, 2014–2016.
6. Finlay, B. B. (1994). Molecular and cellular mechanisms of *Salmonella* pathogenesis. Curr. Top. Microbiol. Immunol., in press.
7. Finlay, B. B., Heffron, F., Falkow, S. (1989). Epithelial cell surfaces induce *Salmonella* proteins required for bacterial adherence and invasion. Science **243**, 940–943.
8. Finlay, B. B., Chatfield, S., Leung, K. Y., Dougan, G., Falkow, S. (1991). Characterization of a *Salmonella choleraesuis* mutant that cannot multiply within epithelial cells. Can. J. Microbiol. **37**, 568–572.
9. Finlay, B. B., Falkow, S. (1989). *Salmonella* as an intracellular parasite. Mol. Microbiol. **3**, 1833–1841.
10. Finlay, B. B., Falkow, S. (1990). *Salmonella* interactions with polarized human intestinal Caco-2 epithelial cells. J. Infect. Dis. **162**, 1096–1106.
11. Finlay, B. B., Gumbiner, B., Falkow, S. (1988). Penetration of *Salmonella* through a polarized Madin-Darby canine kidney epithelial cell monolayer. J. Cell Biol. **107**, 221–230.
12. Finlay, B. B., Ruschkowski, S., Dedhar, S. (1991). Cytoskeletal rearrangements accompanying *Salmonella* entry into epithelial cells. J. Cell Sci. **99**, 283–296.
13. Finlay, B. B., Starnbach, M. N., Francis, C. L., Stocker, B. A. D., Chatfield, S., Dougan, G., Falkow, S. (1988). Identification and characterization of Tn*phoA* mutants of *Salmonella* that are unable to pass through a polarized MDCK epithelial cell monolayer. Mol. Microbiol. **2**, 757–766.
14. Francis, C. L., Ryan, T. A., Jones, B. D., Smith, S. J., Falkow, S. (1993). Ruffles induced by *Salmonella* and other stimuli direct macropinocytosis of bacteria. Nature **364**, 639–642.
15. Francis, C. L., Starnbach, M. N., Falkow, S. (1992). Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low-oxygen conditions. Mol. Microbiol. **6**, 3077–3087.
16. Galán, J. E., Curtiss III, R. (1989). Cloning and molecular characterization of genes whose products allow *Salmonella typhimurium* to penetrate tissue culture cells. Proc. Natl. Acad. Sci. USA **86**, 6383–6387.
17. Galán, J. E., Curtiss III, R. (1990). Expression of *Salmonella typhimurium* genes required for invasion is regulated by changes in DNA supercoiling. Infect. Immun. **58**, 1879–1885.
18. Galán, J. E., Ginocchio, C., Costeas, P. (1992). Molecular and functional characterization of the *Salmonella* invasion gene *invA*: homology of InvA to members of a new protein family. J. Bacteriol. **174**, 4338–4349.
19. Galán, J. E., Pace, J., Hayman, M. J. (1992). Involvement of the epidermal growth factor receptor in the invasion of cultured mammalian cells by *Salmonella typhimurium*. Nature **357**, 588–589.
20. García-del Portillo, F., Foster, J. W., Maguire, M. E., Finlay, B. B. (1992). Characterization of the micro-environment of *Salmonella typhimurium*-containing vacuoles within MDCK epithelial cells. Mol. Microbiol. **6**, 3289–3297.
21. García-del Portillo, F., Zwick, M. B., Leung, K. Y., Finlay, B. B. (1993). *Salmonella* induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells. Proc. Natl. Acad. Sci. USA **90**, 10544–10548.

22. García-del Portillo, F., Pucciarelli, M. G., Jefferies, W. A., Finlay, B. B. (1994). *Salmonella typhimurium* induces selective aggregation and internalization of host cell surface proteins during invasion of epithelial cells. J. Cell Sci. **107**, 2005–2020.
23. Ginocchio, C., Pace, J., Galán, J. E. (1992). Identification and molecular characterization of a *Salmonella typhimurium* gene involved in triggering the internalization of salmonellae into cultured epithelial cells. Proc. Natl. Acad. Sci. USA **89**, 5976–5980.
24. Goldberg, M. B., Rubin, R. H. (1988). The spectrum of *Salmonella* infection. Infect. Dis. Clin. North Am. **2**, 571–598.
25. Groisman, E. A., Ochman, H. (1993). Cognate gene clusters govern invasion of host epithelial cells by *Salmonella typhimurium* and *Shigella flexneri*. EMBO J. **12**, 3779–3787.
26. Isberg, R. R., Falkow, S. (1985). A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K-12. Nature **317**, 262-264.
27. Jones, B. D., Lee, C. A., Falkow, S. (1992). Invasion by *Salmonella typhimurium* is affected by the direction of flagellar rotation. Infect. Immun. **60**, 2475–2480.
28. Jones, B. J., Paterson, H., Hall, A., Falkow, S. (1993). *Salmonella typhimurium* induces membrane ruffling by a growth factor-receptor-independent mechanism. Proc. Natl. Acad. Sci. USA **90**, 10390–10394.
29. Khoramian, F. T., Harayama, S., Kutsukake, K., Pechere, J. C. (1990). Effect of motility and chemotaxis on the invasion of *Salmonella typhimurium* into HeLa cells. Microb. Pathog. **9**, 47-53.
30. Lee, C. A., Falkow, S. (1990). The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. Proc. Natl. Acad. Sci. USA **87**, 4304–4308.
31. Lee, C. A., Jones, B. D., Falkow, S. (1992). Identification of a *Salmonella typhimurium* invasion locus by selection for hyperinvasive mutants. Proc. Natl. Acad. Sci. USA **89**, 1847–1851.
32. Leung, K. Y., Finlay, B. B. (1991). Intracellular replication is essential for the virulence of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **88**, 11470–11474.
33. Liu, S. L., Ezaki, T., Miura, H., Matsui, K., Yabuuchi, E. (1988). Intact motility as a *Salmonella typhi* invasion-related factor. Infect. Immun. **56**, 1967–1973.
34. MacBeth, K. J., Lee, C. A. (1993). Prolonged inhibition of bacterial protein synthesis abolishes *Salmonella* invasion. Infect. Immun. **61**, 1544–1546.
35. Mroczenski, M. J., Di, F. J. L., Cabello, F. C. (1989). Invasion and lysis of HeLa cell monolayers by *Salmonella typhi*: the role of the lipopolysaccharide. Microb. Pathogen. **6**, 143–152.
36. Pace, J., Hayman, M. J., Galán J. E. (1993). Signal transduction and invasion of epithelial cells by *S. typhimurium*. Cell **72**, 505–514.
37. Rosenshine, I., Duronio, V., Finlay, B. B. (1992). Tyrosine protein kinase inhibitors block invasin-promoted bacterial uptake by epithelial cells. Infect. Immun. **60**, 2211–2217.
38. Rosenshine, I., Finlay, B. B. (1993). Exploitation of host signal transduction pathways and cytoskeletal functions by invasive bacteria. Bioessays **15**, 17–24.
39. Ruschkowski, S., Rosenshine, I., Finlay, B. B. (1992). *Salmonella typhimurium* induces an inositol phosphate flux in infected epithelial cells. FEMS Microbiol. Lett. **95**, 121–126.
40. Schiemann, D. A., Shope, S. R. (1991). Anaerobic growth of *Salmonella typhimurium* results in increased uptake by Henle 407 epithelial and mouse peritoneal cells in vitro and repression of a major outer membrane protein. Infect. Immun. **59**, 437–440.
41. Stone, B. J., García, C. M., Badger, J. L., Hassett, T., Smith, R. I., Miller, V. L. (1992). Identification of novel loci affecting entry of *Salmonella enteritidis* into eukaryotic cells. J. Bacteriol. **174**, 3945–3952.
42. Takeuchi, A. (1967). Electron microscope studies of experimental *Salmonella* infection. I. Penetration into the intestinal epithelium by *Salmonella typhimurium*. Am. J. Pathol. **50**, 109–136.
43. Yokoyama, H., Ikedo, M., Kohbata, S., Ezaki, T., Yabuuchi, E. (1987). An ultrastructural study of HeLa cell invasion with *Salmonella typhi* GIFU 10007. Microbiol. Immunol. **31**, 1–11.

MICROBIOLOGÍA SEM **10** (1994), 239–248 **MINIREVIEW**

Chitin synthetases in *Candida albicans*: a review on their subcellular distribution and biological function

José P. Martínez, Daniel Gozalbo*

Sección Departamental de Microbiología, Facultad de Farmacia, Universidad de Valencia

Received 20 April 1994/Accepted 2 August 1994

Summary

In the light of recent genetic advances, some results regarding chitin biosynthetic activities are reviewed in this paper. Genes coding for distinct enzymes displaying chitin synthetase activities have been characterized in *Saccharomyces cerevisiae* as well as in other fungal species including *Candida albicans*. Several activities seem to exist in the cells: (i) one zymogenic, located in cytoplasmic vesicles called chitosomes, although the presence of other types of vesicles with zymogenic activity cannot be completely discarded, and (ii) plasma membrane associated activities (the active enzyme and probably two distinct pools of zymogenic activity). Possible relationships between these activities, if any, remain to be determined. These multiplicity of enzymes is not surprising taking into account that chitin biosynthesis is required during very well defined temporal and spatial events of the cell cycle. A general repair function for one of the chitin biosynthetic activities is proposed as a possible salvage mechanism to warrant cell survival after wall damage has been caused, since chitin appears to be the most suitable polymer to carry out this function due to its particular physico-chemical properties.

Key words: chitin synthetases, *Candida albicans*, chitosomes, plasma membrane, cell wall, chitin

Resumen

En este artículo se revisan algunos aspectos de la actividad quitina sintetasa en hongos, teniendo en cuenta los recientes avances que en el terreno de la genética se han producido en este campo. En *Saccharomyces cerevisiae* y en otras especies, incluyendo *Candida albicans*, han sido identificados

* *Correspondence to*: Daniel Gozalbo. Sección Departamental de Microbiología. Facultad de Farmacia. Universidad de Valencia. Avda. Vicent Andrés Estellés, s/n, 46100 Burjassot, Valencia. Tel.: 96-3864770. Fax: 96-3864770.

varios genes que codifican la síntesis de distintas enzimas con actividad quitina sintetasa. Teniendo en cuenta su localización subcelular y sus características fisiológicas, parecen existir distintas actividades biosintéticas de quitina en las células fúngicas: (i) enzima inactiva (zimógeno), asociada a un tipo particular de microvesículas citoplásmicas denominadas quitosomas (aunque no debe descartarse la existencia de otras estructuras subcelulares que contengan también quitina sintetasa zimogénica), y (ii) actividades asociadas a la estructura de la membrana plasmática (la enzima activa in vivo, así como muy probablemente dos poblaciones distintas de actividad zimógena). Las posibles relaciones, si es que existe alguna, entre todas estas actividades enzimáticas, están todavía por determinar. La existencia simultánea de las mismas en las células no debe sorprender si se tiene en cuenta que es necesaria la síntesis de quitina durante etapas concretas, perfectamente definidas tanto espacial como temporalmente, del ciclo celular. Se propone que una de las actividades biosintéticas de quitina presentes en las células tiene asignada una función reparadora de tipo general, como un posible mecanismo de salvamento para garantizar la supervivencia celular tras producirse daños en la estructura de la pared celular fúngica. Ello se debe a que, a causa de sus características físico-químicas, la quitina parece ser el polímero más adecuado para llevar a cabo esta función de reparación.

Introduction

Several extensive and well documented reviews on chitin synthesis in fungi have been recently published (9, 35, 36). The aim of this work is not to extensively review again this topic, but to present distinct aspects related with the subcellular distribution of chitin biosynthetic activity and the synthesis of the polymer, mainly in *Candida albicans*, in order to re-evaluate the significance of different chitin biosynthetic activities, the relationship between enzyme populations, and their possible function.

Chitin is an insoluble polysaccharide made of ß-(1,4)-linked N-acetylglucosamine units. This biological polymer, which is known to be second more abundant natural substance after cellulose, is one of the structural microfibrillar components of the fungal cell wall (35, 36), structure which maintains the morphologic shape of the cells and that play essential roles in fungal morphogenesis (37, 38) and in the relationships of fungi with the external environment (11, 12, 40).

Chitin, chitin synthesis, and morphogenesis in *Candida albicans*

C. albicans is an opportunistic pathogen that exhibits several morphological shapes (chlamydospores, yeast, pseudohyphae, and hyphae), yet two of these forms (yeast and mycelium) are the most frequently found in nature, depending on different external factors such as composition of culture medium, pH, and temperature. Although factors which influence the form growth in vivo are not defined, the yeast form is associated with the presence of the organisms as a commensal in the normal host, while the hyphal morphology is additionally observed in infection. The ability to form hyphae appears to be one of the important virulence factors of this fungus (31).

The wall of *C. albicans* yeast cells consists of a fibrillar framework mostly made of non-cellulosic ß-glucans, and chitin as minor component (0.6–2.7% of the dry weight of the wall), embedded in a matrix composed mainly of mannoproteins. From a chemical point of view composition of mycelial walls is basically similar to that of yeast cells, although qualitative and quantitative differences in the relative amounts of the distinct wall components have been detected (37, 40). Thus, regarding chitin, mycelial walls contain roughly three-fold more of this polymer than yeast cell walls (14, 16, 40). In any case, there is no correlation between the morphology of fungal cells and the amount of chitin present in their walls, as some dimorphic fungi contain more chitin in the mycelial versus the yeast forms, whereas in other species the opposite situation is observed (35, 36).

In budding yeast, chitin is mainly present in the primary septum between the mother and daughter (bud) cells, and a small amount of chitin is also uniformly dispersed over the entire cell wall. In hyphae chitin is located in the inner layers of the cell wall. In the hyphal apex, chitin appears to be the main component, as well as in the septa that separates cell compartments along the hyphae (35, 36).

Most chitin synthetase activity from the yeast phase of *C. albicans* is found in a zymogenic state, being activated in vitro by proteolysis (6, 7, 14). During germ tube formation and mycelial growth there is an increase in the specific activity of the enzyme. Thus, mycelial cells contain several fold the activity present in yeast (6, 14, 16, 33).

With respect to the subcellular distribution of the enzymatic activity, two locations have been clearly established: (i) plasma membrane, the structure where chitin synthesis takes place, containing both zymogenic and active (non-zymogenic) enzyme (6, 7, 24, 25), and (ii) chitosomes, cytoplasmic microvesicles containing fully zymogenic enzyme which may act as conveyors of chitin synthetase to its final destination to the cell surface (23–25). Chitosomes were first discovered in the yeast form of *Mucor rouxii* and were described and further characterized later in other chitinous fungi (2, 35, 36). The above mentioned subcellular distribution of chitin synthetase (i.e., plasma membrane and chitosomes) is similar in both morphogenetic phases and protoplasts of *C. albicans* (23–25).

During regeneration of protoplasts obtained from *C. albicans*, yeast and mycelial cells, a skeleton of chitin is the first structure appearing externally to the plasma membrane (16, 30). During the regeneration process, which initially leads to the appearance of hyphae-like structures, chitin synthetase activity exponentially raised (16). Chitin is also the first polymer deposited during the apical growth of fungal cells (35, 36).

The formation of the final cell wall structure appears to depend on the establishment of different types of linkages between its distinct molecular components. Chitin seems to play an essential role in the initial steps of this process, although other wall components such as the mannoproteins and glucans appear to play also an important morphogenetic function (12, 37, 38).

Chitin synthetase genes

The genetics of chitin synthetases in fungi have been recently reviewed with great detail by Bulawa (9) and Robbins et al. (34). Hence, here we will only deal with the most relevant features about this topic.

In the budding yeast *S. cerevisiae* three distinct chitin biosynthetic activities have been described so far. *CHS1* and *CHS2* genes encode closely related proteins, chitin synthetase I and II, whose in vitro

activation require proteolytic treatment (10, 32). The third enzyme, chitin synthetase III, is active in vitro without protease treatment, and at least three genes, *CAL1* (=*CSD2*), *CAL2* (=*CSD4*) and *CAL3*, are needed for its activity (8, 39, 41).

These enzymes have different roles in cell physiology. One of them, chitin synthetase I, is required for normal budding in acidic media and seems to have a repair function during cell separation. Chitin synthetase II is involved in septum formation between mother and daughter cells and is also required for cell separation and normal morphology. Chitin synthetase III is responsible for the synthesis of 90% of the cell wall chitin, including the chitin ring at the site of bud emergence and the chitin in the rest of the wall (9).

Although one may expect the existence of similar chitin synthetase activities in *C. albicans*, as this fungus possess a higher genetic and morphological complexity than *S. cerevisiae*, only two genes coding for chitin synthetases have been described so far in *C. albicans* (34). The *CaCHS1* gene was cloned by complementation in *S. cerevisiae chs1* mutant (1). The product of *CaCHS1* gene is membrane-bound and also requires partial proteolysis for its activation. Using the polymerase chain reaction (PCR) a second *CHS* homologue (*CaCHS2*) whose product differs in the biochemical properties, has been detected (13). Expression of this gene is induced several fold during the yeast-to-mycelium transition (13), and homozygous *chs2* mutants of *C. albicans* have no obvious defects in growth (21), although the amount of chitin in hyphal cells is reduced by 20-50%. The products of the *Candida CHS1* and *CHS2* genes are likely zymogenic enzymes similar to that encoded by *Saccharomyces CHS1* and *CHS2* genes (34).

Analysis of genomic DNA from taxonomically different fungal species by PCR technology showed that most species have several chitin synthetase gene homologues, whose sequences, except that of *S. cerevisiae CHS1*, belong to three distinct groups (5). Whether the genes of each group play similar functions is a question that remains to be answered.

Subcellular distribution of chitin biosynthetic activity

Findings emanating from the genetic approach and that were mentioned in the previous section, lead us to reconsider the state of the art on the subcellular distribution of chitin synthetase.

It is known that several activities may be present both in cell-free extracts and subcellular fractions, and that such activities differ in their biochemical properties, being thus difficult to interpret the results obtained. The presence of a partly active enzyme attached to the plasma membrane and of a zymogenic activity bound to microvesicles called chitosomes, is currently accepted (see above). It seems clear that at least three activities are present: (i) active and (ii) zymogenic plasma membrane bound enzymes and (iii) chitosomal chitin synthetase. However, the relationships between the different activities, if any, have been not clearly established yet. Thus, is the zymogenic membrane-bound enzyme precursor of the active one? Do the chitosomes fuse with the plasma membrane? No precursor-product relationship has been found between chitosomes and plasma membrane enzyme. Protein synthesis inhibition does not produces a decay in the chitosomal activity and, in addition, the ratio total activity/zymogenic activity in membrane preparations remains unaffected (24). Hence, the possibility that all three enzymes correspond to distinct gene products cannot be ruled out.

Regardless the purification procedure used to obtain chitin synthetase active preparations, researchers have currently focused on the main pools with activity obtained, whereas other minor peaks or fractions of unknown nature have been often reported as artifacts produced during cell fractionation manipulations. However, this seems to be the simplest interpretation of the different experimental findings.

In this context, Flores-Carreón et al. (20) described two distinct pools of zymogenic activity in toluenized cells of *M. rouxii*, in addition to the active enzyme. Each zymogenic pool was suggested to be differentially located, one associated to the plasma membrane and the other bound to the chitosomal fraction, on the basis of inactivation of the membrane-bound zymogen observed in cells treated at acid pH. However, it is now widely accepted that the plasma membrane bound activity is located at the inner face of the membrane, as it do not result inhibited when protoplasts are treated with glutaraldehyde (6, 15), and chitosomal enzyme might be not activated by exogenous proteases in permeabilized cells (18, 33; Gozalbo, unpublished observations). Consequently, it cannot be dismissed that both zymogenic pools actually represent enzymes attached to the plasma membrane of *M. rouxii* but with a distinct topological location in the membrane structure. In addition, several experiments carried out in *S. cerevisiae* and *C. albicans* point out that in vivo and in vitro activation of zymogenic chitin synthetase are different processes, since active and activated membrane-bound enzymes differ in several properties such as pH-dependent activity kinetics, requirement of free N-acetylglucosamine, and digitonin solubilization properties (18, 22). Evidence for two zymogenic pools of membrane-bound activity in *C. albicans* has been found by treatment of the membrane fraction with digitonin and trypsin under different conditions (26). All the above mentioned findings point to the possibility that more than one gene product with chitin biosynthetic activity is bound to the membrane. In support of this contention there is additional evidence. Digitonin exclusively solubilizes, under particular conditions, the zymogen (but not the in vivo active) membrane-bound form of the enzyme, and after in vitro proteolytic activation, the activated zymogen continues being solubilized by the detergent (22). Besides, centrifugation of distinct digitonin-solubilized preparations, which contain either zymogen, in vivo active enzyme and/or in vitro activated enzyme, on linear density gradients indicates that both zymogen and trypsin-activated enzyme sediment slightly slower than the active enzyme (26). These results suggest that differences exist between the activation processes in vivo and in vitro or, alternatively, that both enzyme activities (active in vivo and zymogenic) correspond to different gene products.

On the other hand, there is a body of experimental data which may suggest the existence of more than one type of microvesicles containing zymogenic chitin synthetase activity (chitosomes). Hanseler et al. (27) found two distinct populations of chitosomes from *Agaricus bisporus* when cell-free extracts were treated with digitonin, proposing that the one released after treatment with the detergent corresponds to chitosomes bound to wall and/or plasmalemma, whereas the other one, that does not require detergent to be released, represents cytoplasmic microvesicles. Kang et al. (28) detected a minor peak of chitin synthetase activity lighter than the activity peak corresponding to the plasma membrane, following centrifugation of a lysate of con A-treated protoplasts (Con A treatment avoids plasma membrane fragmentation during cell lysis) of *S. cerevisiae* on renografin gradients. This lighter peak appeared to be unrelated to the activity bound to plasma membrane and no precursor-product relationship was found for both enzymes. A similar light peak of chitin synthetase, accounting for a minor percentage of the total activity detected, was also reported by Durán et al. (15). Leal-Morales et al. (29) found, in

addition to «classic» chitosomes and plasma membrane-bound chitin synthetase, a second population of microvesicles containing zymogenic activity in *S. cerevisiae*. Similarly, when a partially purified vesicular fraction obtained from *S. cerevisiae* protoplasts lysates was centrifuged on linear sucrose gradients, several minor peaks of activity were observed along with the large peak of chitosomal chitin synthetase (19). Furthermore, two fractions containing chitin synthetase activity, plasma membrane and chitosomes, have been detected both in yeast and mycelial cells as well as in protoplasts of *C. albicans* (23–25). However, during these subcellular fractionation experiments performed following distinct procedures, different zymogenic pools of activity were observed. A possible artifactual origin for these pools was suggested as their detection was dependent on the method used to disrupt the cells (24, 25), yet it is also possible that at least some of these minor fractions exhibiting chitin synthetase activity, reflects genuine subcellular structures which may require particular conditions either for their separation from the major pools of enzyme and their optimal in vitro detection.

From all the above mentioned experimental findings, it can be suggested that some of the minor fractions containing chitin synthetase activity actually correspond to minor subcellular pools, or that are detected as such due to the experimental conditions. The situation initially presented on the subcellular distribution of chitin synthetase could become more complicated, considering that several chitin synthetases, i.e., the active enzyme and possibly two different zymogenic activities, may be bound to the plasma membrane, and in addition, although chitosomes are well defined organelles containing zymogenic enzyme, other subcellular vesicular structures may also possess chitin synthetase activity.

Chitin biosynthesis as a repair function

Taking into account all the above mentioned considerations, one can imagine that each cellular event requiring chitin biosynthesis may involve a chitin synthetase activity with particular properties, such as regulation of the expression, activating/inactivating mechanism, and subcellular location, in order to maintain integrity and accuracy of physiological processes. What it seems clear is that chitin synthetases act where and when the wall structure has been partially degraded by physiological processes to allow the progression of morphological events. In this context, it may be considered that all chitin synthetases have a «repair» function leading to the building of new structures. The physico-chemical properties of chitin, which allow a rapid polymerization of nascent chitin into crystalline microfibrils, makes this polymer the most suitable candidate to carry out such function. In addition, chitin synthesis is a process temporally and spatially well defined to allow deposition of chitin at the specific cell surface sites when the polymer is required and in the amounts needed for proper morphological events. In this context, distinct enzymes may have evolved to perform different specific functions.

On the other hand, it has to be noted that microscopic fungus may live in habitats where a wide variety of organisms including other fungal species and bacteria are also present, and that colonization of new habitats implies a competition to ensure survival. Hydrolytic enzymes with potential activity on the fungal cell wall are widely distributed in the biosphere and consequently may play a role in such competition. In fact, chitinases are commonly found in a wide range of organisms, including bacteria, fungi, higher plants, insects, crustaceans, and some vertebrates. Although a major role of chitinases found in fungi, crustaceans, and insects is modification of the organism's structural constituent chitin,

these hydrolytic enzymes may also function as aggressive agents which interfere with the normal growth of chitinous fungi. Thus, the production of chitinases by plants is considered to be a part of the defense mechanisms against fungal pathogens, and bacteria produce chitinase to digest chitin primarily to utilize it as a carbon and energy source (3, 4, 42).

Of course, chitin-producing microorganisms should be able to mount the adequate countermeasures to respond to this aggression. A possible strategy to avoid the action of hydrolyzing enzymes could be simply to neutralize their effect, by repairing damages in the cell wall structure at the sites where they were produced; in this context, chitin deposition seems to be the more effective system. Thus, the existence of a chitin synthetase activity specialized in this repair function can be envisaged. This enzyme would act as a salvage system to avoid cell destruction as a consequence of the aggression by lytic enzymes. Although probably some parts of the wall are more sensitive to hydrolytic activities than others, this salvage system should be able to act over the entire cell wall. How to ensure this? There are two possibilities: (i) the enzyme is distributed in an inactive (zymogenic) state over the whole surface (plasma membrane) of the cell and is selectively activated at the sites where chitin synthesis is required, or (ii) an intracellular pool of zymogenic enzyme (chitosomes?) specifically moves to the damaged sites of the cell surface to synthesize chitin after its activation.

There is some evidence in favour of the existence of the chitin synthetase repair function:

(i) A network of chitin is the first structure appearing on the cell surface during regeneration of fungal protoplasts. This process occurs independently of the original morphology of cells (yeast or hyphae) from which protoplasts are obtained and the specific cell wall composition of the individual fungal species examined (16, 30, 37).

(ii) Some molecules, such as N-acetylglucosamine and chitodextrines have been described to be stimulators of chitin synthesis in several systems (35). However, these molecules are not incorporated into chitin but they rather represent hydrolytic products from cell wall components, which may stimulate chitin synthesis when cell wall degradation occurs.

(iii) Chemicals, such as calcofluor white, which interferes with chitin crystallization, appear to cause an increase in the amount of chitin synthesized by cells (17).

According to the hypothesis raised above (chitin biosynthesis as a repair function) the yeast-to-mycelium transition of *C. albicans*, which implies an increased level of chitin biosynthesis, may be partly regarded as an adaptative response of the fungus to both in vivo or in vitro aggressive conditions, such as the host immunological system or the starvation period and the incubation temperature shift used to induce germ tube formation. As the mycelium is though to be the most virulent morphologic phase of this fungal species (31), antibodies may interact with cell surface components, particularly with the mannoproteins which act as the most immediate elicitors of the host immune system, in addition of playing an important role in configuring the final cell wall structure (11, 12, 37, 38, 40). Such interaction, may interfere the cell wall construction and consequently induce an increase of chitin synthesis.

Final remarks

Chitin appears to be the most suitable polymer to sustain cell wall strength at the sites where wall structure has been damaged either as a result of physiological processes or external aggression. However,

chitin lacks the ability to build the wall structure by itself, and thus configurate cell morphology. Hence, proper cell wall structuration will imply the interaction between skeletal polymers (glucans and chitin) either among them and with proteins and glycoproteins which, as above stated, may play a modulatory role in this process. Chitin biosynthesis is involved in several physiological events which are accurately regulated both spatially and temporally. This points out to the existence of several chitin biosynthetic activities able to synthesize the polymer at specific cell surface sites to allow proper cellular growth. Such activities should be differentially located and/or regulated as they are involved in distinct cellular processes. In fact, several functionally distinct chitin synthetases are present in *S. cerevisiae* and a similar, or even more complex (in the case of polymorphic fungi) situation may also occur in other fungal species including *C. albicans*.

Acknowledgments

Research in the laboratory of J. P. M. is supported by grants from the FISSS, Ministerio de Sanidad y Consumo (grant no. 93/0801), from the DIGICyT (Programa Sectorial de Promoción General del Conocimiento), Ministerio de Educación y Ciencia (grant no. PM 92-0246), Spain, and from the NATO Collaborative Research Grants Programme (project no. CRG 931457). D. G. was supported by grants from DIGICyT (grant no. PB 90-0424) and from Commission of the European Communities (EC Group on *Candida albicans* Cell Biology and Pathogenicity; C11*.0631.M).

References

1. Au-Young, J., Robbins, P. W. (1990). Isolation of a chitin synthetase gene (*CHS1*) from *Candida albicans* by expression in *Saccharomyces cerevisiae*. Mol. Microbiol. **4**, 197–207.
2. Bartnicki-García, S., Bracker, C. E., Reyes, E., Ruiz-Herrera, J. (1978). Isolation of chitosomes from taxonomically diverse fungi and synthesis of chitin microfibrils *in vitro*. Exptl. Mycol. **2**, 173–192.
3. Berkeley, R. C. W. (1979). Chitin, chitosan and their degradative enzymes. *In* Berkeley, R. C. W., Gooday, G.W., Ellwood, D.C. (ed.), Microbial Polysaccharides and Polysaccharases, pp. 205–236. Academic Press, London.
4. Boller, T. (1985). Induction of hydrolases as a defence reaction against pathogens. *In* Key, J. L., Kosuge, T. (ed.), Cellular and Molecular Biology of Plant Stress, pp. 247–262. Alan R. Liss, New York.
5. Bowen, A. R., Chen-Wu, J. L., Momany, M., Young, R., Szaniszlo, P. J., Robbins, P. W. (1992). Classification of fungal chitin synthases. Proc. Natl. Acad. Sci. USA **89**, 519–523.
6. Braun, P. C., Calderone, R. A. (1978). Chitin synthesis in *Candida albicans*: comparison of yeast and hyphal forms. J. Bacteriol. **135**, 1472–1477.
7. Braun, P. C., Calderone, R. A. (1979). Regulation and solubilization of *Candida albicans* chitin synthetase. J. Bacteriol. **140**, 666–670.
8. Bulawa, C. E. (1992). *CSD2*, *CSD3*, and *CSD4*, genes required for chitin synthesis in *Saccharomyces cerevisiae*: the *CSD2* gene is related to chitin synthases and to developmentally regulated proteins in *Rhizobium* species and *Xenopus laevis*. Mol. Cell. Biol. **12**, 1764-1776.
9. Bulawa, C. E. (1993). Genetics and molecular biology of chitin synthesis in fungi. Annu. Rev. Microbiol. **47**, 505–534.

10. Cabib, E., Silverman, S. J., Shaw, J. A. (1991). Chitin synthetases 1 and 2 from yeast, two isoenzymes with different functions. *In* Latgé, J. P., Boucias, D. (ed.), Fungal Cell Wall and Immune Response. NATO ASI Series vol. H 53, pp. 39–48. Springer-Verlag, Berlin.
11. Calderone, R. A., Braun, P. C. (1991). Adherence and receptor relationships in *Candida albicans*. Microbiol. Rev. **55**, 1–20.
12. Cassone, A. (1989). Cell wall of *Candida albicans*: its function and its impact on the host. *In* McGinnis, M. R., Borgers, M. (ed.), Current Topics in Medical Mycology, vol. 3, pp. 248–314. Springer-Verlag, New York.
13. Chen-Wu, J. L., Zwicker, J., Bowen, A. R., Robbins, P. W. (1992). Expression of chitin synthase genes during yeast and hyphal growth phases of *Candida albicans*. Mol. Microbiol. **6**, 497–502.
14. Chiew, Y. Y., Shepherd, M. G., Sullivan, P. A. (1980). Regulation of chitin synthesis during germ-tube formation in *Candida albicans*. Arch. Microbiol. **125**, 97–104.
15. Durán, A., Bowers, B., Cabib, E. (1975). Chitin synthetase zymogen is attached to the yeast plasma membrane. Proc. Natl. Acad. Sci. USA **72**, 3952–3955.
16. Elorza, M. V., Rico, H., Gozalbo. D.. Sentandreu, R. (1983). Cell wall composition and protoplast regeneration in *Candida albicans*. Antonie van Leeuwenhoek **49**, 457–469.
17. Elorza, M. V., Rico, H., Sentandreu, R. (1983). Calcofluor white alters the assembly of chitin fibrils in *Saccharomyces cerevisiae* and *Candida albicans* cells. J. Gen. Microbiol. **131**, 1577–1582.
18. Fernández, M. P., Correa, J. U., Cabib, E. (1982). Activation of chitin synthetase in permeabilized cells of a *Saccharomyces cerevisiae* mutant lacking proteinase B. J. Bacteriol. **152**, 1255–1264.
19. Flores-Martinez, A., Schwencke, J. (1988). Chitin synthetase activity is bound to chitosomes and to the plasma membrane in protoplasts of *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **946**, 328–336.
20. Flores-Carreón, A., Larriba, G., Sentandreu, R. (1979). Stabilization of chitin synthase of *Mucor rouxii* and detection of two zymogenic pools. FEMS Microbiol. Lett. **8**, 151–155.
21. Gow, N. A. R., Swoboda, R., Bertram, G., Gooday, G. W., Brown, A. J. P. (1992). Key genes in the regulation of dimorphism of *Candida albicans*. *In* Vanden Bossche, H., Odds, F. C., Kerridge, D. (ed.), Dimorphic Fungi in Biology and Medicine, pp. 61–71. Plenum, New York.
22. Gozalbo, D., Dubón, F., Sentandreu, R. (1985). Studies on zymogenicity and solubilization of chitin synthase from *Candida albicans*. FEMS Microbiol. Lett. **26**, 59–63.
23. Gozalbo, D., Dubón, F., Schwencke, J., Sentandreu, R. (1987). Characterization of chitosomes in *Candida albicans* protoplasts. Exptl. Mycol. **11**, 331–338.
24. Gozalbo, D., Dubón, F., Sentandreu, R. (1991). Chitin synthetase activity in *Candida albicans*: subcellular distribution in yeast cells and protoplasts. Mycol. Res. **95**, 513–520.
25. Gozalbo, D., Dubón, F., Sentandreu, R. (1992). Chitin synthetase activity is bound to the plasma membrane and to a cytoplasmic particulate fraction in *Candida albicans* germ tube cells. FEMS Microbiol. Lett. **97**, 255–260.
26. Gozalbo, D., Dubón, F., Sentandreu, R. (1993). Effect of digitonin on membrane-bound and chitosomal chitin synthetase activity in protoplasts from yeast cells of *Candida albicans*. Antonie van Leeuwenhoek **64**, 67–74.
27. Hanseler, E., Nyhlen, L. E., Rast, D. M. (1983). Isolation and properties of chitin synthetase from *Agaricus bisporus* mycelium. Exptl. Mycol. **7**, 17–30.
28. Kang, M. S., Au-Young, J., Cabib, E. (1985). Modification of yeast plasma membrane density by concanavalin A attachment. Application to study of chitin synthetase distribution. J. Biol. Chem. **260**, 12680–12684.
29. Leal-Morales, C. A., Bracker, C. E., Bartnicki-García, S. (1988). Localization of chitin synthetase in cell-free homogenates of *Saccharomyces cerevisiae*: chitosomes and plasma membrane. Proc. Natl. Acad. Sci. USA **85**, 8516–8520.
30. Miragall, F., Rico, H., Sentandreu, R. (1988). Regeneration of the cell wall in protoplasts of *Candida albicans*. A cytochemical study using wheat germ agglutinin and Concanavalin A. Arch. Microbiol. **149**, 286–290.
31. Odds, F. C. (1988). *Candida* and candidosis. A review and bibliography. Bailliere Tindall, London.

32. Orlean, P. (1987). Two chitin synthases in *Saccharomyces cerevisiae*. J. Biol. Chem. **262**, 5732–5739.
33. Ram, S. P., Sullivan, P. A., Shepherd, M. G. (1983). The *in situ* assay of *Candida albicans* enzymes during yeast growth and germ-tube formation. J. Gen. Microbiol. **129**, 2367–2378.
34. Robbins, P. W., Bowen, A. R., Chen-Wu, J. L., Momany, M., Szaniszlo, P. J., Zwicker, J. (1993). The multiple chitin synthase genes of *Candida albicans* and other pathogenic fungi. A review. *In* Vanden Bossche, H., Odds, F. C., Kerridge, D. (ed.), Dimorphic Fungi in Biology and Medicine, pp. 51–59. Plenum, New York.
35. Ruiz-Herrera, J. (1992). Fungal cell wall: structure, synthesis and assembly. C.R.C. Press, Boca Ratón, Florida.
36. Ruiz-Herrera, J., Sentandreu, R., Martínez, J. P. (1992). Chitin biosynthesis in fungi. *In* Arora, D. K., Elander, R. P., Mukerji, K. G. (ed.), Handbook of Applied Mycology, vol. 4, pp. 281–312. Marcel Dekker, New York.
37. Sentandreu, R., Martínez, J. P., Elorza, M. V., Mormeneo, S. (1991). Relationships between dimorphism, cell wall structure and surface activities in *Candida albicans*. *In* Prasad, R. (ed.), *Candida albicans*: Cellular and Molecular Biology, pp. 53–70. Springer-Verlag, New York.
38. Sentandreu, R., Elorza, M. V., Mormeneo, S., Sanjuan, R., Iranzo, M. (1993). Possible roles of mannoproteins in the construction of *Candida albicans* cell wall. *In* Vanden Bossche, H., Odds, F. C., Kerridge, D. (eds.), Dimorphic fungi in biology and medicine, pp. 169–175. Plenum, New York.
39. Shaw, J. A., Mol. P. C., Bowers, B., Silverman, S. J., Valdivieso, M. H., Durán, A., Cabib, E. (1991). The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. J. Cell Biol. **114**, 111–123.
40. Shepherd, M. G. (1987). Cell envelope of *Candida albicans*. Crit. Rev. Microbiol. **15**, 7–25.
41. Valdivieso, M. H., Mol, P. C., Shaw, J. A., Cabib, E., Durán, A. (1991). Cloning of *CAL1*, a gene required for activity of chitin synthase 3 in *Saccharomyces cerevisiae*. J. Cell Biol. **114**, 101–109.
42. Watanabe, T., Oyanagi, W., Suzuki, K., Ohnishi, K., Tanaka, H. (1992). Structure of the gene encoding chitinase D of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class III plant chitinases. J. Bacteriol. **174**, 408–414.

MICROBIOLOGÍA SEM **10** (1994), 249–256

Virulence factors and O groups of *Escherichia coli* strains isolated from cultures of blood specimens from urosepsis and non-urosepsis patients

Miguel Blanco,[1] Jesús E. Blanco,[1] M. Pilar Alonso,[2] Jorge Blanco[1,*]

[1]*Departamento de Microbioloxía e Parasitoloxía, Facultade de Veterinaria, Universidade de Santiago de Compostela*
[2]*Unidade de Microbioloxía, Complexo Hospitalario Xeral-Calde, Lugo*

Received 29 October 1993/Accepted 27 April 1994

Summary

Ninety-six *Escherichia coli* strains isolated from cultures of blood specimens taken from both urosepsis patients (n = 48) and non-urosepsis patients (n = 48) were examined for the production of α-haemolysin (Hly) and cytotoxic necrotizing factor type 1 (CNF1), the expression of P-fimbriae and mannose-resistant haemagglutination (MRHA). Twenty-seven (56%) of the *E. coli* strains from urosepsis showed some of the virulence factors investigated, whereas only 15 (31%) of the strains associated with non-urosepsis possessed virulence factors ($P < 0.05$). By contrast, only 16% ($P < 0.001$) of the faecal isolates from healthy individuals were virulent. Of the bacteremic *E. coli* strains, 56 (58%) belonged to one of 8 serogroups (O1, O2, O4, O6, O8, O9, O18 and O83). Virulence factors were concentrated in strains belonging to serogroups O2, O4, O6, O18 and O83. Thus, 23 (72%) of the 32 strains of these 5 groups showed virulence factors, but only 19 (30%) of the 64 strains belong to other serogroups ($P < 0.001$). The majority of bacteremic O2, O4, O6 and O83 *E. coli* strains were Hly$^+$CNF1$^+$ and expressed P-fimbriae or MRHA type III, whereas the strains of serogroup O18 were Hly$^+$CNF1$^-$ and had P-fimbriae. We conclude that strains from urosepsis show more virulence factors than bacteremic strains isolated from non-urosepsis.

Key words: bacteremia, *Escherichia coli*, pathogenicity, toxins, virulence

* *Correspondence to*: Jorge Blanco. Departamento de Microbioloxía e Parasitoloxía. Facultade de Veterinaria. Universidade de Santiago de Compostela. 27002 Lugo. Tel.: 982-252231. Fax: 982-252195.

Resumen

El objetivo de este estudio es comparar los factores de virulencia y los serogrupos O de las cepas de *Escherichia coli* causantes de urosepsis con los de las cepas asociadas con bacteriemias de otros orígenes. La producción de α-hemolisina (Hly) y del factor necrótico citotóxico tipo 1 (CNF1), la expresión de fimbrias P y de los tipos de hemaglutinación resistente a la manosa (MRHA) III, IVa y IVb, se detectaron más frecuentemente en cepas causantes de urosepsis que en cepas aisladas de otros tipos de sepsis. Concretamente, 27 (56%) de las 48 cepas de urosepsis presentaron al menos uno de los factores de virulencia estudiados, frente a tan sólo 15 (31%) de las 48 cepas aisladas de otros tipos de sepsis ($P < 0,05$). En cambio, solamente el 16% (24/154) de las cepas fecales de control aisladas de personas sanas presentaron factores de virulencia ($P < 0,001$). El 58% (56/96) de las *E. coli* bacteriémicas pertenecieron a ocho serogrupos (O1, O2, O4, O6, O8, O9, O18 and O83), los cuales predominaron tanto entre las cepas aisladas de urosepsis como de sepsis de otros orígenes. Los factores de virulencia se encontraron en las cepas de los serogrupos O2, O4, O6, O18 y O83, ya que 23 (72%) de las 32 cepas de estos cinco serogrupos mostraron factores de virulencia, frente a tan sólo 19 (30%) de las 64 cepas pertenecientes a otros serogrupos ($P < 0,001$). La mayoría de las *E. coli* bacteriémicas de los serogrupos O2, O4, O6 y O83 resultaron ser Hly^+CNF1^+ y expresaban fimbrias P o el tipo MRHA III, mientras que las cepas del serogrupo O18 eran Hly^+CNF1^- y tenían fimbrias P. Se concluye que las *E. coli* bacteriémicas de origen urinario poseen más factores de virulencia que las cepas responsables de sepsis de otros orígenes.

Introduction

Escherichia coli is one of the most common microorganisms found in blood cultures. Bacteria reach the bloodstream from many different sites of infection, especially from the urinary tract (2,16). Although numerous studies have addressed the frequency of virulence factors in *E. coli* cultured from the urine of patients with cystitis and acute pyelonephritis, little is known about the virulence factors of bacteremic *E. coli* (1, 2, 11, 12, 16, 18). Blanco et al. (1) have recently described that *E. coli* isolated from sepsis belong to a limited number of serogroups, mainly O2, O4, O6, O7, O8 and O75, and show virulence factors similar to those shown by strains responsible for urinary tract infections (UTI), such as the production of the α-haemolysin (Hly) and the cytotoxic necrotizing factor type 1 (CNF1) and the expression of P-fimbriae and the mannose-resistant haemagglutination (MRHA) types III, IVa and IVb. In this study we have compared the virulence factors and serogroups of the bacteremic *E. coli* strains that cause urosepsis (bacteremia arising from primary infection of the urinary tract) with those isolated from non-urosepsis.

Materials and methods

Patients and clinical isolates of *E. coli*. Ninety-six *E. coli* strains were isolated from cultures of blood specimens taken from patients treated in the Hospital Xeral-Calde of Lugo and in the Hospital Comarcal da Costa of Burela (Galicia, North-western Spain) between 1989 and 1992. The patients

belonged to different age groups (range 15 days to 92 years). Patients were grouped according to the source of the bacteremia by retrospective review of clinical data obtained from hospital records. Only when strong (> 10^5 bacteria/ml of urine and/or symptoms and signs of UTI) evidence that a simultaneous *E. coli* urinary tract infection was the probable source of the bacteremia it was the infection classified as *E. coli* urosepsis. The patient charts were reviewed before knowing the results of the parameters investigated. From each patient, a single *E. coli* colony was examined. One hundred and fifty-four *E. coli* strains isolated from the stools of 102 healthy individuals from a widely varying age group were used as controls. From each faecal sample five colonies with the typical appearance of *E. coli* were randomly chosen, investigated for production of toxins, MRHA and P-fimbriae, then serotyped. When one healthy person yielded colonies with different toxic phenotypes, MRHA types or serogroups, only one of each was selected for statistical analysis. In individuals in which the five faecal isolates were identical, only one was considered for comparative studies (Table 1). Isolation and identification of *E. coli* was performed by standard bacteriological methods. Strains were stored at room temperature in nutrient broth (Difco) with 0.75% of agar.

Production and detection of toxins. Haemolysis was observed after 24 h of growth on blood agar base medium containing 5% (v/v) washed sheep erythrocytes. For detection of CNF1 the filtrates of cultures treated with mitomycin C were assayed on Vero and HeLa cell monolayers as previously described (4). Seroneutralization assays with CNF1 antiserum were carried out to confirm the production of CNF1 (8).

P-fimbriae and haemagglutination tests. Identification of P-fimbriae was performed by a commercial P-fimbriae-specific agglutination test (PF test, Orion Diagnostica, Finland) (13). Mannose-resistant haemagglutination (MRHA) was determined by the rocked-tile method with human group A, calf, guinea-pig, adult chicken, sheep and pig erythrocytes as previously described (1). Strains were grouped according to their MRHA patterns into the six MRHA types (I to VI) of our classification (1). For both P-fimbriae and haemagglutination tests, bacteria were grown in batch culture in Mueller-Hinton broth at 37°C for 5 days and the pellicle formed was plated on CFA agar and incubated at 37°C for 18 h.

Serotyping. Serotyping of the O antigen was carried out by means of a micro-technique described by Guinée et al. (10) and modified by us, using the 101 antisera listed in a published paper (3). The antisera were absorbed with the corresponding cross-reacting antigens to remove the non-specific agglutinins. The antisera were obtained from the National Institute of Public Health and Environmental Protection (Bilthoven, Netherlands) and Difco Laboratories (Detroit, MI).

Control strains. Reference *E. coli* strains used as positive and negative controls were: C1212-17 (O6:K12:H1, P-fimbriated, MRHA IVa$^+$, Hly$^+$), C1976-79 (O1:K1:H7, P-fimbriated, MRHA IVa$^+$, Hly$^+$), MR48 (O75:K95, MRHA III$^+$, CNF1$^+$, Hly$^+$), MR199 (O6:K13, MRHA III, CNF1$^+$, Hly$^+$) and K12-185 (non-toxigenic strain MRHA$^-$).

Statistical methods. Results were compared by using the χ^2 test with Yates' correction for continuity.

Results and discussion

E. coli is the most common Gram-negative bacillus causing infection of the bloodstream and urinary system (2, 16). Adherence of bacteria to the host epithelium is an important virulence factor; in uropathogenic *E. coli* this is mediated mainly by P-fimbriae. P-fimbriation is characteristic of strains causing upper urinary tract infections in children and adults as well as pyelonephritis leading to urosepsis in adults (12, 13). Besides bacterial adherence, several virulence factors may contribute to the pathogenicity of bacteremic *E. coli*, such as the production of Hly and CNF1 (1, 6). We have previously developed a simple, rapid and economical typing system of *E. coli* strains based on their MRHA pattern that was found to be useful for the presumptive identification of pathogenic strains. MRHA types I and II are expressed only by enterotoxigenic *E. coli* (producing LT and/or STa enterotoxins) with colonization factors CFA/I and CFA/II respectively (5); MRHA III appears in Hly$^+$ strains producing CNF1 without P-fimbriae; MRHA types IVa and IVb are specific for P-fimbriated *E. coli*, some of which also produce CNF1 and/or Hly; and MRHA types V and VI are usually expressed by *E. coli* without virulence factors (5). In the present study the production of Hly and CNF1, the expression of P-fimbriae and MRHA types III, IVa and IVb were all detected more frequently in bacteremic *E. coli* than in strains isolated from the faeces of healthy people. In total, 42 (44%) of the *E. coli* strains isolated from sepsis showed some of the virulence factors investigated in comparison with only 24 (16%) of 154 faecal control strains ($P < 0.001$) (Table 1). Furthermore, 27 (56%) of the 48 *E. coli* strains from urosepsis showed some of the virulence factors investigated, whereas only 15 (31%) of the 48 strains associated with non-urosepsis possessed virulence factors ($P < 0.05$) (Table 2).

TABLE 1
DISTRIBUTION OF VIRULENCE FACTORS IN BACTEREMIC STRAINS AND IN ISOLATES FROM THE FAECES OF HEALTHY PEOPLE

Virulence factors	Number of strains from[a]			
	Blood (n=96)		Faeces (n=154)	
Hly	31	(32)	17	(11)
CNF1	21	(22)	10	(6)
P-fimbriae	20	(21)	7	(5)
MRHA type III	11	(11)	5	(3)
MRHA type IVa	18	(19)	7	(5)
MRHA type IVb	7	(7)	1	(0.6)
With virulence factors	42	(44)	24	(16)

[a]Percentage enclosed in parentheses.

TABLE 2
VIRULENCE FACTORS OF *E. coli* STRAINS ISOLATED FROM PATIENTS WITH UROSEPSIS AND SEPSIS FROM OTHER SOURCES

Virulence factors	Number of bacteremic strains from[a]			
	Urosepsis (n=48)		Non-urosepsis (n=48)	
Hly	19	(40)	12	(25)
CNF1	14	(29)	7	(15)
P-fimbriae	13	(27)	7	(15)
MRHA type III	9	(19)	2	(4)
MRHA type IVa	10	(21)	8	(17)
MRHA type IVb	6	(13)	1	(2)
With virulence factors	27	(56)	15	(31)

[a]Percentage enclosed in parentheses.

E. coli strains from a relatively small number of O serogroups, namely O1, O2, O4, O6, O7, O8, O9, O16, O18 and O75, have been reported to account for most of O-groupable bacteremic strains from different areas of the world (7, 9, 15, 16, 17, 19). These O groups were frequently detected also in *E. coli* strains from urinary tract infections, but they were rarely found in strains isolated from stools of healthy people. The 96 bacteremic *E. coli* isolated in Spain belonged to 24 different serogroups. However, 56 (58%) were from one of these 8 serogroups (O1, O2, O4, O6, O8, O9, O18 and O83) (Table 3). It has been repeatedly suggested that strains belonging to these serogroups possessed specific virulence factors which confer on them their special invasive ability. Our results support this suggestion. Virulence factors occurred especially in the strains of serogroups O2, O4, O6, O18 and O83. Thus, 23 (72%) of the 32 strains of these 5 groups showed some of the virulence factors investigated, but only 19 (30%) of the 64 strains belong to other serogroups ($P < 0.001$) (Table 3). The majority of bacteremic O2, O4, O6 and O83 *E. coli* strains were Hly^+CNF1^+ and expressed either P-fimbriae or the adhesin responsible for MRHA type III, whereas the strains of serogroup O18 were Hly^+CNF1^- and P-fimbriated. We detected adhesins associated with virulence in 81% of Hly^+CNF1^+ strains, in 90% of Hly^+CNF1^- strains and in 17% of Hly^-CNF1^- strains. Therefore, the expression of P-fimbriae and MRHA types III, IVa and IVb were detected in 26 (84%) of 31 toxigenic strains and in 11 (17%) of 65 non-toxigenic bacteremic *E. coli* strains ($P < 0.001$) (Table 4). As in previous studies (1, 6), we found that CNF1 producing strains are Hly^+ and that the majority (75%) of P-fimbriated strains belong to MRHA type IVa.

TABLE 3
RELATIONSHIP OF BACTEREMIC *E. coli* SEROGROUPS WITH VIRULENCE FACTORS

	Number of strains							
	Isolated from					Of MRHA types		With virulence factors
Serogroups	Urosepsis (n=48)	Non-urosepsis (n=48)	Hly$^+$ (n=31)	CNF1$^+$ (n=21)	PF$^+$[a] (n=20)	III (n=11)	IVa or IVb (n=25)	(n=42)
O1	5	6	1	0	3	0	3	3 (27%)
O2	4	4	3	3	3	2	2	4 (50%)
O4	5	1	6	6	3	3	3	6 (100%)
O6	4	4	5	4	2	1	1	5 (63%)
O8	3	4	0	0	0	0	1	1 (14%)
O9	2	4	0	0	0	0	1	1 (17%)
O18	3	3	6	0	5	0	5	6 (100%)
O83	3	1	2	2	0	1	1	2 (50%)
Other[b]	13	10	6	6	2	4	4	10 (43%)
NT[c]	6	11	2	0	2	0	4	4 (24%)

[a] P-fimbriae.
[b] Represented by three or less strains and belonging to serogroups: O5, O11, O14, O15, O20, O21, O22, O25, O50, O75, O78, O84, O117, O134, O141 and O147.
[c] Not typeable with the 101 antisera used.

TABLE 4
P-FIMBRIATION AND MRHA TYPES III, IVa AND IVb IN TOXIGENIC AND NON-TOXIGENIC BACTEREMIC *E. coli* STRAINS

	Number of strains				
			Of MRHA types		With adhesins[b]
Toxic phenotypes	Tested	PF$^+$[a]	III	IVa or IVb	
Hly$^+$ CNF1$^+$	21	6	11	6	17 (81%)
Hly$^+$ CNF1$^-$	10	9	0	9	9 (90%)
Hly$^-$ CNF1$^-$	65	5	0	10	11 (17%)

[a] P-fimbriae.
[b] P-fimbriated or with MRHA type III, IVa or IVb.

Adherence to host cells is regarded as a critical initial step in bacterial colonization and subsequent invasion. Our results strongly support the hypothesis that pathogenic potential of bacteremic *E. coli* infecting urinary tract is closely related to the expression of adhesins that mediate colonization and facilitate subsequent invasion. However, although a substantial proportion of bacteremic strains had at least one of the virulence factors evaluated, 56% were negative; this result suggests that such virulence factors should not be strictly necessary for the development of bacteremia.

While virulence factors appear to contribute to the development of bacteremia, we observed similar mortality regardless of whether infecting isolates had adhesins (P-fimbriae and/or MRHA types III, IVa and IVb) or toxins (Hly and/or CNF1). Like Maslow et al. (14), we found that mortality following *E. coli* bacteremia appeared to be independent of bacterial virulence factors and to be determined primarily by the severity of the host's underlying disease (unpublished results).

We conclude that *E. coli* strains isolated from urosepsis show virulence factors more frequently than bacteremic *E. coli* from non-urosepsis, and that *E. coli* strains that cause sepsis in Spain belong to the same serogroups as bacteremic strains isolated in other geographical areas around the world.

Acknowledgments

This study was supported by grants from FIS (90/0447-2 and 92/5675) and from Xunta de Galicia (XUGA 84301188) and from CICYT (AGF92-0570). M. B. and J. E. B. acknowledge the DGICYT of the Spanish Ministry of Education and Science for FPI research fellowships. We thank M. Cristina Prado, M. Luisa López and Inés Ferreiro for skillful technical assistance.

References

1. Blanco, J., Alonso, M. P., González, E. A., Blanco, M., Garabal, J. I. (1990). Virulence factors of bacteremic *Escherichia coli* with particular reference to production of cytotoxic necrotising factor (CNF) by P-fimbriate strains. J. Med. Microbiol. **31**, 175–183.
2. Blanco, J., Alonso, M. P., Blanco, M., González, E. A. (1991). Mecanismos de patogénesis de *Escherichia coli* causantes de infecciones extraintestinales. Enf. Infec. Microbiol. Clin. **9**, 640–651.
3. Blanco, J., Blanco, M., Alonso, M. P., Blanco, J. E., Garabal, J. I., González, E. A. (1992). Serogroups of *Escherichia coli* strains producing cytotoxic necrotizing factors CNF1 and CNF2. FEMS Microbiol. Lett. **96**, 155–160.
4. Blanco, J., Blanco, M., González, E. A., Alonso, M. P., Garabal, J. I. (1990). Comparative evaluation of three tests for the detection of *Escherichia coli* cytotoxic necrotizing factors (CNF1 and CNF2) using filtrates of cultures treated with mitomycin C. FEMS Microbiol. Lett. **69**, 311–316.
5. Blanco, J., González, E. A., Blanco, M., Garabal, J. I., Alonso, M. P., Jansen, W. H., Guinée, P. A. M. (1989). Prevalence of enterotoxigenic *Escherichia coli* strains in outbreaks and sporadic cases of diarrhoea in Spain. Eur. J. Clin. Microbiol. Infect. Dis. **8**, 396–400.
6. Caprioli, A., Falbo, V., Ruggeri, F. M., Minelli, F., Ørskov, I., Donelli, G. (1989). Relationship between cytotoxic necrotizing factor production and serotype in hemolytic *Escherichia coli*. J. Clin. Microbiol. **27**, 758–761.

7. Cross, A. S., Gemski, P., Sadoff, J. C., Ørskov, F., Ørskov, I. (1984). The importance of the K1 capsule in invasive infections caused by *Escherichia coli*. J. Infect. Dis. **149**, 184–193.
8. De Ryche, J., González, E. A., Blanco, J., Oswald, E., Blanco, M., Boivin, R. (1990). Evidence for two types of cytotoxic necrotizing factor in human and animal clinical isolates of *Escherichia coli*. J. Clin. Microbiol. **28**, 694–699.
9. Devine, D. A., Robinson, L., Roberts, A. P. (1989). Occurrence of K1, K5 and O antigens in *Escherichia coli* isolates from patients with urinary tract infections or bacteremia. J. Med. Microbiol. **30**, 295–299.
10. Guinée, P. A. M., Agterberg, C. M., Jansen, W. H. (1972). *Escherichia coli* O antigen typing by means of a mechanized microtechnique. Appl. Microbiol. **24**, 127–131.
11. Johnson, J. R., Moseley, S. L., Roberts, P. L., Stamm, W. E. (1988). Aerobactin and other virulence factor genes among strains of *Escherichia coli* causing urosepsis: association with patient characteristics. Infect. Immun. **56**, 405–412.
12. Johnson, J. R., Roberts, P. L., Stamm, W. E. (1987). P fimbriae and other virulence factors in *Escherichia coli* urosepsis: association with patients' characteristics. J. Infect. Dis. **156**, 225–229.
13. Kärkkäinen, U., Ikäheino, R., Katila, M. L., Mántyjärvi, R. (1991). P fimbriation of *Escherichia coli* strains from patients with urosepsis demonstrated by a commercial agglutination test (PF TEST). J. Clin. Microbiol. **29**, 221–224.
14. Maslow, J. N., Mulligan, M. E., Adams, K. S., Justis, J. C., Arbeit, R. D. (1993). Bacterial adhesins and host factors: role in the development and outcome of *Escherichia coli* bacteremia. Clin. Infect. Dis. **17**, 89–97.
15. McCabe, W. R., Kaijser, B., Ollings, J., Uwaydah, M., Hanson, L. A. (1978). *Escherichia coli* in bacteremia: K and O antigens and serum sensitivity of strains from adults and neonates. J. Infect. Dis. **138**, 33–41.
16. Ørskov, I., Ørskov, F. (1985). *Escherichia coli* in extra-intestinal infections. J. Hyg. **95**, 551–575.
17. Ørskov, F., Ørskov, I. (1975). *Escherichia coli* O:H serotypes isolated from human blood. Acta Pathol. Microbiol. Scand. B **83**, 585–600.
18. Vázquez, F., Mendoza, M. C., Viejo, G., Méndez, F. J. (1992). Survey of *Escherichia coli* septicemia over a six-year period. Eur. J. Clin. Microbiol. Infect. Dis. **11**, 110–117.
19. Vosti, K. G., Goldberg, L. M., Monto, A. S., Rantz, L. A. (1964). Host parasite interaction in patients with infections due to *Escherichia coli*. The serogrouping of *Escherichia coli* from intestinal and extraintestinal sources. J. Clin. Invest. **43**, 2377–2385.

MICROBIOLOGÍA SEM **10** (1994), 257– 262

Incidence of motile *Aeromonas* spp. in foods

Carmen Pin,* M. Luisa Marín, M. Luisa García, José Tormo,
M. Dolores Selgas, Carmen Casas

*Departamento de Nutrición y Bromatología III, Facultad de Veterinaria,
Universidad Complutense de Madrid*

Received 14 October 1993/Accepted 27 April 1994

Summary

A total of 80 food samples were purchased from local retail consumer shops and examined for the presence of motile *Aeromonas* spp. Of the food categories tested, poultry had the highest incidence, with 100% positive. This was followed by lamb samples, with 60% positive. Raw milk and cheese samples had very low incidence (20%). No motile *Aeromonas* spp. were found in pre-prepared salads. Shellfish, fish, pork and beef samples had incidences of 40%. Most of the strains isolated were *Aeromonas hydrophila*, and for most of the food categories, no *Aeromonas caviae* isolates were obtained.

Key words: *Aeromonas* spp., food, food-borne pathogens

Resumen

Con el fin de determinar la presencia de *Aeromonas* spp. móviles en alimentos, se ha estudiado un total de 80 muestras pertenecientes a diferentes categorías de alimentos, adquiridas en locales de venta al público. La mayor incidencia de *Aeromonas* se detectó en carne de pollo (100% de las muestras fueron positivas). La leche cruda y el queso registraron los niveles más bajos (20%). No se detectó la presencia de *Aeromonas* en ninguna de las muestras de ensaladas analizadas. En marisco, pescado y carnes de cerdo y vacuno la incidencia fue de un 40%. La mayoría de las cepas aisladas pertenecían a *Aeromonas hydrophila*, mientras que *Aeromonas caviae* estaba ausente en la mayoría de los alimentos analizados.

* *Correspondence to*: Carmen Pin. Departamento de Nutrición y Bromatología III (Higiene y Tecnología de los Alimentos). Facultad de Veterinaria. Universidad Complutense de Madrid. 28040 Madrid. Tel.: 91-3943749. Fax: 91-3943743.

Introduction

The significance of *Aeromonas* species as potential intestinal pathogens is controversial. The increasing number of reports in recent years that implicated *Aeromonas* spp. as the etiologic agent of acute diarrhoea suggests that these organisms are more significance than it is currently appreciated. Buchanan and Palumbo (2) suggested that aeromonads, in particular *A. hydrophila* and *A. sobria,* may represent significant «new» food-borne pathogens and hypothesized that foods may play a major role in the dissemination of these microorganisms. So, aeromonads have been isolated from different foods such as meat, sea-food, dairy products and vegetables (6, 10). *Aeromonas* spp. are also commonly isolated from drinking water and chlorinated drinking water (8, 16). However, little is known about the occurrence, growth, behavior and significance of these organisms in food.

The aims of this study were to determine the incidence of motile *Aeromonas* spp. in food samples and to identify the isolated strains to a species level.

Materials and methods

Samples. A total of 80 samples were examined. Fresh fish, shellfish, raw meat, unripened cheese and pre-prepared salads were purchased and collected aseptically in their original packings from different retail consumer shops. Raw milk was collected from a farm. Number of samples in each case is indicated in Table 1.

Enrichment and isolation. Twenty-five grams of each food sample were aseptically removed and placed in Stomacher bags. Subsequently, 225 ml of alkaline peptone water (APW) pH 8.4-8.6 was added, and the sample homogenized in a Stomacher 400 (Colworth, London, UK) for 2 min. The homogenized samples in alkaline peptone water were enriched by incubating them at 30°C for 24 h.

Serial dilutions of each enrichment were then prepared and 0.1 ml amounts were plated on Oxoid *Aeromonas* Ampicillin medium (AAM) (Oxoid, Basingstoke, U.K.). Plates were incubated at 30°C for 24 h.

Confirmation of isolates. Typical *Aeromonas* colonies from AAM (green, opaque, 0.5–1.5 mm in diameter) were tested for oxidase production and ability to ferment glucose.

All oxidase-positive, glucose fermenters were considered presumptive positive, and tested for: ONPG (+), DNase (+), catalase (+), growth at 37°C (+), growth in nutrient broth without NaCl (+), starch hydrolysis (+), fermentation of D-mannitol (+) and i-inositol (–), H_2S production from thiosulfate (–) and urea (–), to confirm motile *Aeromonas* spp.

Adscription to species was carried out following Popoff (14).

Results and discussion

Since 1:10 dilution is the commonly used procedure for the examination of foods (quantitative and isolation purposes), samples with low *Aeromonas* levels might be misjudged. In this way, alkaline

TABLE 1
ISOLATION OF *Aeromonas* spp. FROM FOOD SAMPLES AFTER ENRICHMENT IN ALKALINE PEPTONE WATER

Sample type	Examined samples	Positive samples	
Fish	25	9	(36)[a]
Shellfish	20	9	(45)
Beef	5	2	(40)
Pork	5	2	(40)
Lamb	5	3	(60)
Poultry	5	5	(100)
Raw milk	5	1	(20)
Cheese	5	1	(20)
Pre-prepared salad	5	0	(0)
Total	80	32	(40)

[a] Percentage of positive samples.

peptone water (pH 8.4–8.6) has been recommended as an enrichment broth to enhance the recovery of *Aeromonas* spp. from both environmental and clinical samples (11, 15). Accordingly, we incorporated this enrichment procedure.

The results of the isolation of *Aeromonas* spp. from samples of different types of food are summarized in Table 1. *Aeromonas* spp. were isolated from 40% of the food samples investigated. None of the samples of pre-prepared salad were found to contain *Aeromonas* spp., and samples of raw milk and cheese had the lowest incidence of the organisms (20%). In our study, pre-prepared salads consisted of pre-packaged raw vegetables without mayonnaise, plus one sample containing mayonnaise. Presumably, mayonnaise can diminish the pH of the food,either preventing the growth of, the *Aeromonas* spp. or killing it, if present (6). According to several authors, *Aeromonas* can frequently be found on, raw vegetables and grow on them (3). In contrast, Fricker and Tompsett (5) have found a low incidence of these organisms in salads, and Krovacek et al. (8) have also reported that none of the *Aeromonas* spp. were isolated from vegetables or raw milk samples. This could, however, be attributed to the very low, and initially undetectable, levels of *Aeromonas* in these foods. In fact, Palumbo et al. (12) have also shown that although *Aeromonas* were not detectable in raw milk samples at the time of purchase, the organisms could be isolated after 7 days of storage at 5°C.

The results of the analysis of the 20 raw meat and poultry samples showed that the highest isolate rate (100%) was detected with poultry samples. Fricker and Tompsett (5), Majeed et al. (10) and Krovacek et al. (8) have also found high numbers of *Aeromonas* spp. in poultry, pork, lamb and beef. Drazek et al. (4) have reported a very low incidence of *Aeromonas* in the faecal material of these animals, wich indicates that the organism has entered the food during handling.

Fish had a lower incidence of motile *Aeromonas* spp. than shellfish samples. 36% of the fish samples and 45% of the shellfish samples examined were found to contain *Aeromonas* spp. Similarly, Hudson and De Lacy (6) reported that 34% of the fish samples examined contained motile *Aeromonas* spp., and Abeyta and Wekell (1) found between 70 and 100% of the shellfish samples to be positive.

The distribution of isolates among the species *A. hydrophila, A. sobria* and *A. caviae,* as well as unclassified strains, is shown in Table 2. Strains were designated as «unclassified» when they could not be classified into any of the other three species, because they showed different biochemical patterns.

The species most frequently found in our study was *A. hydrophila*, in agreement with the data of Knöchel and Jeppesen (7). In several occasions, two different *Aeromonas* spp. were obtained from the same sample. Thus, *A. hydrophila, A. sobria* and one unclassified strain were isolated from one poultry sample, and *A. hydrophila, A. sobria* and *A. caviae* were isolated from a pork sample.

TABLE 2
DISTRIBUTION OF SPECIES OF *Aeromonas* IN DIFFERENT FOODS

Sample type	No. of strains	*Aeromonas hydrophila*	*Aeromonas sobria*	*Aeromonas caviae*	*Aeromonas* unclassified
Fish	12	6	1	0	5
Shellfish	12	4	4	3	1
Beef	2	2	0	0	0
Pork	6	2	3	1	0
Lamb	4	1	1	0	2
Poultry	8	2	3	0	3
Raw milk	2	2	0	0	0
Cheese	2	0	0	0	2
Salads	0	0	0	0	0
Total	48	19	12	4	13

Furthermore, the low number of *A. caviae* isolates found in this study is also observed. *A. caviae* isolates were obtained from one pork and three shellfish samples.

The present study shows that a high incidence of *Aeromonas* spp. was found in different types of food. This result is magnified by the fact that motile *Aeromonas* spp. can grow at refrigeration temperatures (13). Majeed et al. (10) reported that growth of *Aeromonas* spp. at 5°C is of considerable practical importance since refrigeration of animal carcasses in the abattoir and the processed meat may no arrest their growth. Similarly, motile aeromonads may multiply on ready-to-eat foods during display in refrigerated cabinets (6).

The present specific public health significance of these findings is unknown since clear well-documented data about the role of *Aeromonas* species as food-borne pathogens are lacking. However, Krovacek et al. (8) reported that most of *Aeromonas* strains isolated from different foods were capable of producing a variety of virulence determinants such as haemolysin, cytotoxin, cytotonic toxin and proteases. Our observations suggest that it should be given adequate attention to the presence of *Aeromonas* species in foods since it may play major role in the etiology of human gastroenteritis outbreaks.

References

1. Abeyta, C. Jr., Wekell, M. M. (1988). Potential sources of *Aeromonas hydrophila*. J. Food Safety **9**, 11–22.
2. Buchanan, R. L., Palumbo, S. A. (1985). *Aeromonas hydrophila* and *Aeromonas sobria* as potential food poisoning species: a review. J. Food Safety **7**, 15–20.
3. Callister, S. M., Agger, W. A. (1987). Enumeration and characterization of *Aeromonas caviae* isolated from grocery store produce. Appl. Environ. Microbiol. **53**, 249–253.
4. Drazek, E. S., Stern, N. J., Joseph, S. (1986). Low incidence of *Aeromonas* spp. in livestock feces. Abstr. Annual Meeting Am. Soc. Microbiol. p. 276.
5. Fricker, C. R., Tompsett, S. (1986). *Aeromonas* spp. in foods: a significant cause of food poisoning? Int. J. Food Microbiol. **9**, 17–23.
6. Hudson, J. A., De Lacy, K. M. (1991). Incidence of motile aeromonads in New Zealand retail foods. J. Food Prot. **54**, 696–699.
7. Knöchel, S., Jeppesen, C. (1990). Distribution and characteristics of *Aeromonas* in food and drinking water in Denmark. Int. J. Food Microbiol. **10**, 317–322.
8. Krovacek, K., Faris, A., Baloda, S. B., Peterz, M., Lindberg, T., Mansson, I. (1992a). Prevalence and characterization of *Aeromonas* spp. isolated from foods in Uppsala, Sweden. Food Microbiol. **9**, 29–36.
9. Krovacek, K., Faris, A., Baloda, S. B., Lindberg, T., Peterz, M., Mansson, I. (1992b). Isolation and virulence profiles of *Aeromonas* spp. from different municipal drinking water supplies in Sweden. Food Microbiol. **9**, 215–222.
10. Majeed, K., Egan, A., MacRae, I. C. (1989). Enterotoxigenic aeromonads on retail lamb meat and offal. J. Appl. Bacteriol. **67**, 165–170.
11. Millership, S. E., Chattopadhyay, B. (1984). Methods for the isolation of *Aeromonas hydrophila* and *Plesiomonas shigelloides* from faeces. J. Hyg. **92**, 145–152.
12. Palumbo, S. A., Maxino, F., Williams, A. C., Buchanan, R. L., Thayer, D. W. (1985). Starch ampicillin agar for the quantitative detection of *Aeromonas hydrophila*. Appl. Environ. Microbiol. **50**, 1027–1030.
13. Palumbo, S. A., Buchanan, R. L. (1988). Factors affecting growth and survival of *Aeromonas hydrophila* in foods. J. Food Safety **9**, 37–51.

14. Popoff, M. (1984). Genus III. *Aeromonas* Kluyver and Van Niel 1936, 398^{AL}. *In* Krieg, N. R., Holt, J. G. (ed.), Bergey's Manual of Systematic Bacteriology, vol. 1, pp. 545–548. Williams and Wilkins, Baltimore.
15. Robinson, J., Beaman, J., Wagewner, L., Burke, V. (1986). Comparison of direct plating with the use of enrichment culture for isolation of *Aeromonas* spp. from faeces. J. Med. Microbiol. **22**, 315–317.
16. Van der Kooij, D., Hijnen, W. A. M. (1988). Nutritional versatility and growth kinetics of an *Aeromonas hydrophila* strain isolated from drinking water. Appl. Environ. Microbiol. **54**, 2842–2851.

MICROBIOLOGÍA SEM **10** (1994), 263–272

Different responses of the marine diatom *Phaeodactylum tricornutum* to copper toxicity

Susana Reiriz, Ángeles Cid, Enrique Torres, Julio Abalde, Concepción Herrero*

Laboratorio de Microbiología, Facultad de Ciencias, Universidad de La Coruña

Received 10 March 1994/Accepted 27 July 1994

Summary

Different responses of the marine diatom *Phaeodactylum tricornutum* (Bohlin) to toxic copper concentrations were investigated. Besides the classical variables applied to toxicity studies in microalgae, such as growth or chlorophyll *a* content, other variables analyzed by flow cytometry were used. Toxic effects due to copper concentration were observed. Cell density reached in the stationary phase was reduced to 50% in cultures with 20 mg Cu/l, with respect to control cultures without copper. Cell light scatter properties (related to cell volume and intracellular granularity) and chlorophyll *a* fluorescence of microalgal cells were determined by flow cytometry analysis at the beginning of growth, 1 h after copper exposure, and when cultures reached the stationary phase (72 h). After 1 h of exposure to metal, no differences were observed, but when cultures reached the stationary phase, a gradual increase in the variables analyzed by flow cytometry was observed as the copper concentration increased. The increase in chlorophyll *a* fluorescence detected by flow cytometry was not correlated with an increase in the cell content of this photopigment, thus indicating an inhibitory effect of copper on photosystem II.

Key words: marine microalgae, *Phaeodactylum tricornutum*, copper, toxicity, flow cytometry

Resumen

En el presente trabajo se han estudiado diferentes respuestas de la diatomea marina *Phaeodactylum tricornutum* (Bohlin) frente a la toxicidad ejercida por diferentes concentraciones de cobre. Además de

* *Correspondence to*: Concepción Herrero. Laboratorio de Microbiología. Facultad de Ciencias. Universidad de La Coruña. Campus da Zapateira, s/n. 15071 La Coruña. Tel.: 981-280788. Fax: 981-104129.

las variables clásicas utilizadas en los estudios de toxicidad con microalgas, se han utilizado otras variables analizadas por citometría de flujo. Se han observado efectos tóxicos producidos por las concentraciones de cobre utilizadas. La densidad celular alcanzada en la fase estacionaria se redujo en un 50% en los cultivos con 20 mg Cu/l, con respecto a la observada en los cultivos sin cobre. Mediante citometría de flujo se analizó el volumen y granularidad celular, además de la fluorescencia emitida por la clorofila *a* después de 1 h de exposición al cobre y en la fase estacionaria del crecimiento (72 h). Después de 1 h de exposición al metal no se observaron diferencias, mientras que en la fase estacionaria se observó un aumento gradual de las variables analizadas por citometría de flujo, a medida que la concentración de cobre aumentaba. El incremento observado en la fluorescencia de la clorofila *a* detectada por citometría de flujo no estaba correlacionado con el contenido celular de este fotopigmento, lo que parece indicar un efecto inhibitorio del cobre sobre el fotosistema II.

Introduction

Heavy metals are among the most toxic pollutants to living organisms in aquatic environments. The effects of heavy metals on aquatic organisms have been extensively studied, and algae have received much of the attention. Initial interest in studying toxicity focused on lethal effects. However, knowledge of sublethal effects is essential to understand the long-term consequences of pollution in ecosystems, and the need for convenient methods and variables to assess pollutant toxicity has become obvious. Microalgae have already been used as biological indicators for this purpose (12, 14, 20).

It has been confirmed that inhibition of growth and photosynthesis, as well as other variables closely related to photosynthesis, such as ATP formation, radioactive carbon assimilation, oxygen evolution and algal fluorescence induction phenomena, reflect the toxic effects of pollutants (17, 24, 29). Nevertheless, other variables are less known because their study is difficult, especially *in vivo* assays. Flow cytometry (FCM) has been introduced as an alternative to more traditional techniques of analyzing cells in cultures and from natural populations (10, 13, 18, 27, 28). In FCM, simultaneous measurements of individual particle cellular volume, fluorescence and light scatter properties are directly applicable in aquatic research. In the case of phytoplankton, the pigmented cells can be detected and identified by their autofluorescence and light scattering properties (10). Since then, FCM could be used in phytoplankton toxicological studies.

Copper, an essential trace metal for microalgae, can be toxic to microalgae at high concentrations. In this study, the effect of high copper concentrations on light scatter properties and chlorophyll *a* fluorescence of the marine diatom *Phaeodactylum tricornutum* (Bohlin) has been investigated by FCM. Growth, a classical variable used in phytotoxicity work, and cellular chlorophyll *a* content were also studied.

Materials and methods

The marine diatom *P. tricornutum* (obtained from J. Fábregas, Department of Microbiology and Parasitology, University of Santiago de Compostela, Spain) was cultured in batch conditions in seawater filtered through a 0.45 µm Millipore filter, autoclaved at 121°C for 20 min and enriched with the modified culture medium of Fábregas et al. (3) without EDTA and copper. Stock cultures were incubated at 18 ± 1°C in cyclic light (light:dark, 12h:12h) provided by Mazda Fluor C7 TF 40 fluorescent tubes (68 µmol photon/m²/s).

Experiments were carried out in Kimax tubes containing 40 ml of culture medium. Test tubes and other glassware used were acid washed (nitric acid 10% v/v) and sterilized.

Experimental aliquots were taken from a stock culture in exponential phase to give equal initial cell concentrations in each subculture. The initial cell density was 0.6 x10^6 cells/ml. All experimental treatments were carried out in triplicate.

Copper was added to the cultures from a copper stock solution. This stock solution was prepared by dissolving Cl_2Cu in distilled and sterilized water. Various volumes of copper stock solution (1 g Cu/l) were added to the cultures. Control cultures (without copper) were also included. Because the cupric ion can be precipitated or complexed in the enriched seawater , free copper was determined in each culture before inoculation. Seawater enriched with culture medium and different copper concentrations was filtered through 0.45 µm Millipore-MF filters. Filtrates were passed through a column of Ca-Chelex. The column was eluted with HNO_3 and eluates from the column were measured by atomic absorption spectrophotometry (AAS) (4). Free copper concentrations obtained in the different cultures were: 7, 20 and 28 mg Cu/l.

Growth of the algal cultures was determined daily by counting culture aliquots in a Coulter Counter ZM with a 70 µm orifice. Growth rate (µ), expressed in day^{-1} (d^{-1}), was calculated by the usual formula:

$$\mu = (\ln N_t - \ln N_o) / (t - t_o)$$

where N is cellular density at time t after copper exposure and time is expressed in days.

Cellular pigments were extracted in 90% acetone at 4°C, in dark conditions. Chlorophyll *a* was determined spectrophotometrically; its concentration in each case was calculated by using the formula of Jeffrey and Humphrey (11).

Light scatter properties of the cells and chlorophyll *a* fluorescence were determined by FCM, using a FACScan flow cytometer (Becton Dickinson Instruments, San Jose, CA), equipped with an argon excitation laser (488 nm). Forward light scatter (FSC), which can be correlated with the volume of the cell, and the right-angle light scatter (SSC), which can be correlated with the complexity of the cellular cytoplasm, were measured. Red chlorophyll *a* fluorescence emission was collected in the FL3 (>600 nm) channel.

For each cytometric variable investigated (cell volume, complexity and chlorophyll *a* fluorescence), 10^4 events (cells) were analyzed. Measurements were carried out at the beginning of growth after 1 h of exposure to copper, and when the cultures reached the stationary phase (72 h). All the measurements were carried out in triplicate and data were statistically analyzed by the LYSIS II program (Becton Dickinson); this software was also used to obtain cell volume, complexity and chlorophyll *a* fluorescence distribution histograms.

TABLE 1
CELLULAR DENSITY, GROWTH RATE AND CHLOROPHYLL *a* CONTENT IN *Phaeodactylum tricornutum* CULTURES WITH DIFFERENT COPPER CONCENTRATIONS

[Cu] (mg/l)	Maximum number of cells (10^6cells/ml)[a]	Growth rate (d^{-1})[b]	Chlorophyll *a* content (pg/cell)[a]
0	3.12 ± 0.21	0.55	0.36 ± 0.02
7	2.12 ± 0.19	0.42	0.35 ± 0.01
20	1.61 ± 0.12	0.33	0.32 ± 0.02
28	1.07 ± 0.11	0.19	0.34 ± 0.02

[a] Data are expressed in mean values ± standard deviation.
[b] Day^{-1}.

Results

The experiments showed an inhibitory effect due to copper concentration on the growth of *P. tricornutum* (Table 1). Copper reduced the growth of *P. tricornutum*, leading to lower cell density throughout the experimental period. Stationary phase was reached after 72 h with the maximum cellular density in control cultures (3.12 x 10^6 cells/ml) significantly higher than densities obtained in the treated cultures ($P < 0.01$) (Table 1). Maximum growth rate was also found in control cultures, with 0.55 d^{-1}, whereas cultures treated with 7, 20 and 28 mg Cu/l reached 0.42, 0.33 and 0.19 d^{-1}, respectively.

Cell content of chlorophyll *a* of *P. tricornutum* at the stationary phase, calculated from spectrophotometric data are shown in Table 1. Significant variations in the chlorophyll *a* content per cell were not observed, with values between 0.36 and 0.32 pg/cell reached in control cultures and cultures with 20 mg Cu/l, respectively.

Changes in cellular volume, complexity and autofluorescence obtained by FCM are shown in Fig. 1, 2 and 3. After 1 h copper exposure differences in FSC were not observed among the cultures (Fig. 1A). When the cultures reached the stationary phase (Fig. 1B), FSC gradually increased as copper concentration increased. The peak-channel in control cultures was 272; peak-channel obtained in cultures with 7 and 20 mg Cu/l was not significantly different to control cultures, whereas in cultures with 28 mg Cu/l, peak-channel reached a significantly higher value (it was 355).

SSC showed the same pattern that FSC, without differences in the first hour of copper exposure (peaks channels between 81 and 86) (Fig. 2A). When the cultures reached the stationary phase (Fig. 2B), an increase in SSC was observed with the higher copper concentration used. Control cultures, and cultures with 7 and 20 mg Cu/l showed similar levels in SSC to those obtained after 1 h of exposure to copper (they were 80 and 71, respectively); whereas culture with 28 mg Cu/l showed a higher peak channel level, with a value of 184.

After one hour of copper exposure, there were no differences in the chlorophyll *a* fluorescence (FL3) among the cells of all the cultures assayed, with values in the peak-channel between 76 and 88 (Fig. 3A). At the stationary phase, control cultures and those with 7 mg Cu/l showed similar levels in the autofluorescence, but a different histogram for the autofluorescence was obtained in cultures with 20 mg Cu/l at the stationary phase (Fig. 3B), showing two peaks, one with a peak-channel of 85, similar to that showed after 1 h of copper exposure and other showing a higher peak channel (it was 227). The cultures with 28 mg Cu/l showed a higher level in the autofluorescence, with only a peak-channel with a value of 229 (Fig. 3B).

Discussion

The toxicity of heavy metals depends upon their chemical speciation. Various ionic forms of a metal characterized by different valency states, may be differentially toxic to a test algae. Illumination, temperature conditions, and nutrient concentrations are essential factors in microalgae cultures and can affect their response to different environmental toxicants. Likewise, heavy metal toxicity largely depends upon algal population density: the denser the population the more numerous the cellular sites available, leading to decreased toxicity (22). The effects of heavy metals on microalgae have been assayed in a great variety of both metal concentrations and cellular densities. In the present experiments, relatively high cellular densities were used, but after previous assays this density was found to be the

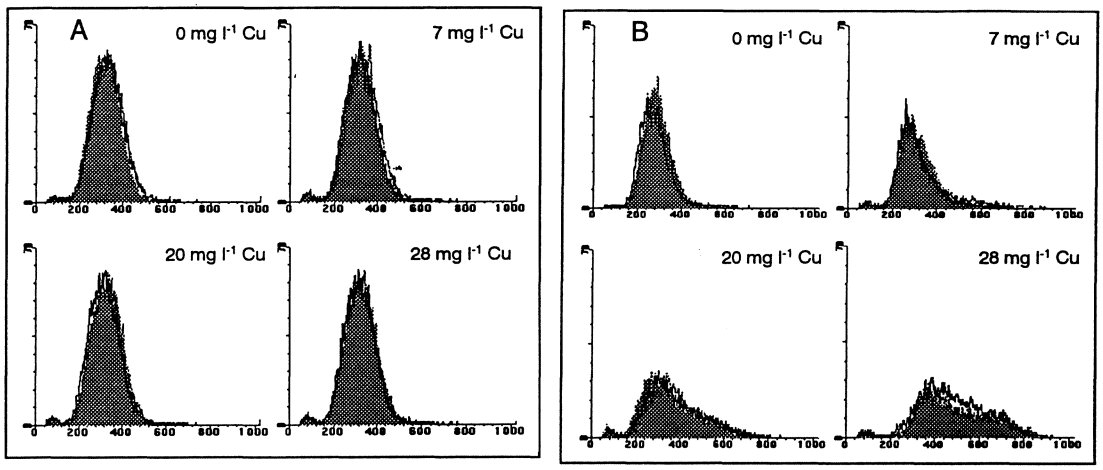


FIG. 1. Histograms of FSC (related to cellular volume) in *Phaeodactylum tricornutum* cultures after exposure to different copper concentrations. *X*-axis represents the FSC channel and *Y*-axis the number of cells. (A) after 1 h exposure; (B) at the stationary phase.

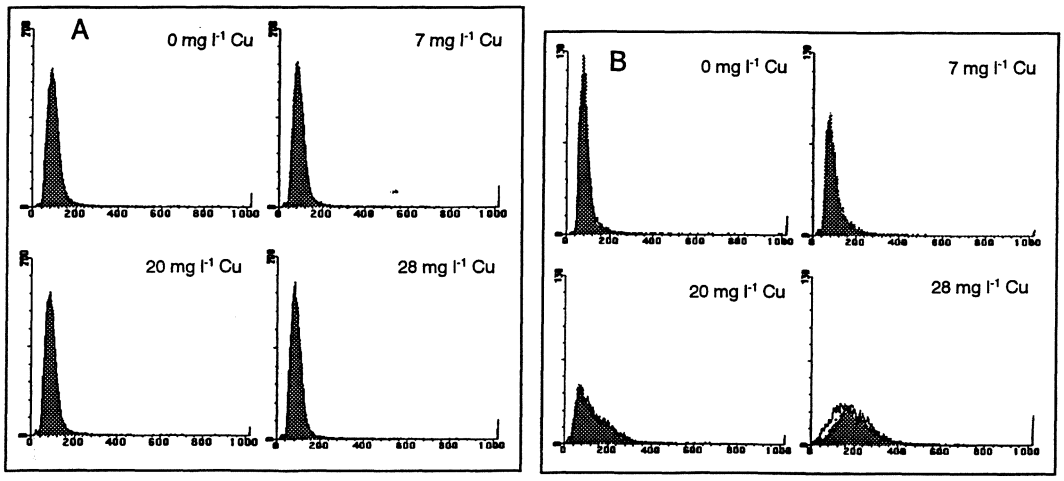


FIG. 2. Histograms of SSC (related to cellular complexity) in *Phaeodactylum tricornutum* cultures after exposure to different copper concentrations. *X*-axis represents the SSC channel and *Y*-axis the number of cells. (A) after 1 h exposure; (B) at the stationary phase.

optimum for the analysis to be performed. Therefore, copper concentrations used were also relatively high in comparison to those reported by other authors. However, similar and even higher copper concentrations have been used by Lustigman (13) in studies with *Dunaliella tertiolecta*.

When copper is added to the enriched seawater, a part can be precipitated, or retained by different components of the culture medium with chelating capability. Measurements of copper in the uninoculated medium after filtering and passing through Chelex-100, showed that effectively a percentage of copper added to seawater enriched with the culture medium was not free. Part of the copper was insoluble and was retained by the filter; another fraction of copper was bound in complexes (or absorbed on colloidal matter) and a third fraction of copper was free. With this technique, the copper able to bind to Chelex-100 or copper labile to Chelex-100 was measured. This fraction contains free copper and copper from complexes with a high dissociation constant to free copper (5).

Some species of single-cell algae respond to toxic levels of metals by depressed cell division rates and increased cellular size; this apparent uncoupling of cytokinesis from other metabolic processes has been noted with microalgal cells exposed to copper (7). It has also been reported that microalgal exponential growth is delayed by sublethal concentrations of copper (9, 28). Our results agree with these reports, because growth decreased and size increased when copper was increased in the culture medium.

Short-term effects of pollutants on populations of organisms may result in the inhibition of growth, cell division or metabolic functions. On the long-term, these impairments may result in such effects as

changes in the species composition of communities. Numerous studies have dealt with changes in growth rate and chemical composition of unicellular algae resulting from exposure to changing environmental conditions, whereas cellular features have been less considered.

FCM has been introduced as an alternative to more traditional techniques to analize cells in cultures and from natural populations (10, 13, 18, 28). In flow cytometry, simultaneous measurements of individual particle cellular volume, fluorescence and light scatter properties are directly applicable in aquatic research. In the case of phytoplankton, the pigmented cells can be detected and identified by their autofluorescence and light scattering properties (10).

Our results show that when the cultures exposed to copper reached the stationary phase, FSC, directly related to cell volume, increased in contrast to control cells. Increase in cellular volume was directly related to copper concentration (Fig. 1B). Fisher and Frood (6) reported that cells of the diatom *Asterionella japonica* swelled considerably when exposed to copper in contrast to control cells, with swelling and distortion generally increasing with dosage and time; growth of *A. japonica* was also depressed by copper.

When the cultures reached the stationary phase, a gradual increase in SSC was observed as copper concentration increased (Fig. 2B). Electron microscopy analysis (unpublished data) shows ultrastructural changes, mainly in lysosomes and vacuoles, agree with changes observed by other authors in other microalgal species (26). Rachlin et al. (21) reported that the blue-green alga *Plectonema boryanum* exposed to copper

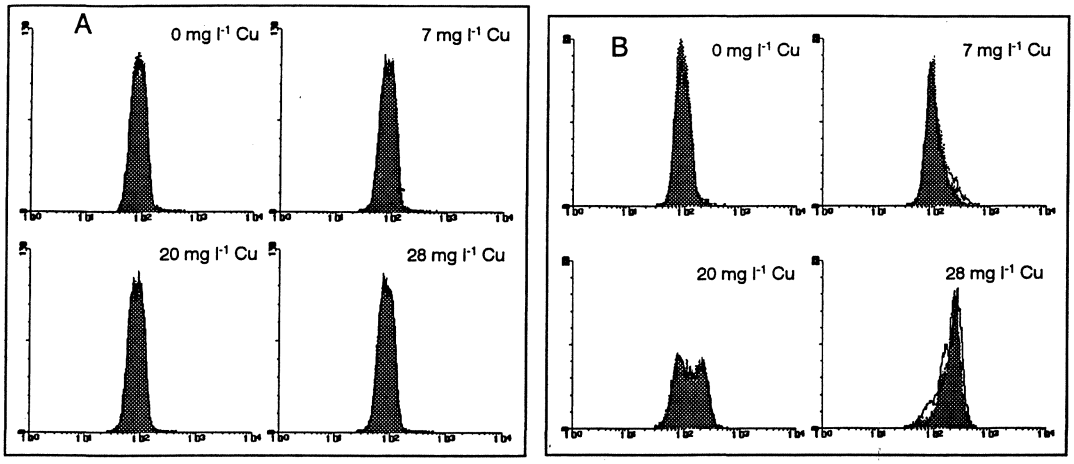


FIG. 3. Histograms of cellular autofluorescence in *Phaeodactylum tricornutum* cultures after exposure to different copper concentrations. *X*-axis represents the cellular autofluorescence channel and *Y*-axis the number of cells. (A) after 1 hour exposure; (B) at the stationary phase.

revealed an increase in cell volume and the appearance of electron-dense granular bodies. Morphometric analysis of algae exposed to low levels in short-term experiments showed that in the diatom *Melosira granulata* there was an increase in vacuole volume, and in the blue-green alga *Anacystis cyanea* there was an increase in the number of cell inclusions containing reserve materials (25). Electron-dense inclusions were observed in the vacuoles of *Scenedesmus quadricauda* exposed to copper (1). The occurrence of the electron-dense inclusions was correlated significantly with the copper concentration. In the case of *Chlorella vulgaris*, an increase in copper concentration was followed by a slight increase in the occurrence of the membranous organelles, of electron-dense inclusions and of inclusion density (1).

Chlorophyll *a* fluorescence is particularly sensitive to the functioning of photosystem II (PS II). Measurement of the fluorescence of chlorophyll *a* in intact algal cells provides information on the absorption, distribution and utilization of energy in photosynthesis (8, 19). Algal fluorescence induction phenomena were used to study phytotoxicity (15, 24). Chlorophyll *a* fluorescence monitored by the single-laser based flow cytometer is the maximum fluorescence when the PS II reaction centers are locked in the Q_A^- state (30). It is known that the inhibition of the electron flow in the PS II reaction center at the donor side induces a decrease in the chlorophyll a fluorescence, while if the inhibition is produced in the acceptor side of PS II, an increase in the chlorophyll *a* fluorescence is observed (2, 16, 23). According to Samson et al. (23), chlorophyll *a* fluorescence results obtained by FCM (Fig. 3B) show that copper's inhibitory effect on PS II activity is located on its oxidizing side, probably because copper inactivates some PS II reaction centers. The observed increase in chlorophyll *a* fluorescence is not due to a higher cell chlorophyll *a* content in these affected cells (Table 1).

Our results showed that copper concentrations assayed affected growth, cellular volume, complexity and autofluorescence of *P. tricornutum*, and that the FCM is a powerful tool to study physiological disturbances caused by toxic agents on microalgal cells.

Acknowledgments

This work was supported in part by a grant from Dirección General de Investigación Científica y Técnica (DGICyT), Madrid, Spain (PB89-0546). Part of this work was supported by a grant to S. Reiriz from Xunta de Galicia.

References

1. Bastien, C., Côte, R. (1989). Effect of copper on the ultrastructure of *Scenedesmus quadricauda* and *Chlorella vulgaris*. Int. Revue Ges. Hydrobiol. **74**, 51–71.
2. Butler, W. L. (1977). Chlorophyll fluorescence as a probe for electron transfer and energy transfer. *In* Trebst, A., Avron, M. (eds.), Encyclopedia of Plant Physiology, New Series, vol. 5, pp. 149–167. Springer-Verlag, Berlin.
3. Fábregas, J., Herrero, C., Abalde, J., Liaño, R., Cabezas, B. (1986). Biomass production and biochemical variability of the marine microalga *Dunaliella tertiolecta* (Butcher) with high nutrient concentrations. Aquaculture **53**, 187–199.

4. Figura, P., McDuffie, B. (1977). Characterization of the calcium form of Chelex-100 for trace metal studies. Analyt. Chem. **49**, 1950–1953.
5. Figura, P., McDuffie, B. (1980). Determination of labilities of soluble trace metal species in aqueous environmental samples by anodic stripping voltametry and Chelex column and batch methods. Anal. Chem. **52**, 1433–1439.
6. Fisher, N. S., Frood, D. (1980). Heavy metals and marine diatoms: influence of dissolved organic compound on toxicity and selection for metal tolerance among four species. Mar. Biol. **59**, 85–93.
7. Fork, D. C., Mohanty, P. (1986). Fluorescence and other characteristics of blue-green algae (cyanobacteria), red algae and cryptomonads. *In* Godvindjee, J. A., Fork, D. C. (ed.), Light Emission by Plants and Bacteria, pp. 451–496. Academic Press, London.
8. Hawkins, P. R., Griffiths, D. J. (1982). Cupric ion tolerance in four species of marine phytoplankton. Bot. Mar. **25**, 31–33.
9. Herzenberg, L. A., Sweet, R. G., Herzenberg, L. A. (1976). Fluorescence activated cell sorting. Sci. Am. **234**, 108–117.
10. Horan, P. K., Wheeless, L. L. Jr. (1977). Quantitative single cell analysis and sorting. Science **198**, 149–157.
11. Jeffrey, S. W., Humphrey, G. F. (1975). New spectrophotometric equations for determining chlorophylls *a, b,* c_1 and c_2 in higher plants, algae and natural phytoplankton. Biochem. Physiol. Pflanz. **167**, 191–194.
12. Kruth, H. S. (1982). Flow cytometry: rapid biochemical analysis of single cells. Analyt. Biochem. **125**, 225–242.
13. Lustigman, B. K. (1986). Enhancement of pigment concentrations in *Dunaliella tertiolecta* as a result of copper toxicity. Bull. Environ. Contam. Toxicol. **37**, 710–713.
14. Moody, R. P., Weinberger, P., Greenhalgh, R. (1983). Algal fluorimetric determination of the potential phytotoxicity of environmental pollutants. *In* Nriagu, O. (ed.), Aquatic Toxicology, vol. 13, pp. 504–512. Wiley-Interscience, New York.
15. Murthy, S. D. S., Sabat, S. C., Mohanty, P. (1989). Mercury-induced inhibition of photosystem II activity and changes in the emission of fluorescence from phycobilisomes in intact cells of the cyanobacterium, *Spirulina platensis*. Plant Cell Physiol. **30**, 1153–1157.
16. Overnell, J. (1976). Inhibition of marine algal photosynthesis by heavy metals. Mar. Biol. **38**, 335–342.
17. Paau, A.S., Uro, J., Cowles, J. R. (1978). Applications of flow cytometry to the study of algal cells and isolated chloroplast. Exp. Bot. **29**, 1011–1020.
18. Papageorgiou, G. (1975). Chlorophyll fluorescence: an intrinsic probe of photosynthesis. *In* Godvindjee, J. A. (ed.), Bioenergetics of Photosynthesis, pp. 319–371. Academic Press, New York.
19. Phinney, D. A., Cucci, T. L. (1989). Flow cytometry and phytoplankton. Cytometry **10**, 511–521.
20. Rachlin, J. W., Jensen, T. E., Baxter, M., Jani, V. (1982). Utilization of morphometric analysis in evaluating response of *Plectonema boryanum* (Cyanophyceae) to exposure to eight heavy metals. Arch. Environ. Contam. Toxicol. **11**, 323–333.
21. Rai, L. C., Gaur, J. P., Kumar, H. D. (1981). Phycology and heavy-metal pollution. Biol. Rev. **56**, 99–151.
22. Samson, G., Morissette, J. C., Popovic, R. (1988). Copper quenching of the variable fluorescence in *Dunaliella tertiolecta*. New evidence for a copper inhibition effect on PS II photochemistry. Photochem. Photobiol. **48**, 329–332.
23. Samson, G., Popovic, R. (1988). Use of algal fluorescence for determination of phytotoxicity of heavy metals and pesticides as environmental pollutants. Ecotoxicol. Environ. Safety **16**, 272–278.
24. Sicko-Goad, L. (1982). A morphometric analysis of algal response to low dose, short-term heavy metal exposure. Protoplasma **110**, 75–86.
25. Stauber, J. L., Florence, T. M. (1987). Mechanism of toxicity of ionic copper and copper complexes to algae. Mar. Biol. **94**, 511–519.
26. Steeman-Nielsen, E., Wium-Anderson, S. (1970). Copper ions as poison in the sea and in freshwater. Mar. Biol. **6**, 93–97.

27. Trask, B. J., Van Der Engh, G. J., Elgershuizen, J. H. B. W. (1982). Analysis of phytoplankton by flow cytometry. Cytometry **2**, 258–267.
28. van Coillie, R., Couture, P., Visser, S. A. (1983). Use of algae in aquatic ecotoxicology. *In* Nriagu, O. (ed.), Aquatic Toxicology, vol. 13, pp. 488-502. Wiley-Interscience, New York.
29. Wong, S. L., Beaver, J. L. (1980). Algal bioassays to determine toxicity of heavy metals mixtures. Hydrobiologia **74**, 199–208.
30. Xu, C., Auger, J., Godvindjee, J. A. (1990). Chlorophyll *a* fluorescence measurements of isolated spinach thylakoids obtained by using single-laser-based flow cytometry. Cytometry **11**, 349–358.

MICROBIOLOGÍA SEM **10** (1994), 273–278

Utilization of some phenolic compounds by *Azotobacter chroococcum* and their effect on growth and nitrogenase activity

Mohamed H. Abd-Alla*

Department of Botany, Faculty of Science, Assiut University, Assiut, Egypt

Received 19 July 1993/Accepted 25 June 1994

Summary

Azotobacter chroococcum MH1 was grown in a mannitol and nitrogen free medium supplemented with *p*-hydroxybenzoic acid, resorcinol, catechol or vanillic acid as a sole carbon source. Growth and nitrogenase activity of *p*-hydroxybenzoic acid were supported by 8, 6 and 4 mM of *p*-hydroxybenzoic acid, resorcinol and catechol, respectively. The generation time of 1.71 h in *p*-hydroxybenzoic acid did not differ from a generation time of 1.64 h, when grown in mannitol. The compound *p*-hydroxybenzoic acid was utilized rapidly. However, the decomposition of other phenolic compounds tested proceeded slowly. These results suggested that phenolic compounds released during biodegradation of plant wastes could be utilized as carbon sources for both growth and nitrogen fixation of *Azotobacter chroococcum*.

Key words: *Azotobacter chroococcum*, biodegradation, nitrogenase activity, phenolic compounds

Resumen

Se hizo crecer *Azotobacter chroococcum* MH1 en un medio carente de manitol y de nitrógeno suplementado con ácido *p*-hidroxibenzoico, resorcinol, catecol o ácido vanílico como única fuente de carbono. Tanto el crecimiento como la actividad nitrogenasa se determinaron a concentraciones de 8, 6 y 4 mM de ácido *p*-hidroxibenzoico, resorcinol y catecol, respectivamente. El tiempo de generación de 1,71 h, en ácido benzoico, no difería del de 1,64 h cuando el crecimiento se hacía en medio suplementado con manitol. Además, se observó que la rápida utilización del ácido *p*-hidroxibenzoico contrastaba con

* *Correspondence to*: Mohamed H. Abd-Alla. Department of Botany. Faculty of Science. Assiut University. 71516 Assiut. Egypt.

la lenta descomposición de otros compuestos fenólicos. Estos resultados sugerían que los compuestos fenólicos que se liberan en la biodegradación de desechos de plantas podían ser utilizados como fuente de carbono, tanto para el crecimiento como para la fijación de nitrógeno, por *Azotobacter chroococcum*.

Introduction

Plant residues which are low in nitrogen, but rich in decomposable carbon, offer the potential for dinitrogen fixation (8). This process can become particularly important in natural, low input systems where dinitrogen fixation can make a major contribution to the overall nitrogen economy. Generally this requires the cooperation of diazotroph with the second organism capable of degrading the cellulose and hemicellulose of the plant residues (1). Phenolic compounds (1-3 %) such as hydroxy- and methoxy-benzoic and cinnamic acid are found in decaying plant residues, soil and roots (6, 12, 23). They may also be synthesized by soil microorganisms (7) or formed during humification (10). Growth of *Azotobacter* in soil and its ability to fix nitrogen could be influenced by the amount of available phenolic compounds. The available information on the ability of members of genus *Azotobacter* to utilize phenolic compounds is limited (9, 11, 18).

The aim of this study was to throw light on the effect of different phenolic compounds as sole sources of carbon and energy on both growth and nitrogenase activity of *Azotobacter chroococcum* isolated from straw-amended soil.

Materials and methods

Culture and cultural conditions. Samples of *Azotobacter chroococcum* were isolated from different straw-amended soils and identified as *A. chroococcum* (17, 22). Cultures were maintained in mannitol- and nitrogen-free medium (3).

Degradation of phenolic compounds. The ability of the isolates to utilize phenolic compounds (*p*-hydroxybenzoic acid, resorcinol, vanillic acid and catechol) was studied in agar medium free of nitrogen and mannitol. The phenolic compound agars (10 mM) were prepared as by Henderson (13). The degradation of phenolic compounds was assessed by adding 5 ml of 1.0% (w/v) solution of ferric chloride–potassium ferricyanide (20). A blue colour developed in non-inoculated agar medium containing phenolic compounds. The presence of yellowish-green zones in agar medium was interpreted as an evidence of the degradation of these compounds.

Growth conditions. For growth on phenolic substances, cells were grown in nitrogen and mannitol free medium to which filter sterilized substances were amended at concentrations of 2, 4, 6, 8 and 10 mM. After inoculation with 1 ml of bacterial suspension (ca. 10^6 CFU/ml), cultures were incubated at 30°C in a rotatory shaker at 125 rpm for 5 days. Growth was measured turbidometrically at 540 nm after 12, 24, 36, 48 and 60 h of culture. To determine generation time, cells were grown in nitrogen and mannitol free medium containing phenolic compounds at their optimal concentrations. Mannitol and nitrogen free medium was used as a control. Viable counts were made by plate counts in Brown's agar medium (3).

Nitrogenase activity. To estimate nitrogenase activity, cells were put into a closed system (556 ml mannitol bottles) and incubated under air containing 10% acetylene. After 2 h, gas samples of 10 ml were taken with syringe and put into another closed system containing 2 ml of oxidant solution (80 ml of 0.05 M $NaIO_4$, 10 ml of 0.005 M $KMnO_4$, at pH 7.5 adjusted with KOH, diluted to 100 ml). The closed system was agitated vigorously on a rotatory shaker at 300 rpm for 90 min at room temperature. Then, 250 µl of 4 M $NaAsO_2$ and 250 µl of H_2SO_4 were added, mixing to destroy excess of oxidant. One ml of Nash reagent (150 g of ammonium acetate, 3 ml of acetic acid, and 2 ml of acetyl acetone, diluted to 1 litre) was added and the absorbance at 412 nm was determined after 60 min. Standards containing known amounts of ethylene were carried through the analysis at the same time as the samples (15).

Determination of residual phenolic substances. The determination of residual phenolic substances was carried out by centrifugation of growth medium at 3000 xg for 15 min. Following centrifugation the supernatant was allowed to stand for 24 h at −15°C, for extracellular protein precipitation, and thereafter the precipitate was removed by centrifugation at 3000 xg for 15 min. Residual phenol was determines using 1 ml samples of the supernatant mixed with 5 ml of bidistilled water followed by the addition of 1 N Folin-Ciocalteau reagent and immediate mixing. Three minutes after, 0.5 ml of a saturated Na_2CO_3 solution was added and mixed thoroughly (21). The resulting blue color was measured 2 h later at 660 nm using a Spectronic 2000 spectrophotometer (Bausch and Lomb).

Results

Ability of isolates to utilize phenolic compounds. Isolates of *Azotobacter chroococcum* were tested for their ability to utilize phenolic compounds. All of the isolates completely decomposed *p*-hydroxybenzoic acid. While isolate MH1 decomposed all other tested phenolic compounds, other isolates were not so effective.

Growth on phenolic compounds. Isolate MH1 was grown in liquid Brown's medium (devoid of mannitol) containing *p*-hydroxybenzoic acid, resorcinol, catechol and vanillic acid as sole carbon source. At 8 mM, *p*-hydroxybenzoic acid supported maximal growth (Fig. 1, A). Growth was inhibited at higher concentration (10 mM). Concentration of 6 mM resorcinol yielded a higher cell abundance (Fig. 1, B). Substrate concentrations above 6, 4 and 2 mM inhibited growth on resorcinol, catechol and vanillic acid respectively (Fig. 1, B, C and D).

Mean generation times for the selected isolate for growth on phenolic substances ranged from approximately 1.71 h to more than 10.6 h. The generation time was 1.64 h when grown in mannitol (Table 1).

Nitrogenase activity. Nitrogenase activity was detected when *Azotobacter chroococcum* MH1 was cultured in medium supplemented with *p*-hydroxybenzoic acid, resorcinol and catechol for 24 h and 48 h, respectively. On the contrary, no nitrogenase activity was detected in medium containing vanillic acid (Table 1).

The utilization of phenolic compounds was determined during growth periods. It was apparent that the isolate mainly degraded *p*-hydroxybenzoic acid (Table 2), and this compound was depleted after more than 60 h. On the other hand, the utilization of other phenolic compounds, proceeded slowly. These data indicate that about 85 % of *p*-hydroxybenzoic acid was utilized after 60 h. However, only 47, 33 and 30% of resorcinol, catechol and vanillic acid were degraded after 60 h, respectively.

TABLE 1
EFFECT OF PHENOLIC COMPOUNDS ON GENERATION TIME AND NITROGENASE ACTIVITY OF *Azotobacter chroococcum* MH1

Substance	Concentration (mM)	Generation time (h)	Nitrogenase activity (nmol ethylene/ml.h)	
			24 h	48 h
Mannitol (control)	10	1.64	265	640
p-Hydroxybenzoic acid	8	1.71	235	590
Resorcinol	6	3.87	68	185
Catechol	4	5.11	27	89
Vanillic acid	2	10.67	0	0

TABLE 2
UTILIZATION OF PHENOLIC COMPOUNDS DURING GROWTH OF *Azotobacter chroococcum* MH1

Compound	Remaining phenol (mM) in the growth medium Hours after inoculation			
	0	24	48	60
p-Hydroxybenzoic acid	8	5	3.8	1.2
Resorcinol	6	5.5	4.6	3.2
Catechol	4	1.8	1.6	1.4
Vanillic acid	2	1.8	1.6	1.4

Discussion

Apparently, *Azotobacter chroococcum* MH1 isolated from decomposing straw was effective in the decay of a wide range of phenolic substances as sole carbon source.

Bacteria utilizing some phenolic compounds seem to be limited to a small number of organisms which include *Mycobacterium* sp. (4), *Pelobacter acidigallici* (19) and *Rhodococcus* sp. (2).

Reduction of acetylene to ethylene confirmed that the cells had nitrogenase activity. Several years ago, it was demonstrated that *Azotobacter vinelandii* not only fixes dinitrogen, but it also grows at the expense of several aromatic compounds (9, 18). Recently, Wu et al. (24) failed to detect phenolic compounds-dependent nitrogenase activity in *Azotobacter vinelandii*. Cells were cultured in soil extracts and growth was supported by phenolic compounds. The

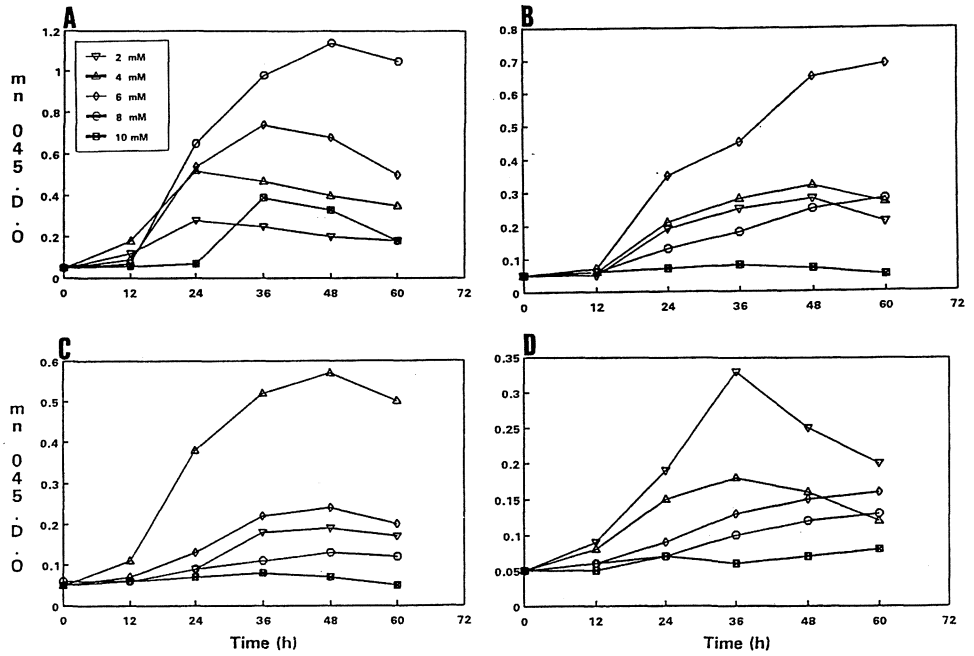


FIG. 1. Growth of *Azotobacter chroococcum* MH1 on *p*-hydroxybenzoic acid (A), resorcinol (B), catechol (C) and vanillic acid (D).

absence of detectable nitrogenase activity was ascribed to the presence of combined nitrogen on the soil and repression of nitrogen fixation (5, 14). If so, it would suggest that phenolic compounds-supported nitrogen fixation by *Azotobacter* in natural environments may be limited to nitrogen poor soils.

Results of present work indicate that some phenolic compounds would be expected to fuel nitrogen fixation. These results agree with recent findings obtained by Peterson and Peterson (16), who reported that *p*-hydroxybenzoate supported nitrogenase activity of *Azotobacter vinelandii*.

References

1. Abd-Alla, M. H., Omar, S. A., Abdel-Wahab, A. M. (1992). The role of cellulose-decomposing fungi in nitrogenase activity of *Azotobacter chroococcum*. Folia Microbiol. **37**, 215–218.
2. Armstrong, S., Patel, T. R. (1992). Utilization of 1,3,5-trihydroxybenzene (phloroglucinol) by a soil isolate, *Rhodococcus* species BPC-8. J. Basic Microbiol. **32**, 3–11.
3. Brown, M. E., Burlingham, S. K., Jackson, R. M. (1962). Studies on *Azotobacter* species in soil. I. Comparison of media and techniques for counting *Azotobacter* in soil. Plant Soil **17**, 309–319.
4. Brune, A., Schink, B. (1990). Pyrogallol-to-phloroglucinol conversion and other hydroxy-hydroxyl-transfer reaction catalyzed by the cell extract of *Pelobacter acidigallici*. J. Bacteriol. **172**, 1070–1076.
5. Buhler, T., Sann, R., Monter, U., Dingler, C., Kuhla, J., Oelze, J. (1987). Control of dinitrogen fixation in ammonium-assimilating cultures of *Azotobacter vinelandii*. Arch. Microbiol. **148**, 247–251.
6. Dalton, B. R., Blum, U., Weed, S. B. (1989). Plant phenolic acid in soils: sorption of ferulic and by soil components sterilized by different techniques. Soil Biol. Biochem. **21**, 1011–1018.
7. Flaig, W. (1971). Organic compounds in soil. Soil Science **111**, 19–33s.

8. Gibson, A. H., Halsall, D. M., Roper, M. M. (1988). Nitrogen fixation associated with residue breakdown. *In* Bothe, H., DeBruijin, F. N., Newton, W. E. (ed.), Nitrogen Fixation—100 Years After, pp. 753–758. Gustav Fischer, Stuttgart.
9. Groseclose, E. E., Ribbons, D. W. (1981). Metabolism of resorcinylic compounds by bacteria: New pathway for resorcinol catabolism in *Azotobacter vinelandii*. J. Bacteriol. **146**, 460–466.
10. Haider, K., Martin, J. P., Filip, Z. (1975). Humus biochemistry. *In* Paul E. A., McLarren, A. D. (ed.), Soil Biochemistry, pp. 195–244. Marcel Dekker, New York.
11. Hardisson, C., Sala-Trepat, J. M., Stainer, R. Y. (1969). Pathways for the oxidation of aromatic compounds by *Azotobacter chroococcum*. J. Gen. Microbiol. **58**, 1–110.
12. Hartley, R. D., Whitehead, D. C. (1985). Phenolic acid in soil and their influence on plant growth and soil microbial process. *In* Vaughan, D., Malcom, R. E. (ed.), Soil Organic Matter and Biological Activity, pp. 109–149. Martinus Nijhoff, Dordrecht.
13. Henderson, M. E. K. (1961). Isolation, identification and growth of some soil Hyphomycetes and yeast-like fungi which utilise aromatic compounds related to lignin. J. Gen. Microbiol. **26**, 149–154.
14. Klugkist, J., Haaker, H. (1984). Inhibition of nitrogenase activity by ammonium chloride in *Azotobacter vinelandii*. J. Bacteriol. **157**, 148–151.
15. LaRue, T. A., Kurz, W. G. W. (1973). Estimation of nitrogenase using a colorimetric determination for ethylene. Plant Physiol. **51**, 1074–1075.
16. Peterson, J. B., Peterson, A. L. (1988). *para*-Hydroxybenzoate supported nitrogen fixation in *Azotobacter vinelandii* strain OP (13705). Can. J. Microbiol. **34**, 1271–1275.
17. Rennie, R. J. (1981). A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. **27**, 8–14.
18. Sala-Trepat, J. M., Evans, W. C. (1971). The *meta* cleavage of catechol by *Azotobacter* sp. Eur. J. Biochem. **20**, 400–413.
19. Samain, E., Albagnace, G., Bubourguier, H.-C. (1986). Initial steps of catabolism of trihydroxybenzenes in *Pelobacter acidigallici*. Arch. Microbiol. **144**, 242–244.
20. Sundman, V., Näse, L. (1971). A simple plate test for direct visualisation of biological lignin degradation. Paperji ja puu **53**, 67–71.
21. Swain, T., Hillis, W. E. (1959). The phenolic constituents of domestica. I. The quantitative analysis of phenolic constituents. J. Sc. Food Agric. **10**, 63–68.
22. Tchan, T. T., New, P. B. (1984). *Azotobacteriaceae*. *In* Krieg, N. R., Holt, J. G. (ed.), Bergey's Manual of Systematic Bacteriology, vol. 1, pp. 220–229. Williams and Wilkins, London.
23. Whitehead, D. C., Dibb, H., Hartley, R. D. (1983). Bound phenolic compounds in water extract of soils, plant roots and leaf litter. Soil Biol. Biochem. **15**, 133–136.
24. Wu, F. J., Moreno, J., Vela, G. R. (1987). Growth of *Azotobacter* on soil nutrients. Appl. Environ. Microbiol., **53**, 489–494.

MICROBIOLOGÍA SEM **10** (1994), 279– 284

Otitis externas infecciosas: etiología en el área de Terrassa, métodos de cultivo y consideraciones sobre la otomicosis

Miquel Bayó,[1] Montserrat Agut,[2,*] M. Àngels Calvo[2]

[1]*Laboratorio de análisis M. Bayó Boada, Terrassa*
[2]*Departament de Patologia i Producció Animals, Facultat de Veterinària, Universitat Autònoma de Barcelona*

Recibido 23 octubre 1993/Aceptado 27 abril 1994

Summary

The etiology of infections of the outer ear was studied in the area of Terrassa (Barcelona) over a six-year period (1987-1992). A total of 1419 samples of ear discharge were processed. Traditional culture media were used to isolate the microorganisms as well as a modified culture medium prepared by us (APA). Usual methods were used to identify the microorganisms. The results show *Pseudomonas aeruginosa* to be the most frequently isolated microorganism. 76.5% of the otitis studied were unimicrobial and the presence of moulds or yeast was noted in 6.9% of samples. Otomycosis (5.9%) is one of the major pathological processes of the outer ear, and *Aspergillus niger* is the prime causal agent.

Key words: otitis, *Pseudomonas aeruginosa*, otomycosis, *Aspergillus niger*, *Proteus mirabilis*

Resumen

Se estudió la etiología de la otitis externa en el área de Terrassa (Barcelona) durante un período de seis años (1987-1992). Se procesaron un total de 1419 muestras de exudado ótico. Para el aislamiento de los microorganismos se realizó una siembra en placas sobre los medios de cultivo habituales y en uno

* *Correspondencia*: Montserrat Agut. Departament de Patologia i Producció Animals. Unitat de Microbiologia i Immunologia. Facultat de Veterinària. Universitat Autònoma de Barcelona. 08193 Bellaterra (Barcelona). Tel.: 93-5811748. Fax: 93-5812006.

modificado por nosotros (APA). La identificación de los microorganismos se realizó por métodos habituales. El microorganismo aislado con más frecuencia fue *Pseudomonas aeruginosa*. El 76,5% de las otitis estudiadas fueron unimicrobianas, observándose la presencia de hongos filamentosos o levaduras en un 6,9% de las muestras. Las otomicosis (5,9%) ocupan un lugar importante entre los procesos patológicos del oído externo, siendo *Aspergillus niger* el principal agente causal.

Introducción

Los procesos inflamatorios activos que afectan al oído externo son conocidos como otitis externas, pudiendo ser alérgicos o infecciosos. La etiología de estos últimos abarca todo tipo de bacterias, hongos y virus (1, 7, 9). La prevalencia de determinados organismos depende de la zona geográfica, del clima, de los hábitos del paciente y de las condiciones higiénico-sanitarias, aunque *Pseudomonas aeruginosa* parece ser el microorganismo aislado más frecuente (1, 3, 4, 5).

Ciertas personas, por ejemplo, individuos con alergias, psoriasis, eccema o dermatitis seborreica tienen una propensión particular a desarrollar una otitis externa. Los factores que predisponen a sufrir este proceso incluyen la entrada de agua o de agentes irritantes (laca o tinte del cabello) en el conducto auditivo y los traumatismos provocados durante la higiene del conducto con aplicadores de algodón (1).

Hemos efectuado un estudio retrospectivo de las otitis externas en nuestro laboratorio en un período de seis años e intentado establecer un patrón etiológico de la zona de Terrassa, incidiendo especialmente en los procesos producidos por hongos (otomicosis) y en aquéllos en los que coexisten bacterias y hongos.

Materiales y métodos

Se han estudiado 1419 muestras del área comarcal de Terrassa (Barcelona) correspondientes a un período de seis años (1987–1992).

En todos los casos se realizó una extensión sobre portaobjetos, que se tiñó por el método de Gram. Asimismo se procedió, por este orden, a la siembra sobre placas de agar triptosa-soja (TSA), agar sangre (BA), agar Sabouraud-gentamicina y medio APA. Los medios de cultivo se prepararon en nuestros laboratorios, siendo el medio APA (agar *Pseudomonas aeruginosa*) una modificación de otros similares (6), consistente en una base formada por peptona de gelatina (22,0 g), agar (17,0 g), sulfato potásico (8,5 g), cloruro magnésico (1,0 g), bromuro de cetil-trimetil amonio (0,3 g) y agua destilada (c.s.p. 1 litro), la cual se deja humectar durante 10 min, transcurridos los cuales se lleva a ebullición y se le añaden 10 ml de glicerol estéril; se esteriliza al autoclave y finalmente se distribuye en placas de Petri (20 ml/placa).

La incubación de las placas de BA, TSA y APA se efectuó a 37°C durante 48 h, y a 28°C las de Sabouraud-gentamicina, realizándose lecturas a los 7, 14, 21 y 28 días. La identificación bacteriana se realizó mediante métodos habituales (7), recurriéndose en algunos casos al uso de micrométodos

comercializados (API) (10). La identificación de los hongos filamentosos se llevó a cabo teniendo en cuenta las características macro y microscópicas de los cultivos (10).

Resultados

Del total de 1419 muestras procesadas, un 95,2% (1351) fueron positivas en el cultivo microbiológico, aislándose de una a tres especies distintas de bacterias u hongos en cada una de ellas (Tabla 1).

El examen del frotis directo (Gram) puso de manifiesto la existencia de un componente inflamatorio-infeccioso en la mayoría de los casos: un 86,7% de los cultivos positivos mostraba la presencia de leucocitos polimorfonucleares en el examen microscópico. Este examen fue especialmente útil en la detección de hongos: el 95,2% de las muestras en que se demostró mediante cultivo la existencia de hongos, se había observado previamente su presencia mediante la tinción de Gram.

El microorganismo aislado con mayor frecuencia fue *Pseudomonas aeruginosa* (73,6%), seguido de otras especies en porcentajes muy inferiores (Tabla 2). Tal resultado se mantuvo en el caso de infecciones producidas por una o más especies bacterianas o fúngicas, difiriendo únicamente en los 13 cuadros en que intervinieron una bacteria y un hongo. En ellas, la bacteria más frecuente fue *Proteus*

TABLA 1
OTITIS EXTERNAS SEGÚN TIPO Y NÚMERO DE MICROORGANISMOS AISLADOS

Especie/biotipo	Número de casos	Porcentaje
Otitis unimicrobianas	1033	76,5
Otitis dimicrobianas	208	15,4
Otitis unifúngicas	70	5,2
Otitis trimicrobianas	17	1,2
Otitis bacterio-micóticas	13	1,0
Otomicosis difúngicas	10	0,7
Total	1351	100,0

TABLA 2
ESPECIES AISLADAS EN CULTIVO DE EXUDADO ÓTICO

Especie	Número de aislamientos[a]	Porcentaje
Pseudomonas aeruginosa	1190	73,6
Proteus mirabilis	127	7,9
Staphylococcus aureus	63	3,9
Aspergillus niger	52	3,2
Escherichia coli	23	1,4
Citrobacter freundii	21	1,3
Enterobacter cloacae	20	1,2
Enterococcus faecalis	18	1,1
Serratia marcescens	18	1,1
Candida albicans	17	1,1

[a] Se omiten las especies aisladas en menos de un 1,0% de las muestras.

mirabilis (53,8%), seguida de *Staphylococcus aureus* (30,8%) y *de Pseudomonas aeruginosa* (15,4%). Cabe destacar que en las otitis bacterio-micóticas la especie fúngica aislada más frecuentemente fue *Aspergillus niger*.

Discusión

La demostración del agente etiológico en un 95,2% de los casos remitidos significa un porcentaje muy alto, comparable a los de otros autores (3, 4, 5). La razón de este hecho creemos que radica en la evidencia de los signos clínicos que acompañan a las otitis externas, junto al hábito cada vez más extendido por el que el clínico decide apoyarse en el laboratorio para obtener diagnósticos más precisos.

La realización sistemática de frotis sobre portaobjetos y su examen microscópico se confirma como técnica de gran valor (5), que aporta datos importantes sobre el componente inflamatorio, el tipo de bacterias visualizadas (morfología y Gram), la presencia o no de hifas, conidios o levaduras, etc. A nuestro parecer debe incluirse en el informe final, a fin de situar cada aislamiento concreto en su contexto.

Un 18,3% de los cultivos positivos correspondieron a otitis multimicrobianas (de ellas, un 15,4% dibacterianas [Tabla 1]). Esta proporción es parecida a la hallada en algunos estudios (4), pero inferior a la de otros (5).

Pseudomonas aeruginosa es el principal agente etiológico de las otitis externas en nuestra zona, así como en la mayoría de estudios efectuados (3, 4, 5, 7). Su intervención en tres cuartas partes de los procesos de este tipo nos hizo incluir el medio APA entre los utilizados sistemáticamente para el cultivo de exudados óticos. Contiene cetrimida, un compuesto de amonio cuaternario, al que son especialmente resistentes las cepas de *Pseudomonas aeruginosa*, que inhibe el crecimiento de la práctica totalidad de especies, incluidas otras del género *Pseudomonas*, actuando las sales de potasio y magnesio como activadoras de la producción de pigmentos. Su selectividad puede suprimir el aislamiento secundario de la cepa cultivada en agar sangre o TSA y sus características diferenciales llevarnos a una identificación presuntiva antes de las 24 h de la recepción de la muestra.

El 5,9% de los procesos son otomicosis, dato concordante con otros estudios (5) y opuesto a otros en los que, a nuestro entender, se obtienen cifras muy superiores (6). Esto último puede ser debido al hecho de que se cultivan muestras al azar, sin patología evidente, aislándose gran cantidad de hongos saprófitos del oído normal, que no intervienen como microorganismos productores de otitis. Otros investigadores obtienen cifras muy inferiores (4, 10), producto quizás de unas condiciones socio-geográficas distintas o de un tratamiento inadecuado de las muestras. En estos procesos es *Aspergillus niger* el microorganismo más destacado, dato concordante con otros estudios (8), junto a otras especies del mismo género y a algunas levaduras del género *Candida*.

Encontramos 13 casos de otitis con asociación de bacterias y hongos o levaduras, a los que denominamos otitis bacterio-micóticas, por entender que se trata, si no de una entidad distinta, sí de una forma específica de presentación de este cuadro infeccioso, a menudo de curso clínico más intenso (8). La sucesión bacteria-hongo-bacteria es bien conocida en muchos procesos patológicos y se sabe que resulta influenciada por el estado del huésped, el tratamiento antimicrobiano, etc., pero la coexistencia de ambos tipos de microorganismo infeccioso no es tan habitual. Observamos en estos casos que *Pseudomonas aeruginosa* cede su primer puesto a *Proteus mirabilis*, seguido de *Staphylococcus aureus*. Si bien es conocida la insistente presencia de *P. mirabilis* en las otitis mixtas (3, 4, 8), este hecho no deja de ser sorprendente y abre nuevos interrogantes sobre la relación bacteria–hongo en estos casos.

Bibliografía

1. Bertrán, J. M. (1983). Otitis. Medicine (Madrid) **57**, 11–22.
2. Blondiau, P. (1959). Le rôle des champignons dans la genèse de l'otite externe. Acta Otolaryng. Belg. **13**, 375–390.
3. Doroghazi, R. M., Nadol, J. B., Hyslop, N. E., Baxer, A. S., Axelrod, L. (1981). Invasive external otitis. Report of 21 cases and review of the literature. Am. J. Med. **71**, 603–614.

4. García Martos, P., Delgado, D., Díaz, J., Ávalos, E., Marín, P. (1991). Etiología de la otitis externa en la ciudad de Cádiz. Enf. Infec. Microbiol. Clin. **9**, 260–261.
5. García Martos, P., Marín Casanova, P., Rodríguez Sarmiento, M. T., García Herruzo, J. (1980). Microbiología de las otitis medias crónicas. Inmunologika **3**, 50–56.
6. King, C. O., Ward, M. K., Raney, D. E. (1954). Two simple media for the demonstration of pyocyne and fluorescein. J. Lab. Clin. Med. **44**, 301–307.
7. Lennette, E. H., Balows, A., Hausler, W. J., Shadomy, H. J. (1987). Manual de Microbiología Clínica. 4ª ed. Panamericana, Buenos Aires.
8. Martín, A. M., Canut, A., Muñoz, S., Pescador, C., Gómez, J. L. (1989). Otomicosis: presentación de quince casos. Enf. Infec. Microbiol. Clin. **7**, 248–251.
9. Otto, L. A., Pickett, M. J. (1976). Rapid method for identification of Gram-negative, non-fermentative bacilli. J. Clin. Microbiol. **3**, 566–575.
10. Rippon, J. W. (1988). The Pathogenic Fungi and the Pathogenic Actinomycetes. 3ª ed. Saunders, Philadelphia.

MICROBIOLOGÍA SEM **10** (1994), 285–296

A direct membrane filter method for enumerating somatic coliphages in drinking water

M. Carmen Alonso, José M. Sánchez, Miguel A. Moriñigo, Juan J. Borrego*

Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga

Received 15 March 1994/Accepted 22 July 1994

Summary

The application of a simple membrane filter method to enumerate specific somatic bacteriophages of *Escherichia coli*, using *E. coli* C as host strain, from drinking water samples was studied. The efficiency of the method using cellulosic membrane filters, samples pre-treated with magnesium ions and Tween 80 added to agar medium-host cell lawns ranged from 68.9 to over 112%, depending on the phage content of the sample. To avoid the pre-treatment of the sample with magnesium salts, electropositive-charged filters of cellulosic ester (HA-PEI and HA-Nalco) and Virosorb-1MDS filters were tested in conjunction with the simple membrane filter method. The electropositive filters showed wide bacteriophage recovery rate intervals depending on the sample treatment, ranging between 31.4 and 96.2% for ester-type filters, and a mean recovery lower than 2.2% for Virosorb filters. On the other hand, it was proved that the use of Tween 80 as an eluent improved somatic coliphage recovery rates for all the filters tested. In short, this methodology provides a rapid analysis (6-8 h) of the somatic coliphages from drinking water using the membrane filtration technique.

Key words: somatic coliphages, drinking water, membrane filtration, enumeration method

* *Correspondence to*: Juan J. Borrego. Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga. Campus Universitario Teatinos, 29071 Málaga. Tel.: 95-2131893. Fax: 95-2132000.

Resumen

Se ha estudiado la aplicación del método de filtración por membrana para la rápida enumeración de colifagos somáticos, usando *Escherichia coli* C como cepa hospedadora, a partir de muestras de agua de bebida. La eficiencia del método, utilizando filtros de ésteres de celulosa, muestras con iones magnesio y Tween 80 añadido a la capa de agar base, varió entre el 68,9 y más del 112%, dependiendo del título inicial de colifagos. Para evitar el pretratamiento de la muestra con sales de magnesio se probaron filtros cargados positivamente (HA-PEI, HA-Nalco y Virosorb). Estos filtros electropositivos mostraron un amplio rango de recuperación de fagos, entre el 31,4 y el 96,2% para los filtros de ésteres, y menos del 2,2% para los filtros Virosorb. Por otro lado, se ha comprobado que la recuperación fágica aumenta con el uso del eluyente Tween 80 en todos los filtros comparados. En resumen, esta metodología es útil para el análisis rápido y sencillo de colifagos somáticos a partir de agua de bebida, utilizando la técnica de filtración por membrana.

Introduction

The most frequent waterborne diseases related to contaminated—or inadequately treated—drinking waters are viral acute gastroenteritis and infectious hepatitis (9, 19). The etiological agents for these diseases are often described as enteric viruses, from of several taxonomic groups, including Picornaviridae, Adenoviridae, Caliciviridae, Astroviridae, Coronaviridae and Reoviridae (8, 18).

Sanitary safety of drinking water has been generally evaluated measuring certain microorganisms, named indexes (22), related to fecal waste and health-hazards associated with the presence of pathogens. However, the efficiency of index microorganisms in assessing the risk of viral disease has been questioned, because enteric viruses are more persistent in natural waters (11), and more resistant to water disinfection processes than conventional index organisms such as total coliforms, fecal coliforms and *Escherichia coli* (4, 24).

On the other hand, clear and direct epidemiological evidences on the role of enteric viruses in waterborne disease have not been established yet (9, 17). It is important to note that a practical virus methodology is difficult to establish because the standard culture and enumeration techniques of enteric viruses are too laborious, expensive, and time-consuming (23).

Because of their viral nature, bacteriophages may simulate the inactivation kinetics of enteric viruses to disinfectants, better than bacteria, and thus certain bacterial viruses have been considered as adequate and reliable viral indexes in drinking waters (13, 16). In 1990, the Spanish Sanitary Reglamentation on the survey of drinking water established the analysis of fecal bacteriophages as a complementary test to check the microbiological quality of the finished supply waters (5).

Several methods have been developed to detect and enumerate bacteriophages from water samples. The most frequently used are the Double-Agar-Layer method (1), the Simple-Agar-Layer method (14), the ACART technique (31), the Most-Probable-Number method (20), and the VIRADEL technique using negatively-charged filters (26), or positively-charged filters (6, 27). Recently, a simple membrane filter method to concentrate and enumerate F-specific RNA coliphages from water has been developed by Sobsey et al. (30), which consists in the passing of the water sample supplemented with magnesium ions (100 ml or more) through a cellulose ester filter (0.45 μm pore size). The filter with adsorbed phages is then placed upside-down onto lawns of host bacteria (*Salmonella typhimurium* WG49) in a standard medium supplemented with Tween 80 and tetrazolium violet. This method is similar to VIRADEL technique but the elution step is avoided.

The purpose of this study was to apply the simple membrane filter method for the concentration and enumeration of somatic coliphages (using *E. coli* C as the host strain) from drinking water. In addition, an evaluation of different filters and conditions of the method was also carried out in comparison with the standard methodology.

Materials and methods

Samples. Drinking water samples were obtained from the drinking-water treatment plant of Málaga (EMASA, El Atabal, Málaga, Spain). The samples were taken aseptically using sterile amber glass bottles of 1,000 ml capacity, and transported to the laboratory in isothermic containers maintained at 4°C. Water samples were dechlorinated by adding 75 mg of sodium thiosulfate/l. Samples of dechlorinated tap water were seeded with indigenous coliphages from raw sewage collected from a wastewater pipe discharge located on the Huelin beach (Málaga, Spain).

Microorganisms and culture conditions. *Escherichia coli* C (ATCC 13706) was used as host strain for the somatic coliphage analysis. To compare the efficiency of the simple membrane filter method using different phage groups, five phage-host systems were used, including T6 (ATCC 11303-B6), T7 (ATCC 11303-B7) and Φx174 (ATCC 13706-B1) with *E. coli* C, and MS-2 (ATCC 15597-B1) and Qß (ATCC 15597-B2) with *E. coli* C-3000 (ATCC 15597). All the bacterial hosts were grown in Trypticase-Soy broth (TSB, BBL) at 36 ± 1°C in a shaker for 6–8 h.

Direct phage analysis. All the samples were processed in parallel by the Double-Agar-Layer (DAL) and Simple-Agar-Layer (SAL) techniques, analyzing a total amount of the sample of 10 and 100 ml, respectively. Modified Scholtens agar (15) containing 0.7 and 1.1% of agar were used as top and bottom layers in the DAL technique. The medium was supplemented with 1.25 mM of magnesium chloride, 5 mM of calcium chloride (10) and 0.03% of 2, 3, 5-triphenyl tetrazolium chloride (30). Petri dishes containing Phage Agar Concentrate (PCA) (agar 1.2%) (14) supplemented with calcium and magnesium ions was used to perform the SAL technique. For the DAL technique, 1 ml of water sample or dilutions and 0.2 ml of the bacterial host culture in exponential growth in TSB were added to tubes containing 3 ml of soft agar (0.7% of agar) melted and maintained at 45°C. The mixture was poured onto

MSA plates, and after solidification, the Petri dishes were inverted and incubated at 36±1°C for 12–14 h. The SAL technique was performed as previously described by Grabow and Coubrough (14), and 100 ml of sample were poured onto five Petri dishes (140 mm diameter).

Simple membrane filter method. To enhance phage adsorption to the electronegative-charged filters, the samples were supplemented with 0.05 M of magnesium chloride, and pH was adjusted at 7.0–7.2. The medium used in conjunction with this technique is MSA supplemented with 3 g/l of Tween 80. The filter types used were the following: (i) cellulose ester filters (type HA, Millipore Ibérica, Madrid, Spain); (ii) the same filters treated with the cationic polymers, polyethylenimine (PEI) (Sigma Chemical Co., St. Louis, MO) at 0.05% and Nalco 7111 (Leachchem Industries Inc., Titusville, FL) at 0.1%, following the methods described by Preston et al. (25) and Borrego et al. (6); and (iii) electropositive filters Virosorb 1-MDS (AMF Cuno, Meriden, CN).

Specified volumes (100 ml) of the samples tested were vacuum-filtered through 47 mm-diameter membrane filters (0.45 μm pore size). Five-replicate trials per filter type were used for each sample or condition. Membranes with adsorbed phages were removed from the filter holder and placed upside-down onto the top layer in which the host bacteria were previously grown. Petri dishes were incubated with membrane at 36±1°C for 6–8 h and developed lysis plaques were counted as clear zones on a dark red background formed by the tetrazolium stained bacterial lawn.

To determine the efficiency of this method in the recovery of somatic coliphages, controls using DAL and SAL techniques were carried out. In addition, the adsorption rate to the filters was tested by counting the coliphages in the filtrate.

Results

Comparative efficiency recovery of the different membrane filters. A series of experiments was performed to determine the efficiency of the various membrane filters used in this study in the recovery of somatic coliphages. Volumes of 100 ml of seeded tap water (pH 7.0–7.2) with 0.05 M of magnesium chloride with different phage levels were used in all the experiments, following basically the methodology specified by Sobsey et al. (30). As it can be seen in Table 1, all the methods tested increase their sensitivity when the somatic coliphage levels decrease; even at levels of coliphages not detectable by the DAL technique (lower than 10 PFU [plaque-forming units] per 100 ml) all the methods are capable to quantify the numbers of coliphages present in the sample.

In these conditions the SAL technique was the most efficient method to recover low numbers of somatic coliphages from the samples. Among the methods based on the filtration of 100 ml of sample through different filter brands, the best results were obtained using electronegative cellulosic filters (HA type), which recover 68.9% of the coliphages present (considering as 100% the titer obtained by the SAL technique). The filters treated with the cationic polymers PEI and Nalco recovered 31.4 and 36.6%, respectively. The efficiency of Virosorb 1-MDS filters was very low (less than 2.5%), and in addition, lysis plaques were poorly visualized on the top layer, since the thickness of the filter hampered the optimal development of the bacterial lawn.

TABLE 1
COMPARISON OF THE SOMATIC COLIPHAGE RECOVERY EFFICENCY OF THE DIFFERENT MEMBRANE FILTERS

Sample titer range[a]	Phage recovery per 100 ml of sample[b]				
		Membrane filtration through			
	SAL technique	HA filters[c]	HA-PEI filters	HA-Nalco filters	Virosorb filters
50–100	37±14.0	26.3±0.9	10.0±2.2	15.3±4.5	0.7±0.9
10–50	24±17.3	16.8±1.1	8.7±2.0	8.6±2.7	<0.5
<10	16±7.0	10.0±0.8	5.5±1.5	4.3±1.2	<0.5
Average	25.7±10.6	17.7±8.2	8.1±2.3	9.4±5.5	<0.6±0.3
Recovery percent	100	68.9	31.4	36.6	<2.2

[a]Coliphage titer in the sample was calculate by DAL method and expressed as PFU per 100 ml.
[b]Mean ± standard deviation of five experiments, replicated three-fold.
[c]Millipore filters type HAWGO47 (47 mm diameter, 0.45 μm pore size).

To compare the different filter types, an ANOVA-one way analysis was performed. The results obtained indicate a significant difference (at 95% confidence level) between the SAL technique and the filtration through HA-PEI, HA-Nalco and Virosorb filters (F-Scheffer coefficients of 6.29, 5.37 and 12.79, respectively), and also between the filters HA and Virosorb (F-Scheffer coefficient of 5.96).

Effect of magnesium ions on the phage adsorption. To compare the effect of the magnesium ion on the adsorption and recovery of somatic coliphages to several types of membrane filters, a series of experiments were designed in parallel using dechlorinated tap water with or without 0.05 M magnesium chloride. The virus adsorption to membrane filters depends on the type of filters and the addition of magnesium ions to the samples (Table 2). Somatic coliphages were adsorbed efficiently to electronegative filters (HA type) from tap water supplemented with magnesium with rates higher than 85%, whilst only 64.2% adsorption rate was achieved for the same filters in samples without ion addition. On the contrary, electropositive filters, HA-PEI and HA-Nalco, were more efficient in coliphage adsorption with samples without magnesium (99.4 and 97.2% versus 66.1 and 76.4%, respectively).

Recovery rates of somatic coliphages from the adsorbed-filters varied between 16.2% for HA-PEI (samples with magnesium addition) and 96.2% for HA-Nalco (samples without magnesium). In all cases, the addition of magnesium improves the phage recovery from the electronegative filter and decreases the recovery percentages from electropositive filters.

TABLE 2
EFFECTS OF $MgCl_2$ ON ADSORPTION AND RECOVERY OF SOMATIC COLIPHAGES USING SEVERAL MEMBRANE FILTERS

Experiment No.		Sample titer[a]		Virus adsorption (%)[b]			Virus recovery (%)[c]		
		DAL	SAL	HA	HA-PEI	HA-Nalco	HA	HA-PEI	HA-Nalco
1	+ Mg	20	28	72.5	57.1	96.4	106.9	40.3	106.9
	– Mg	40	20	60.4	100	97.5	23.3	100	121.1
2	+ Mg	<10	3	90.0	100	95.0	>92.3	0	>15.4
	– Mg	20	10	66.6	100	100	4.5	22.2	32.5
3	+ Mg	90	45	94.2	41.1	37.7	49.9	8.4	12.8
	– Mg	20	26	65.5	98.1	94.2	23.2	137.7	131.9
Average	+ Mg	<40	25.3	85.6±11.5	66.1±30.4	76.4±33.5	>83.0±29.6	16.2±21.3	>45.0±53.6
	– Mg	26.7	18.7	64.2±3.3	99.4±1.1	97.2±2.9	17.0±10.8	86.6±58.9	96.2±52.8

[a]Expressed as PFU/100 ml.

[b]Adsorption rate = 100 – (phage titer in filtrate/phage titer in the sample by direct assay) × 100.

[c]Considering 100% the recovery obtained by the reference method (direct assay).

Influence of Tween 80 on the elution of coliphages adsorbed to membrane filters. Several experiments were carried out to test the influence of Tween 80 on the elution of adsorbed coliphages, which were previously filtered on different membrane filters. One hundred-ml volumes of dechlorinated tap water at pH 7.5, seeded with indigenous coliphages from raw sewage were treated with 0.05 M magnesium chloride and filtered through each membrane filter type. Three replicates for each experiment and conditions were performed. The filters were then applied upside-down on the agar-layer containing host lawns Petri dishes with and without Tween 80. The maximal efficiency in the elution and recovery of adsorbed-coliphages was obtained from the HA-type electronegative filters in petri dishes supplemented with Tween 80 (112.8% of the recovery obtained with the reference method, SAL technique, and 100 ml of sample processed) (Table 3). In these same conditions, the other filters, HA-PEI and HA-Nalco, showed lower efficiency in the phage recovery, with rates of 91.7 and 57.3%, respectively. All the filters used lost elution capability and recovered a low number of phages with plates containing medium without Tween 80, since none of the methods achieved recovery rates higher than 85%.

Application of the simple membrane filter method to several morphological phage groups. Several phage lysates were used to test the influence of the phage morphology on the efficiency of the

TABLE 3
INFLUENCE OF TWEEN 80 ADDED TO THE BOTTOM AGAR LAYER ON THE ELUTION OF SOMATIC COLIPHAGES ADSORBED ON DIFFERENT MEMBRANE FILTERS

Experiment No.	Sample titer[a]	Filters					
		HA		HA-PEI		HA-Nalco	
		+ Tween	– Tween	+ Tween	– Tween	+ Tween	– Tween
1	23	24	18	24	14	15	9
2	11	18	14	8	5	8	1
3	25	26	20	32	21	17	17
4	13	15	12	9	6	5	5
5	25	23	19	29	22	17	15
6	19	21	15	16	11	10	9
7	4	11	5	7	2	1	0
8	19	22	16	10	13	9	8
9	18	17	12	9	8	8	6
Mean ± SD[b]	17.4±7.0	19.7±4.8	14.6±4.6	16.0±9.8	11.3±6.9	10.0±5.4	7.8±5.7
Standard error	2.3	1.6	1.5	3.3	2.3	1.8	1.9
Recovery (%)	100	112.8	83.5	91.7	64.9	57.3	44.6
One group T-test		3.197		1.429		1.22	
Prob. (one tail)		0.0063[c]		0.0955[c]		0.1282	

[a]Calculated using SAL technique and expressed as PFU/100 ml.
[b]Mean ± standard deviation.
[c]Probability (one tail). Significant at 95% confidence level.

simple membrane filter method. Stock lysates of each bacteriophage were diluted in autoclaved distilled water to achieve a mean titer below 100 PFU per 100 ml. Three-replicate samples of 100 ml of the diluted bacteriophage lysate were analyzed by each membrane filter tested (HA, HA-PEI, and HA-Nalco) following the experimental protocol above mentioned (only magnesium chloride was added to the HA filters). The method was compared to the phage recovery obtained by two direct assay methods, the DAL technique (10 ml total volumes tested) and the SAL technique (100 ml total volumes tested). The results of phage adsorption rates and virus recoveries are given in Table 4. F-specific coliphages (MS-2 and Qß) present high adsorption rates in all the filters used, ranging from

TABLE 4

APPLICATION OF THE SIMPLE MEMBRANE FILTER METHOD TO RECOVER DIFFERENT MORPHOLOGICAL BACTERIOPHAGE GROUPS

		Virus adsorption (%)[b]			Virus recovery (%)[c]			Relative recovery (%)[d]		
Bacteriophage	Sample titer[a]	HA	HA-PEI	HA-Nalco	HA	HA-PEI	HA-Nalco	HA	HA-PEI	HA-Nalco
MS-2	384±86.9	100	100	99.7	>15.6	2.0	7.8	>15.6	2.0	7.8
Qß	46.5±29.8	96.8	100	51.7	45.2	0.7	15.0	46.7	0.7	29.2
Φx174	62.1±27.2	97.6	82.3	37.2	17.2	20.9	7.5	17.6	25.4	20.2
T6	69.0±29.0	100	100	90.6	14.0	10.6	12.1	14.0	10.6	13.6
T7	14.0±11.4	100	100	82.7	100	140.5	76.2	100	140.5	88.9

[a] Mean ± standard deviation of the maximal titer of phage obtained by any reference method (direct assay by DAL or SAL techniques).
[b] Adsorption rate = 100 – (phage titer in filtrate/phage titer in the sample by direct assay) x 100.
[c] Considering 100% the recovery obtained by the reference method (direct assay).
[d] Percent obtained considering the adsorption rate.

96.8 to 100%, except for HA-Nalco filters and Qß coliphage with a mean adsorption rate of 51.7%. However, virus recoveries were low, with a maximum of 45.2% (HA filter) and a minimum of 0.7% (HA-PEI filter). High adsorption rates were also obtained for the other phages tested, except for Φx174 and HA-Nalco filters (37.2%). Only T7 coliphages showed a high recovery rates for all the filters, ranging between 76.2 and 140.5% for HA-Nalco and HA-PEI filters, respectively.

Discussion

Bacteriophages against *E. coli* have been considered a reliable and useful indicator of bacterial and viral contamination of water (21, 28). For this reason, a simple, rapid and accurate method for phage enumeration is necessary.

Methods to concentrate bacteriophages from aquatic environments have been adapted from those used for animal virus concentration. Standard methodology for virus detection and recovery from natural waters consists in the adsorption of the viral particles to microporous filters and subsequent elution of the adsorbed virus into a small volume of eluent (3). The filters used can possess net negative or positive surface charge, although for optimal adsorption of viruses to electronegative filters, the adjustment of the water at about pH 3.5, or the addition to samples prior to filtration of di- or trivalent cations, such as magnesium chloride or aluminium chloride (12), is required. However, several phages may be inactivated at acid pH or as a result of pH changes (26). Electropositive filters possess a substantial advantage over electronegative filters, since viruses can be adsorbed on their surfaces over a broader pH range without the addition of salts, and the pre-treatment of the water is not required prior to virus adsorption (7). The results obtained from the application of these filters suggested that the content of dissolved organic compounds in natural waters significantly affect virus adsorption to electropositive filters (29). However, an additional problem arises with the elution process, since several studies showed that the conventional eluents used for animal viruses recovered low numbers of bacteriophages (2).

To simplify the elution step used for the coliphage concentration from water, Sobsey et al. (30) have proposed the application of filters containing adsorbed phages directly to the surface of agar medium with host bacteria and polysorbate (Tween 80) to enhance the phage elution from the filters.

Low sensitivity in the phage enumeration was obtained by the use of the double-agar-layer technique at low levels of coliphages in the sample (between 0 and 50 PFU per 100 ml). On the contrary, the SAL technique and the simple membrane filter method were more efficient (Table 1). However, the simple membrane filter method with Virosorb 1-MDS filters showed low reliability both in the recovery of somatic coliphages and in the clear lysis plaques visualization. The mean recovery of somatic coliphages obtained in this study by the use of electronegative cellulosic ester filters (68.9%) is similar to the recovery range obtained by Sobsey et al. (30), in the same experimental conditions, which varied between 27% and 97%, with a mean of 49%. In the case of the HA-PEI filters, the results obtained were very similar to those reported by Borrego et al. (6), using the same filters but with the VIRADEL technique. For HA-Nalco filters the recovery percentages obtained in this study are higher (36.6%) than those obtained with the VIRADEL technique (mean of 9.3%). These findings may be explained by the fact that the adding magnesium ions to the water and processing the sample through a cationic polymer-

treated filter is likely to create a situation were both the filter surface and the phages are more highly positively charged. This might actually lead to reduce phage adsorption, as it can be observed in Table 2.

Experiments carried out to determine the adsorption efficiency of somatic coliphages on the different membrane filters tested show a direct relationship between the addition of magnesium ions to the sample and the type of filter used. Thus, the addition of magnesium increases significantly (paired t-value = 3.35; $P = 0.078$) both adsorption and recovery rates with electronegative HA-type filters. On the contrary, higher adsorption and recovery rates were obtained with positively charged filters (HA-PEI and HA-Nalco) using samples without magnesium addition. The highest adsorption rate was achieved by HA-PEI filters with 99.4% (sample without magnesium ions), whilst the maximal recovery of somatic coliphages was obtained by HA-Nalco filters (96.2%) in samples without the addition of magnesium ions (Table 2).

Recently, it was reported that Tween 80 improved the recovery of coliphages adsorbed to filters (2, 30). In the present study, a significant relationship was established with the use of Tween 80 added to agar medium-host lawns on somatic coliphage elution using HA and HA-PEI filters (t-values of 3.19 and 1.43, respectively). On the contrary, no significant increase in the recovery rate of coliphages was obtained with the use of Tween 80 in the assay with HA-Nalco filters (t-value = 1.22, $P = 0.1282$).

The application of the simple membrane filter method to recover different phage groups yielded variable results (Table 4). F-specific RNA coliphages (MS-2 and Qß) tested were poorly recovered, between 15.5 and 45.2%, using HA filters. These results do not agree with those obtained by Sobsey et al. (30), although the different bacterial strains used as host in both studies may be an important factor to explain these differences. For these phages, the use of modified membrane filters, by addition of cationic polymers, decreased phage recovery to less than 15.0%. The exact effect of these cationic polymers on the isometric phages is not completely known, Several unconcluded studies carried out by us (data not presented) suggest a potential toxicity of the cationic polymers (PEI and Nalco) on the recovery of F-specific RNA coliphages. On the other hand, only coliphage T7 showed an optimal recovery rate with the use of the present methodology with all the filter types.

In summary, the simple membrane filter technique as described by Sobsey et al. (30) seems also to be an adequate method to enumerate somatic coliphages from water samples containing a low number of phages, which are undetected by the direct assay using the DAL technique, or for its use to verify the efficiency of the drinking water treatment processes. The application of this technique by the use of filters treated with cationic polymers to avoid the pre-treatment of the sample with magnesium ions, may be of interest in urgent situation, because it is more rapid and easier. The efficiency of the filters charge-modified by treatment with cationic polymers is higher with samples without the addition of divalent salts. On the other hand, the addition of Tween 80 to the agar medium as eluent agent improves the recovery of somatic coliphages in all cases.

References

1. Adams, M. H. (1959). Bacteriophages. Wiley Interscience, New York.
2. Armon, R., Arella, M., Payment, P. (1988). A highly efficient second-step concentration technique for bacteriophages and enteric viruses using ammonium sulfate and Tween 80. Can. J. Microbiol. **34**, 651–655.

3 Bitton, G. (1980). Adsorption of viruses to surfaces: Technological and ecological implications. *In* Bitton, G., Marshall, K. C. (ed.), Adsorption of Microorganisms to Surfaces, pp. 331-374. John Wiley & Sons, New York.

4. Bitton, G., Farrah, S. R., Montague, C. L., Akin, E. W. (1986). Viruses in drinking water. Environ. Sci. Technol. **20**, 216–222.
5. BOE (1990). Reglamentación técnico-sanitaria para el abastecimiento y control de calidad de las aguas potables de consumo público. Gaceta de Madrid **226**, 27488–27497.
6. Borrego, J. J., Cornax, R., Preston, D. R., Farrah, S. R., McElhaney, B., Bitton, G. (1991). Development and application of new positively charged filters for recovery of bacteriophages from water. Appl. Environ. Microbiol. **57**, 1218–1222.
7. Chang, L. T ., Farrah, S. R., Bitton, G. (1981). Positively charged filters for virus recovery from wastewater treatment plant effluents. Appl. Environ. Microbiol. **42**, 921–924.
8. Christensen, M. L. (1989). Human viral gastroenteritis. Clin. Microbiol. Rev. **2**, 51–89.
9. Craun, G. F. (1988). Surface water supplies and health. J. Am. Wat. Wrks. Ass. **80**, 40–52.
10. García-Páez, I. (1988). Estudio de métodos de detección y aislamiento de bacteriófagos específicos de *Bacteroides* sp. y *Escherichia coli* a partir de aguas polucionadas. Tesis de licenciatura, Universidad de Málaga, 256 pp.
11. Geldenhuys, J. C., Pretorius, P. D. (1989). The occurrence of enteric viruses in polluted water, correlation to indicator organisms and factors influencing their numbers. Wat. Sci. Tech. **21**, 105–109.
12. Gerba, C. P. (1984). Applied and theoretical aspects of virus adsorption to surfaces. Adv. Virus Res. **30**, 133–138.
13. Grabow, W. O. K. (1986). Indicator system for assessment of the virological safety of treated drinking water. Wat. Sci. Tech. **18**, 159–165.
14. Grabow, W. O. K., Coubrough, P. (1986). Practical direct plaque assay for coliphages in 100-ml samples of drinking water. Appl. Environ. Microbiol. **52**, 430–433.
15. Havelaar, A. H., Hogeboom, W. M. (1983). Factors affecting the enumeration of coliphages in sewage and sewage-polluted waters. Antonie van Leeuwenhoek **49**, 387–397.
16. Havelaar, A. H., Nieuwstad, T. J. (1985). Bacteriophages and fecal bacteria as indicators of chlorination efficiency in biologically treated wastewater. J. Wat. Pollut. Control Fed. **57**, 1084–1088.
17. Hejkal, T. W., Keswick, B. H., Labelle, R., Gerba, C. P., Sánchez, Y., Dreesman, G., Hafkin, B., Melnick, J. L. (1982). Viruses in a community water supply associated with an outbreak of gastroenteritis and infectious hepatitis. J. Am. Wat. Wrks. Ass. **74**, 318–321.
18. IAWPRC Study Group On Health Related Water Microbiology (1991). Bacteriophages as model viruses in water quality control. Wat. Res. **25**, 529–545.
19. Keswick, B. H., Gerba, C. P., Rose, J. B., Toranzos, G. A. (1985). Detection of rotavirus in treated drinking waters. Wat. Sci. Tech. **17**, 1–6.
20. Kott, Y. (1966). Estimation of low numbers of *Escherichia coli* bacteriophage by use of the Most Probable Number method. Appl. Microbiol. **14**, 141–144.
21. Kott, Y. (1984). Coliphages as a reliable enteric virus indicators. *In* Melnick, J. L. (ed.), Viruses in Water, pp. 171–174. S. Karger, Basel.
22. Mossel, D. A. A. (1982). Marker (index and indicator) organisms in food and drinking water. Semantics, ecology, taxonomy and enumeration. Antonie van Leeuwenhoek **48**, 609–611.
23. Nupen, E. M., Bateman, B. W. (1985). The recovery of viruses from drinking-water by means of an in-line electropositive cartridge filter. Wat. Sci. Tech. **17**, 63–69.
24. Payment, P., Armon, R. (1989). Virus removal by drinking water treatment processes. CRC Crit. Rev. Environ. Control **19**,15–21.
25. Preston, D. R., Farrah, S. R., Bitton, G. (1989). Removal of viruses from tapwater by fiberglass filters modified with a combination of cationic polymers. Wat. Sci. Tech., **21**, 93–98.
26. Seeley, N. D., Primrose, B. (1979). Concentration of bacteriophages from natural waters. J. Appl. Bacteriol. **46**, 103–116.

27. Shields, P. A., Ling, T. F., Tjatha, V., Shah, D. O., Farrah, S. R. (1986). Comparison of positively charged membrane filters and their use in concentrating bacteriophages in water. Wat. Res. **20**, 145–151.
28. Simkova, A., Cervenka, I. (1981). Coliphages as ecological indicators of enteroviruses of various water systems. Bull. World Health Org. **59**, 611–618.
29. Sobsey, M. D., Glass, J. S. (1984). Influence of water quality on enteric virus concentration by microporous filter methods. Appl. Environ. Microbiol. **47**, 956–960.
30. Sobsey, M. D., Schwab, K. J., Handzel, T. H. (1990). A simple membrane filter method to concentrate and enumerate male-specific RNA coliphages. J. Am. Wat. Wrks. Ass. **82**, 52–59.
31. Wentsel, R. S., O'Neill, P. E., Kitchens, J. F. (1982). Evaluation of coliphage detection as a rapid indicator of water quality. Appl. Environ. Microbiol. **43**, 430–434.

MICROBIOLOGÍA SEM **10** (1994), 297–304

Aplicación de métodos inmunológicos al análisis de bacterias biolixiviadoras

Orquídea Coto,* Ana I. Fernández, Teresa León, Dagoberto Rodríguez

Departamento de Microbiología, Facultad de Biología, Universidad de La Habana, Ciudad de La Habana, Cuba

Recibido 20 enero 1994/Aceptado 30 junio 1994

Summary

Pure cultures of *Thiobacillus ferrooxidans* and mixed cultures of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* isolated from the Matahambre mine (Cuba) were used to fit immunodiffusion and immunoelectron microscopy to the study of iron oxidizing bacteria. The possibilities, advantages and limits of those techniques have been studied from both the identification and the serological characterization points of view. Finally, the efficiency of these methods was tested by applying them to the identification of microorganisms from acidic waters from the mine.

Key words: immunodiffusion, immunoelectron microscopy, *Thiobacillus ferrooxidans*, bioleaching

Resumen

Utilizando cultivos simples de *Thiobacillus ferrooxidans* y cultivos mixtos de *Thiobacillus ferrooxidans* y *Leptospirillum ferrooxidans*, aislados del yacimiento de Matahambre (Cuba), se han puesto a punto las técnicas de inmunodifusión doble y de inmunomicroscopía electrónica de transmisión para estudiar bacterias ferrooxidadoras. Se han analizado y valorado sus posibilidades y limitaciones, tanto desde el punto de vista de la identificación como de la caracterización serológica. Finalmente, se ha comprobado la eficiencia de estas técnicas aplicándolas a la identificación de microorganismos de aguas ácidas procedentes de la propia mina.

* *Correspondencia*: Orquídea Coto. Departamento de Microbiología. Facultad de Biología. Universidad de La Habana. Ciudad de La Habana. Cuba.

Introducción

Las bacterias que colonizan las aguas ácidas de drenaje de los yacimientos mineros forman una población muy heterogénea. Entre las bacterias lixiviadoras, *Thiobacillus ferrooxidans* es la más predominante entre un número de microorganismos quimiolitótrofos y heterótrofos. *Leptospirillum ferrooxidans* es una de las bacterias ferrooxidadoras que con mas frecuencia acompaña a *Thiobacillus ferrooxidans* en los ambientes mineros (2, 8). Sin embargo, muchos de los microorganismos que existen en estos hábitats están aún sin conocer, a causa de las dificultades que presentan para su cultivo, aislamiento e identificación.

La identificación y clasificación de bacterias se realiza tradicionalmente mediante estudios morfológicos y bioquímicos, aunque más recientemente se han comenzado a utilizar los métodos genéticos, serológicos y químicos (6), que destacan por su eficiencia, alta sensibilidad y rapidez. Sin embargo, el empleo de métodos serológicos con bacterias lixiviadoras no se ha generalizado hasta el momento.

Con el objetivo de valorar las ventajas de los métodos serológicos como procedimiento para la identificación y caracterización de estos microorganismos, en este trabajo se describe la puesta a punto de la técnica de inmunodifusión doble (IDD) para este tipo de bacterias. Los resultados se relacionan y comparan con los obtenidos mediante inmunomicroscopía electrónica de transmisión (IMET), empleando antisueros obtenidos de conejos, a partir de cultivos simples de *Thiobacillus ferrooxidans* y cultivos mixtos de *Thiobacillus ferrooxidans* y *Leptospirillum ferrooxidans*. Además, se estudian las relaciones antigénicas entre *Thiobacillus ferrooxidans* y otros géneros aislados del mismo hábitat y de otros hábitats no afines.

Materiales y métodos

Cepas bacterianas. Se utilizaron bacterias ferrooxidadoras presentes en aguas ácidas del yacimiento minero de Matahambre (Cuba), aisladas por pases sucesivos de medio líquido (11) a medio sólido (7). Una vez logradas las colonias ámbar, característica principal de estas bacterias sobre medio con agar, se volvieron a inocular en medio líquido. Dos de estos cultivos, denominados 8H y 22poli, resultaron ser cultivos puros. Otro de ellos (24Ha) se comprobó que era un cultivo mixto de *Thiobacillus ferrooxidans* y *Leptospirillum ferrooxidans* (4).

La bacteria *Thiobacillus ferrooxidans*, cepa ATCC 23270, fue donada por el Dr. Bauer. Paralelamente se emplearon dos *Thiobacillus thiooxidans* previamente identificados (4) y seis bacterias no relacionadas, donadas por el Laboratorio de Bacteriología de la Universidad de La Habana: *Escherichia coli*, *Pseudomonas aeruginosa*, *Azospirillum brazilense*, *Flavobacterium suaveolans*, *Salmonella enteritidis* y *Xanthomonas campestris*.

Preparación del inmunógeno. Las bacterias 8H, 22poli y la cepa ATCC 23270 de *Thiobacillus ferrooxidans*, así como el cultivo mixto 24Ha, fueron inoculados en matraces Erlenmeyer con 500 ml de medio líquido (11), y se mantuvieron en agitación durante 7 días a 37°C. Las células se recogieron por filtración a través de membranas Millipore de 0,45 µm, y se lavaron tres veces con 5 ml de agua ácida,

pH 1,8, dejándolas reposar durante 30 min para eliminar el hierro residual. La concentración se estimó por lectura de la absorbancia a 540 nm y, para la preparación de antisueros, se llevó a una densidad óptica (DO) de 0,4 ó 0,8 unidades.

Preparación de antisueros. Se utilizaron 5 conejos de aproximadamente 2 kg de peso. Dos de ellos se inmunizaron con *Thiobacillus ferrooxidans* ATCC 23270, otros dos con *Thiobacillus ferrooxidans* 8H y 22poli, y el quinto con cultivo mixto de *Thiobacillus ferrooxidans* y *Leptospirillum ferrooxidans* (24Ha). Los conejos fueron inoculados semanalmente con 1 ml del inmunógeno correspondiente (a 0,4 ó 0,8 unidades de densidad óptica, según el caso), emulsionado con adyuvante de Freund, 1:1, completo (C) o incompleto (I), de acuerdo con el siguiente esquema:

Inoculación	1	2	3	4	5	6	7	8
Día	1	7	14	28	35	42	49	79
Dosis (DO)	0,4	0,4	0,8	0,8	0,8	0,8	0,8	0,8
Adyuvante	C	I	I	I	–	–	–	–

La primera inoculación se realizó con células inactivadas por calor. Se empleó la vía subcutánea para las 3 primeras inoculaciones y la vía intravenosa para las 5 restantes. La sangre fue recogida por punción cardiaca y los antisueros (As) correspondientes (As23270, As8H, As22poli, As24Ha) fueron conservados en congelación. La inoculación se complementó con una nueva inyección de recuerdo, 4 semanas más tarde.

Inmunodifusión doble. Las inmunodifusiones se realizaron según el procedimiento previamente descrito (9). Se emplearon dos preparaciones del antígeno:

(i) *Tratamiento ultrasónico*: 0,5 ml de la suspensión bacteriana en tampón fosfato, pH 8,2, se sometió a tratamiento con ultrasonido a 20 kHz, en baño de hielo y durante 4 min.

(ii) *Tratamiento enzimático*: la suspensión bacteriana se centrifugó y el sedimento celular fue tratado con lisozima en tampón hipertónico (25 mM sacarosa, 25 mM Tris, 25 mM EDTA, pH 8), durante 48 h.

Inmunomicroscopía electrónica. La inmunomicroscopía electrónica con anticuerpos marcados con oro coloidal se realizó por el método indirecto (10), usándose como conjugado IgG de cabra anti-IgG de conejo marcado con oro coloidal de 5 nm de tamaño de partícula. Los títulos de los As23270, As22poli, As24Ha y As8H se determinaron mediante el establecimiento del umbral de dilución, más allá del cual no se obtenía marcaje. Cada antígeno se enfrentó a distintas diluciones (desde 1:10 hasta 1:3000) de su antisuero homólogo.

Para el estudio de la aplicabilidad de este procedimiento a un caso real, se utilizaron cultivos primarios de muestras de agua de diferentes niveles del yacimiento, concentrados por filtración a través de membranas Millipore de 0,45 μm, e inoculados en medio Touvinen y Kelly (11) y en medio tiosulfato (3). Se mantuvieron 7 y 15 días, respectivamente, en agitación a 35°C, hasta apreciar el cambio de coloración. Las células fueron recogidas, resuspendidas en agua ácida, pH 1,8, y enfrentadas a los antisueros; posteriormente se observaron al microscopio electrónico de transmisión.

TABLA 1
REACCIONES Ag-As ESTUDIADAS POR IDD

As	Título	Número de bandas observadas frente a Ag			
		2321	22poli	8H	24Ha
2321	1:32	3	2	2	0
22poli	1:32	2	3	2	0

Resultados y discusión

La titulación de los antisueros 23270 y 22poli por IDD produjo un título de 1:32 (Fig. 1). Al enfrentar estos antisueros a sus antígenos homólogos tratados, tanto enzimática como ultrasónicamente, no se pudo apreciar ninguna diferencia entre ambos tratamientos en cuanto al numero de bandas, pero sí en cuanto a la intensidad de éstas (datos no mostrados). Las bandas de precipitación fueron más intensas frente al antígeno tratado con lisozima, lo que puede indicar que la sonicación provoca cierta desnaturalización de las estructuras antigénicas (5). Las reacciones cruzadas entre el As 23270 y los antígenos (Ag) 22poli, 8H y 24Ha mostraron menos bandas de precipitación que la reacción homóloga; lo mismo sucedió con el As anti 22poli al reaccionar con sus antígenos heterólogos (Tabla 1).

Por otra parte, al enfrentar el As anti 23270 a su antígeno homólogo y a los antígenos heterólogos 8H y 22poli, se observó la aparición de un saliente o «espuela» en la interacción de la banda de precipitación de 22poli y la de 23270, y la de la primera y la de 8H (Fig. 2). Esto es señal de una identidad parcial entre el antígeno 22poli y los otros dos; algunos de los determinantes antigénicos presentes en los extractos son comunes, pero 22poli posee estructuras antigénicas diferentes a las presentes en 8H y 23270. Este resultado es muy significativo, al indicar que *Thiobacillus ferrooxidans* 22poli es un serotipo

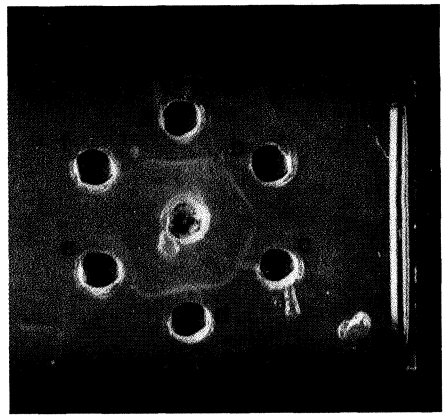


FIG. 1. Titulación del As 23270 frente a su antígeno correspondiente. Dilución del antisuero: **a**: 1/2; **b**: 1/4; **c**: 1/8; **d**: 1/16; **e**: 1/32; **f**: 1/64.

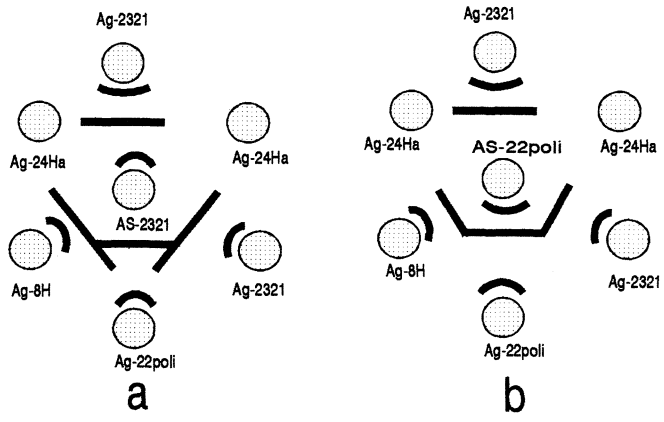


FIG. 2. Caracterización inmunológica de diferentes cultivos de *T. ferrooxidans* por IDD. Los As anti 23270 (a) y anti 22poli (b) se han enfrentado a sus Ag homólogos y a otros heterólogos.

diferente de los *T. ferrooxidans* 8H y 23270. Resultados similares fueron observados por Arredondo y Jerez (1) al estudiar serológicamente diversas cepas de *Thiobacillus ferrooxidans*. Sin embargo, cuando se enfrentó el As anti 22poli a su antígeno homólogo y a los heterólogos 23270 y 8H, se observó que el encuentro de las bandas de precipitación era diferente, y no presentaba cruzamientos (Fig. 2); el As anti 22poli reconoció todos los determinantes antigénicos en los extractos 8H, 22poli y 2321; el Ag 22poli presentó identidad con 2321 y con 8H.

Cuando ambos antisueros anti *Thiobacillus ferrooxidans* (As23270 y As22poli) se enfrentaron al Ag heterólogo 24Ha, no se detectaron bandas de precipitación (Fig. 2). Dado que el Ag 24Ha contiene a la vez *Thiobacillus ferrooxidans* y *Leptospirillum ferrooxidans*, es posible que este resultado se deba a que el número de células del primero, en el cocultivo, sea inferior a la requerida para su detección por IDD. Por otro lado, cuando el antisuero anti 24Ha se enfrenta a sus antígenos homólogos no se detectan bandas de precipitación, pero éstas sí son visibles cuando se enfrenta a los Ag heterólogos de

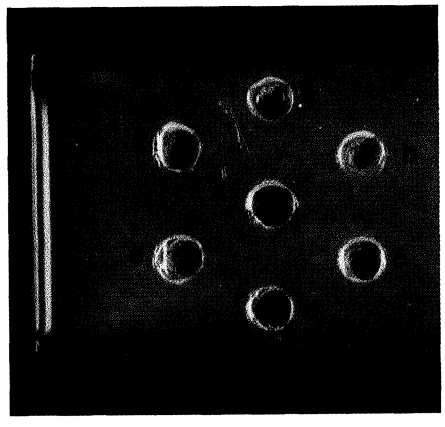


FIG. 3. IDD del As anti 24Ha frente a distintas fuentes antigénicas: **a**, **b** y **f**: Ag 24Ha; **c**: Ag 8H; **d**: Ag 23270; **e**: Ag 22poli.

TABLA 2
RELACIONES ANTIGÉNICAS CRUZADAS DEL As2321 CON OTROS GÉNEROS, ESTUDIADAS POR IMET

Antígeno	Dilución del antisuero		
	1:5	1:10	1:20
Thiobacillus thiooxidans	–	–	–
24Ha / forma bacilar	+	+	+
24Ha / forma espiralada	–	–	–
Escherichia coli	–	–	–
Pseudomonas aeruginosa	–	–	–
Flavobacterium suaveolans	+	+	–
Azospirillum brazilense	–	–	–
Salmonella enteritidis	–	–	–
Xanthomonas campestris	+	+	–

Thiobacillus ferrooxidans 23270, 22poli y 8H (Fig. 3). Este último resultado confirma la hipótesis anterior: en este caso los antígenos proceden de monocultivos y la concentración celular en ellos es mayor, lo que permite la observación de la reacción. La posibilidad de que pudieran darse interferencias entre ambos antígenos que impidieran su adecuada difusión en el gel parece mucho menos viable.

Paralelamente se realizó un estudio de la cinética de producción de Ac en los animales de experimentación inoculados con los inmunógenos 23270, 22 poli y 24Ha; los resultados se muestran en las Figuras 4a, 4b y 4c. Con el esquema de inmunización propuesto se detectaron Ac contra *T. ferrooxidans* a partir de los 12 días de comenzada la inmunización; a los 20 días el título era de 1:500 (Fig. 4a), siendo ya suficientemente bueno para el trabajo de IMET. Este esquema de inmunización también resultó efectivo para *Leptospirillum ferrooxidans* ya que permitió obtener un As que reaccionó en IMET hasta una dilución de 1:2000. Mantener la inmunización más allá de los dos meses no reportó beneficio alguno en cuanto al título de anticuerpo.

La cinética de producción de Ac de las formas bacilar y espiralada del inmunógeno 24Ha fue diferente (Fig. 4b y 4c) y la misma se inició mucho antes en el caso de la forma bacilar.

El estudio de las reacciones serológicas entre los cultivos de *Thiobacillus ferrooxidans* y otros géneros diferentes mediante IMET se muestran en la Tabla 2. El As anti 23270 reconoció la forma bacilar del cocultivo 24Ha pero no la forma espiralada, ni siquiera a menores diluciones del As. También reaccionó con *Flavobacterium suaveolans* y con *Xanthomonas campestris*, si bien únicamente hasta la dilución 1:10 del antisuero, por lo que no resulta significativo; su posible interferencia en los estudios de detección e identificación de *Thiobacillus ferrooxidans* es insignificante ya que estas bacterias no se encuentran en las aguas ácidas de las minas, hábitat natural de las bacterias oxidadoras del hierro.

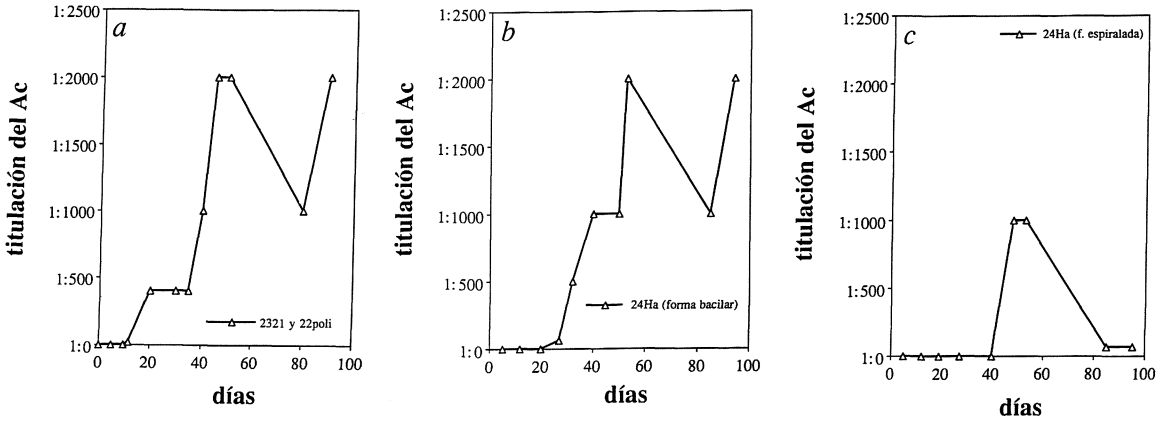


FIG. 4. Cinética de producción de antisueros seguida por IMET. **a**: inmunización con cultivos puros de *T. ferrooxidans* 23270 y 22poli; **b**: inmunización con cocultivo 24Ha y seguimiento de la respuesta contra la forma bacilar; **c**: inmunización con cocultivo 24Ha y seguimiento de la respuesta contra la forma espiralada.

El «screening» de *T. ferrooxidans* mediante IMET en 8 muestras de aguas ácidas de las minas permitió contrastar la validez de esta técnica en un caso práctico. En tres de esas muestras se detectó la presencia de esta bacteria; en otras cuatro muestras se observaron bacilos que no reaccionaron con el As empleado, y en la muestra restante se detectó una mezcla de dos bacterias, una de ellas de morfología bacilar que reaccionó con el As anti 23270, y la otra, de forma espiralada y en ocasiones curvada, que no fue reconocida por el antisuero citado.

En resumen, estos resultados resaltan la importancia de la IMET en el estudio y la caracterización de estas bacterias, al ofrecer una doble información: morfológica (aspecto, tamaño, presencia de flagelos, etc.) y serológica. Igualmente, facilita la detección rápida de *T. ferrooxidans* en muestras naturales. Por otro lado, el estudio que se presenta demuestra también la utilidad de la técnica de IDD para este tipo de investigaciones, ya que permite diferenciar fenotipos entre los distintos *T. ferrooxidans*, aspecto que no alcanza a detectar la IMET. La complementación de estas dos técnicas inmunológicas aportará grandes ventajas en la identificación y caracterización de las bacterias oxidadoras del hierro.

Bibliografía

1. Arredondo, R., Jerez, C. A. (1989). Specific dot-immunobinding assay for detection and enumeration of *Thiobacillus ferrooxidans*. Appl. Environ. Microbiol. **55**, 2025–2029.
2. Brauckmann, M. B. (1986). Untersuchungen zur Biozonose des Bergwesks biotops. «Atter Lager». Erzbergwesk Remmlsberq. Erzmetall. **39**, 613–617.
3. Bryant, R. D., McGroarty, K. M., Costerton, J. W., Laishley, E. J. (1983). Isolation and characterization of a new acidophilic *Thiobacillus* species (*T. albertis*). Can. J. Microbiol. **29**, 1159–1170.

4. Coto, O., Fernández, A. I., León, T., Rodríguez, D. (1992). *Thiobacillus ferrooxidans* detection using immunoelectron microscopy. Microbiología SEM **8**, 76–81.
5. Hughes, D. E. (1961). The desintegration of bacteria and other microorganisms by the MSE-mollar ultrasonic desintegrator. J. Biochem. Microbiol. Techn. Eng. **3**, 405–409.
6. Jones, D., Krieg, N. R. (1984). Serology and chemiotaxonomy: bacterial classification. *In* Tansill, B. (ed.) Bergey's Manual of Systematic Bacteriology, pp. 15–18. Williams and Wilkins, Baltimore.
7. Jerez, C. A., Peirano, I., Chamorro, D., Campos, G. (1986). Immunological and electrophoretic differentiation of *Thiobacillus ferrooxidans* strains. *In* Lawrence, R. W., Branion, R. M. R., Ebner, H. G. (ed.), Fundamental and Applied Biohydrometallurgy, Proc. Intern. Symp. Vancouver, pp. 443–456. Elsevier, Amsterdam.
8. Markosyan, G. E. (1972). A new acidophilic iron bacterium *Leptospirillum ferrooxidans*. Biol. Zh. Arm. **25**, 26–32.
9. Ouchterlony, O., Nelsson, W. (1978). Immunodifussion and immunoelectrophoresis. *In* Weir, D. M. (ed.), Handbook of Experimental Immunology, pp. 655–706. Blackwell, Oxford.
10. Rodríguez, D. (1988) Inmunomicroscopía electrónica de las bacterias fitopatógenas. *In* Métodos Serológicos en Fitobacteriología, pp. 39–94. Publicaciones de la Universidad de La Habana, La Habana.
11. Tuovinen, O. H., Kelly, D. P. (1974). Studies on the growth of *Thiobacillus ferrooxidans*. Arch. Microbiol. **98**, 351–364.

MICROBIOLOGÍA SEM **10** (1994), 305–310

Conservación de bacterias acidófilas

Ana Velasco,[1] Paloma Lorenzo,[1] María I. de Silóniz,[2] Julián Perera[1,*]

[1]*Departamento de Bioquímica y Biología Molecular,* [2]*Departamento de Microbiología, Facultad de Ciencias Biológicas, Universidad Complutense de Madrid*

Recibido 7 febrero 1994/Aceptado 27 abril 1994

Summary

The particular difficulties found in the growth of cultures of acidophilic bacteria led us to compare different standard long-term storage methods and evaluate the results we had from two representative acidophilic bacteria: a mesophile, *Thiobacillus ferrooxidans* ATCC 23270, and a thermophile, *Sulfolobus* BC.

Key words: long-term storage, acidophiles, *Thiobacillus ferrooxidans*, *Sulfolobus*

Resumen

Las peculiares características del crecimiento de cultivos de bacterias acidófilas, presenta una serie de dificultades que hacen aconsejable disponer de un método fiable de conservación a largo plazo. En este trabajo se realiza un estudio comparativo de diferentes procedimientos de conservación aplicados al caso particular de estas bacterias, representadas en una estirpe mesófila (*Thiobacillus ferrooxidans* ATCC 23270) y otra termófila (*Sulfolobus* BC).

* *Correspondencia*: Julián Perera. Depto. de Bioquímica y Biología Molecular. Facultad de Ciencias Biológicas. Universidad Complutense de Madrid. 28040 Madrid. Tel.: 91-4490379. Fax: 91-2433362.

Introducción

La conservación de cultivos bacterianos no constituye generalmente un problema importante. Para muchos casos existen procedimientos adecuados y eficaces, que permiten conservar microorganismos durante largos períodos de tiempo y recuperarlos en cultivo de manera sencilla y eficaz (2). En otros casos, sin embargo, los métodos de conservación y recuperación no están todavía tan firmemente establecidos. Uno de estos casos es el de las bacterias acidófilas, las cuales, debido a sus peculiares características de crecimiento, no se acomodan bien a los métodos de conservación habituales.

Thiobacillus ferrooxidans es una bacteria acidófila y quimiolitótrofa que utiliza la oxidación de hierro ferroso a hierro férrico, y la de ciertos compuestos reducidos de azufre como fuente de energía para el crecimiento (1). *Sulfolobus* BC es una bacteria heterótrofa y quimiolitótrofa facultativa, termoacidófila, capaz de utilizar la oxidación de minerales sulfurosos (pirita y calcopirita) como fuente de energía para el crecimiento (6).

En ambas bacterias, la conservación siempre ha representado un grave problema, pues suele llevar asociada una pérdida de la capacidad de oxidación de hierro, responsable del metabolismo quimiolitotrófico y, por tanto, de la obtención de energía. Además, la biomasa obtenida en los cultivos de estas bacterias es muy inferior a la de la mayoría de los microorganismos, hecho mucho más evidente en el caso de *T. ferrooxidans*. Esto obliga a mantener de forma continua la reserva del cultivo bacteriano mediante subcultivos en crecimiento, en volúmenes grandes, medio fresco y durante períodos largos de tiempo, lo que representa un gran consumo de tiempo y esfuerzo. De ahí el interés que tiene el desarrollo de un método para la conservación de estos microorganismos durante largos períodos de tiempo. Con este fin, hemos llevado a cabo un estudio comparativo de distintos métodos estándar de conservación de cultivos con microorganismos acidófilos, evaluando los resultados obtenidos.

Materiales y métodos

Para el desarrollo de este trabajo se ha escogido un microorganismo representante de bacterias acidófilas mesófilas (la estirpe 23270 de *Thiobacillus ferrooxidans* de la American Type Culture Collection, ATCC) y otro de termófilas (la estirpe BC de *Sulfolobus*)(6). Como control se utilizó la estirpe JM103 de *Escherichia coli* (5). Se hizo crecer cada estirpe en su correspondiente medio óptimo. *T. ferrooxidans* ATCC 23270 crecía en medio mínimo de sales (0,1 g/l K_2HPO_4, 0,2 g/l $(NH_4)_2SO_4$, 0,4 g/l $MgSO_4$, 0,1 g/l KCl), a pH 2,0, suplementado con $FeSO_4$ hasta 50 mM (4), a 35°C y en agitación (150 rpm). *Sulfolobus* BC, en solución salina 9K (3) [3 g/l $(NH_4)_2SO_4$, 0,1 g/l KCl, 0,5 g/l K_2HPO_4, 0,5 g/l $MgSO_4$, 0,01 $Ca(NO_3)_2$], pH 2,0, con calcopirita al 1% y a 65°C. *E. coli* JM103 fue cultivada a 37°C en medio LB (10 g/l triptona, 5 g/l extracto de levadura, 5 g/l NaCl).

Dado el bajo rendimiento celular que se consigue con estos cultivos, se trató de evaluar el efecto de la concentración celular del inóculo en el mantenimiento y recuperación del cultivo. Para ello se utilizó un inóculo procedente de un cultivo en crecimiento normal y otro a partir de un concentrado de células conseguido por filtración del primero. La filtración se llevó a cabo a través de membranas de 0,45 µm de poro. En algún caso (conservación en glicerol), las células retenidas se resuspendieron en el mismo medio de cultivo, en un volumen que permitió lograr una concentración de 20:1 respecto al cultivo

original para *T. ferrooxidans* y de 10:1 para *Sulfolobus*. En otros casos, las células retenidas en el filtro se utilizaron directamente para el inóculo del medio de conservación. Esta etapa de concentración no se llevó a cabo en el caso del cultivo control de *E. coli*, cuya biomasa, muy superior a la de los otras dos bacterias, no debería de plantear ningún problema para la recuperación del cultivo tras la conservación.

Los cultivos así preparados se utilizaron para inocular diferentes medios de conservación (2), tal como se indica:

Medio «agua ácida»: cuando el inóculo usado fue el propio cultivo celular, se le añadieron dos volúmenes de agua acidulada con H_2SO_4 hasta pH 2,0. Cuando el inóculo consistía en células procedentes de la filtración, éstas se suspendieron directamente en agua acidulada.

Medio «glicerol»: la suspensión de células (normal o concentrada) se llevó a una concentración de glicerol del 35%.

Medio «leche desnatada»: al cultivo celular se le añadieron dos volúmenes de leche desnatada (marca Pascual). Cuando se usaron como inóculo las células retenidas tras filtración, éstas fueron directamente suspendidas en la leche.

Como métodos de conservación alternativos se utilizaron, además, la congelación directa del cultivo en nitrógeno líquido y la liofilización.

Los distintos viales se conservaron a diferentes temperaturas (2): ambiente, 4°C, –20°C y –70°C. En todos los casos las muestras se prepararon por triplicado, de tal forma que pudieran ser recuperadas a tres tiempos diferentes (1, 3 y 6 meses) para poder evaluar el efecto del tiempo de conservación sobre la eficacia de cada método.

Para la recuperación de las diferentes muestras, se inoculó cada vial de conservación en proporción 3:10 en medio fresco específico para cada cultivo bacteriano:

Medio mínimo de sales a pH 2,0. Suplementado con 50 mM $FeSO_4$, para *Thiobacillus ferrooxidans* ATCC 23270.

Solución salina 9K a pH 2,0. Suplementada con 5 mM $Na_2S_4O_6$, 2 mM NaCl, 0,2% casaminoácidos para *Sulfolobus* BC. Aunque el medio de cultivo en el que normalmente se hace crecer *Sulfolobus* BC sólo contiene calcopirita como fuente de energía, la recuperación es algo más rápida y eficaz cuando el medio 9K se suplementa con tetrationato, como fuente de energía, y casaminoácidos, como fuente de carbono.

Medio LB, para *E. coli*.

Los cultivos se hicieron crecer a la temperatura adecuada en cada caso: 35°C para *Thiobacillus ferrooxidans* ATCC 23270, 65°C para *Sulfolobus* BC y 37°C para *E. coli*. La recuperación se consideró positiva cuando al cabo del tiempo predeterminado (7 días para *T. ferrooxidans*, 15 días para *Sulfolobus*) pudo detectarse crecimiento celular (mediante observación al microscopio), y, además, en el caso de *T. ferrooxidans*, oxidación de hierro ferroso en el cultivo (aparición de coloración naranja por precipitación de hierro férrico).

Resultados y discusión

Los efectos de los diferentes métodos de conservación sobre la viabilidad de las células de *T. ferrooxidans* quedan resumidos en la Tabla 1. El agua ácida a pH 2,0 parece ser un método de conservación óptimo, a corto y a largo plazo, para *T. ferrooxidans*, ya que en todos los casos (recupe-

TABLA 1
SUPERVIVENCIA DE LAS CÉLULAS DE *Thiobacillus ferrooxidans* DESPUÉS DE HABER SIDO SOMETIDAS A DIFERENTES PROCESOS DE CONSERVACIÓN

Condiciones de conservación			Crecimiento		
Medio de conservación	Inóculo	Temperatura	1 mes	3 meses	6 meses
Agua ácida pH 2,0	cultivo	4°C	+	+	+
	células	4°C	+	+	+
Glicerol 35%	cultivo	–20°C	+	–	–
		–70°C	+	–	–
	células	–20°C	+	–	–
		–70°C	+	–	–
Leche desnatada	cultivo	4°C	+	+	+
		–70°C	+	+	+
	células	4°C	–	–	–
		–70°C	–	–	–
Liofilizado	cultivo	20°C	–[a]	–[a]	–[a]
Nitrógeno líquido	cultivo	–70°C	–	–	–

[a] Tras tiempo largo, resultaba +.

ración al cabo de 1, 3 y 6 meses de conservación) las células presentaban capacidad oxidante de ferroso a férrico y una velocidad de crecimiento semejante a la de un cultivo «fresco». Las células de *T. ferrooxidans* conservadas en glicerol al 35% mantienen su viabilidad a corto plazo, mientras que a tiempos de conservación superiores al mes ya no presentan viabilidad.

La leche desnatada es un buen medio de conservación, pero siempre que el inóculo de células contenga solución salina a pH 2,0. Cuando las células son resuspendidas directamente en leche, no muestran viabilidad en el intento de recuperación. Muy probablemente, el abandono de las condiciones de pH próximas a 2,0 es un factor determinante en la pérdida de viabilidad de estas células. Por el contrario, la precipitación de diferentes componentes de la leche que tiene lugar cuando ésta se añade al cultivo (en medio salino a pH 2,0), podría eliminar algún producto de la solución que resultara tóxico para *T. ferrooxidans* en el momento de la recuperación del cultivo, y que, sin embargo, estaría presente cuando las células se resuspenden directamente en leche.

Los otros dos métodos de conservación, liofilización y congelación en nitrógeno líquido, no resultan adecuados para la conservación de *T. ferrooxidans*. Las células sometidas a liofilización son capaces de recuperar su viabilidad, pero requieren un largo período de tiempo para la recuperación del cultivo. La eficacia de esta recuperación parece aumentar bastante con ciertos tratamientos del cultivo antes y después del proceso de liofilización, tal y como se ha descrito recientemente (7).

La viabilidad celular de *Sulfolobus* BC en los diferentes medios de conservación se indica en la Tabla 2. Independientemente del método de conservación, la biomasa del inóculo parece ser importante, ya que sólo se consigue una recuperación aceptable en los experimentos realizados a partir del concentrado celular, exceptuando el caso de la leche desnatada, por las circunstancias descritas.

TABLA 2
SUPERVIVENCIA DE LAS CÉLULAS DE *Sulfolobus* BC DESPUÉS DE HABER SIDO SOMETIDAS A DIFERENTES PROCESOS DE CONSERVACIÓN

Condiciones de conservación			Crecimiento		
Medio de conservación	Inóculo	Temperatura	1 mes	3 meses	6 meses
Agua ácida pH 2,0	cultivo	4°C	–	–	–
	células	4°C	+	+	+
Glicerol 35%	cultivo	–20°C	–	–	–
		–70°C	–	–	–
	células	–20°C	+	+	+
		–70°C	+	ND[a]	+
Leche desnatada	cultivo	4°C	–	–	–
		–20°C	–	–	–
		–70°C	–	–	–
	células	4°C	–	–	–
		–20°C	–	–	–
		–70°C	–	–	–
Liofilizado	cultivo	20°C	–	ND	–
Nitrógeno líquido	cultivo	–70°C	+	+	+

[a] ND, no determinado.

El agua ácida a pH 2,0 parece ser un buen medio de conservación, a corto y a largo plazo, en *Sulfolobus* BC, así como la congelación del cultivo en nitrógeno líquido. No obstante, resulta difícil explicar el hecho de que la conservación en N_2 líquido realizada a partir de un inóculo directo de cultivo permita la recuperación de la viabilidad celular, mientras que la conservación del mismo inóculo directo en agua ácida a pH 2,0 no lleve a la recuperación del cultivo. Es posible que el motivo sea la diferente temperatura de conservación, 4°C o –70°C, utilizada en cada caso. El efecto que tiene la conservación a 4°C sobre una bacteria termófila parece ser mucho más perjudicial que su congelación.

Finalmente, el glicerol al 35% parece ser un buen medio de conservación, siempre que el inóculo sea un concentrado celular. La recuperación de la capacidad de crecimiento del cultivo en este medio de conservación es algo más lenta que la del cultivo fresco.

Las células de *E. coli* JM103 no presentan ningún problema para recuperar su viabilidad después de haber sido sometidas a los diferentes procesos de conservación.

Bibliografía

1. Kelly, P. D., Harrison, A. P. (1989). Genus *Thiobacillus*. *In* Staley, J. T., Bryant, M. P., Pfennig, N., Holt, J. G. (ed.), Bergey's Manual of Systematic Bacteriology, vol. 3, pp. 1842–1858. Williams and Wilkins, Baltimore.
2. Lapage, S. P., Shelton, J. E., Mitchell, T. G., Mackenzie, A. R. (1970). Culture collection and the preservation of bacteria. *In* Norris, J. R., Ribbons, D. W. (ed.), Methods in Microbiology, vol. 3A, pp. 135–228. Academic Press, London.
3. Lindström, E. B., Shelin, H. M. (1989). High efficiency of plating of the thermophilic sulphur-dependent archaebacterium *Sulfolobus acidocaldarius*. Appl. Env. Microbiol. **55**, 3020–3021.
4. Lorenzo, P., Silóniz, M. I., Perera, J. (1990). Comparison of quantitative analytical methods in evaluating acidophilic iron- and sulphur-oxidizing microorganisms liquid cultures. Microbiología SEM **7**, 53–56.
5. Messing, J., Crea, R., Seeburg, P. H. (1981). A system for shotgun DNA sequencing. Nucleic Acid Res. **9**, 309–321.
6. Norris, P.R. (1990). Acidophilic bacteria and their activity in mineral sulphide oxidation. *In* Ehrlich, H. L., Brierley, C. L. (ed.), Microbial Mineral Recovery, pp. 3–54. McGraw Hill, New York.
7. Wakao, N., Konno, J., Sakurai, Y., Shiota, H. (1990). Survival of freeze-dried *Thiobacillus ferrooxidans* NCIB 8455 after long-term storage. J. Gen. Appl. Microbiol. **36**, 283–286.

MICROBIOLOGÍA SEM **10** (1994), 311– 314

The Spanish Type Culture Collection of microorganisms (CECT)

Federico Uruburu

Colección Española de Cultivos Tipo (CECT), Burjassot, Valencia

The foundation of the Colección Española de Cultivos Tipo (Spanish Type Culture Collection) was planned by Prof. J. R. Villanueva, who started to collect and maintain microbial strains in 1960. Most of these initial strains came from the fungal collection of Dr. C. Ramírez and the yeast collection of Dr. E. Feduchy. Others came from the Phytopathological Station of Oeiras (Portugal) and were deposited by Prof. Villanueva and Dr. J. A. Leal. These initial strains were also offered to the Spanish microbiologists who asked for them.

For eight years, The CECT was located in the Instituto Jaime Ferrán de Microbiología, of the Spanish National Research Council (CSIC), and the Instituto de Biología Celular, also of the CSIC, in Madrid. In 1968 the CECT moved, with Prof. Villanueva, from the CSIC in Madrid to the University of Salamanca and carried on with its task in the Department of Microbiology of the Faculty of Biology in Salamanca. In 1974 the microbial strains of the CECT were transferred to the Department of Biology of the University of the Basque Country, in Bilbao, and I became its new curator. For six years the CECT was housed in Bilbao and in 1976 it published the first edition of its catalogue of strains, in which, 200 bacterial strains, 63 actinomycete strains, 86 filamentous fungal strains and 87 yeast strains were included, they being 436 strains in total.

In 1980 the collection moved from Bilbao to Valencia and, since that date, CECT has been housed in the University of Valencia, at the Department of Microbiology of the Faculty of Biological Sciences. In 1990 the third edition of the CECT's catalogue was published. The catalogue gives details of the strains (source, characteristics, special features, applications, etc.), as well as incubation temperatures, recipes for growth, media and references.

In 1991 the 800 yeast strains of the Microbial Collection Yeast Cultures (MCYC) of the Department of Microbiology, Agronomic School of Madrid, founded in 1958 by Prof. J. Santa María, were transferred to the CECT.

* *Correspondence to*: Federico Uruburu. Colección Española de Cultivos Tipo. Departamento de Microbiología. Facultad de Ciencias Biológicas. Universidad de Valencia. 46100 Burjassot. Tel.: 96-3864612. Fax: 96-3864372.

Organization of the CECT

I continue as curator of the CECT. The laboratory work related with the CECT operations is performed by Prof. M. D. García-López, who has been in charge of this task since 1965, and Ms. C. Belloch, research assistant belonging to the CSIC, working full time in the collection.

The CECT belongs to the University of Valencia (Spain) and is housed in building A of the Faculty of Biological Sciences in Burjassot, just outside of the city of Valencia. In a few months it will move to its own laboratories in a new building. The collection is also associated with the Spanish Society for Microbiology, which provides with some financial support. Other supporting Spanish institutions are the "Comisión Interministerial de Ciencia y Tecnología", of the Ministry of Education and Science, and also the CSIC.

In 1977 the CECT joined the World Federation of Culture Collections (WFCC) and it is listed as no. 412 in both the World Directory of Collections of Microorganisms (WDC) and in the World Data Centre (WDC). In 1983 the CECT joined the European Culture Collections' Organization (ECCO). Prof. Uruburu belongs to the United Kingdom Federation of Culture Collections (UKFCC), as well. In 1988 the CECT was integrated in the Microbial Information Network Europe (MINE) as the Spanish node.

Functions of the CECT

Most of the CECT strains are maintained by freeze-drying methods, and some are either cryo-preserved or maintained by subculture methods. The current number of strains maintained in the CECT is about 3000 (bacterial strains including actinomycetes, fungal and yeast strains). Those strains include organisms of industrial importance, taxonomic type strains, organisms used for assays, testing, teaching, biochemical and genetic research, and strains of general scientific interest. The number of human, animal and plant pathogenic strains is very limited.

The main function of the CECT is to maintain and to distribute microbial strains which are ordered by telephone, letter, fax or electronic mail by a wide range of users. Only orders coming from profit-making laboratories or industry are charged a fee of 5000 PTA per strain (about 25 Sterling pounds). The number of strains distributed by the CECT has increased considerably every year. Most orders come from university departments, centres of the Spanish National Research Council and from hospitals. The pharmaceutical industry is the most important customer among the profit-making institutions.

The CECT is an International Depository Authority under the Budapest Treaty for patent purposes. The collection may also provide information on different microbial aspects. If the CECT receives orders for strains that are not yet deposited in the collection, it makes a search for these strains, and order them through other culture collections. Due to the international airmail postal regulations, strains coming into Spain from some foreign countries can only be sent by airmail if they are addressed to the CECT. This is because in 1982 the CECT obtained special authorization from the Spanish postal authorities to receive strains by airmail from outside Spain.

The CECT provides an identification service for various kinds of microorganisms. Owing to the research work within the Department of Microbiology, the CECT is especially qualified for the identification of Gram-negative bacteria, mainly *Vibrio* sp., lactic acid bacteria and yeast.

Ordering of strains

All orders and other correspondence should be addressed to: Colección Española de Cultivos Tipo. Departamento de Microbiología. Facultad de Ciencias Biológicas. Dr. Moliner, 50. 46100 Burjasot (Valencia), Spain. Orders can also be made by telephone: 96-3864612 or by telefax: 96-3864372.

Use of the catalogue

The CECT catalogue is primarily divided into two parts. The first comprises an alphabetical list of bacteria, and the second a similar list for fungi. The bacterial names are given according to the "Approved List of Bacterial Names" (AL), edited by V. B. D. Skerman, V. McGovan and P. H. A. Sneath and published by the American Society for Microbiology in 1989. Names which have been validly published or listed since 1980 in the International Journal of Systematic Bacteriology are annotated VP. A name which has not been validly published appears without annotation. Yeast nomenclature follows the reference "The Yeast: A Taxonomic Study" edited by N. J. Kreger van Rij and published by Elsevier Science Publishers, Amsterdam, in 1984. The names of the filamentous fungi are given according to usual fungal nomenclature.

The arrangement of the strain data is as follows: Scientific name of the species with authors and date of valid publication where known, and annotated with AL or VP in the case of bacterial names (see above). The name is followed by a list of legitimate synonyms and alternate names whenever exist. The strains are referred to by their number in the CECT collection; this is followed by an indication of the strain taxonomic status (**T** for type), its serovar, biovar, other taxonomic groupings or remarks.

Next it follows a list of other collection numbers and strains names which refer to this strain. The year of accession into the CECT appears on a new line, followed by a history of the strain beginning with the depositor in the CECT and, when known, the date when the depositor received the strain (separates by ,); the direction of deposits is indicated by an arrow (<).

When there has been a change in the name under which an organism was received or previously preserved in the CECT, the current name is given followed by the original source of the strain and country of isolation, if known. Further information including genotype, plasmid and phage date, special properties and applications are then listed, also resistance (**:R**) and sensitivity (**:S**) to named antibiotics and any nutritional requirements. The method of conservation in the CECT is indicated; LY (lyophilized), AG (agar slants) and LD (L-drying).

Finally, the recommended media (number) and conditions for cultivation are given at the right. For composition of media see "List of Media". An example of the layout is given below.

Corynebacterium ammoniagenes (Cooke and Keith 1927) Collins 1987 VP

syn: *Brevibacterium ammoniagenes* (Cooke and Keith 1927) Breed 1953 AL

96 **T** NCIB 8143; NCTC 2398 (no longer available in NCTC catalogue, 1989); ATCC 6871; DSM 20306; Cooke 9.6
1973 NCIB < ATCC NCTC < J. V. Cooke Until 1989 preserved as *Brevibacterium ammoniagenes*
Stool of infant Production of L-isoleucine, US patent 3,262,861 LY
3, 30C

Other Spanish Culture Collections

Also functioning in Spain is the Microbiological Collection of *Rhizobium* Cultures located in the Department of Microbiology, Escuela Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid, 28040 Madrid. It specializes in *Rhizobium* strains. Various Spanish microbiology departments and laboratories maintain specialized microbial collections related with their scientific research, e.g., halophilic and related bacteria in the Department of Microbiology, Faculty of Pharmacy, Sevilla, Department of Microbiology, Faculty of Pharmacy, Granada, and Department of Microbiology, Faculty of Medicine, Alicante; Enterobacteria, *Clostridium*, halophilic bacteria and others in Centre for Biological Research of the CSIC in Madrid; wine yeast in the Institute of Industrial Fermentations of the CSIC in Madrid; *Escherichia coli* strains in the Microbiological Section of the Institute of Cytological Research in Valencia; *Streptomyces* and other actinomycete strains in the Department of Microbiology, University of Oviedo; *Aspergillus* and other filamentous fungal strains in the Unit of Microbiology and Immunology, Faculty of Veterinary, Autonomous University of Barcelona (Bellaterra), and the Department of Microbiology, Faculty of Medicine, University Rovira Virgili (Reus).

In addition, there is the Spanish Collection of Genetic Engineering Data, which is located at the Centre for Biological Research, CSIC, Velázquez 144, Madrid. Dr. Miguel Vicente and Dr. Ángeles Sacristán are in charge of this collection, which is a compilation of data on plasmids, cosmids, bacteriophages, microorganisms important in genetic engineering, cell lines, genetic libraries and recombinant vectors available through the collection. A catalogue containing these data and the source of the information and biotic materials has been published under the title "Vectores Cedig".

The European Culture Collection Organization (ECCO)

In 1981, at the 4th International Conference of Culture Collections, the curators of the major service collections in Europe agreed that it was essential to collaborate and trade ideas and information about all aspects of culture collections activities. Accordingly, ECCO was launched, and in 1982 its first meeting took place at the Deutsche Sammlung von Mikroorganismen (DSM), at that time situated in Göttingen, and presently located in Braunschweig, FRG. Since that inaugural meeting ECCO has met each year in different countries.

ECCO membership is open to representatives of any microbial resource centres that provide a professional service on demand and without restriction, that accept cultures for deposits, that produce catalogues, and that are housed in countries with microbiological societies affiliated to the Federation of European Microbiological Societies (FEMS). Additionally member culture collections must be affiliated to the World Federation for Culture Collections (WFCC). ECCO is itself an affiliated member of the Federation of European Microbiological Societies and of the World Federation for Culture Collections. ECCO has stimulated much activity among its members, and has undoubtedly encouraged a European approach in many issues.

Reference

1. Dawson, C., Belloch, C., G.-López, M. D., Uruburu, F. (ed.). (1990). Spanish Type Culture Collection, Catalogue of Strains, 3rd Edition. University of Valencia, Burjassot, Spain.

Trends in Microbial Ecology

Proceedings of the 6th International Symposium on Microbial Ecology

Dirigido por R. Guerrero, C. Pedrós-Alió

Spanish Society for Microbiology, 1993. Barcelona. xxii + 718 pp. ISBN 84-604-7996-X.

Al año de la celebración en Barcelona del 6th International Symposium on Microbial Ecology, septiembre 1992, aparece el libro de "Proceedings" del congreso, que contiene las ponencias y conferencias invitadas. Los directores, R. Guerrero y C. Pedrós-Alió, que ya consiguieran un notable éxito en la organización de la reunión, han culminado aquella empresa con la publicación de *Trends in Microbial Ecology*, una obra de atractiva presentación y excelente calidad científica, en la que han participado investigadores de un bien ganado prestigio.

Los Simposios Internacionales de Ecología Microbiana se iniciaron en Dunedin, Nueva Zelanda, en el año 1977. Desde entonces se vienen celebrando cada tres años y, tanto por el número de participantes como por la calidad de la ciencia representada, se han convertido en acontecimientos científicos de primera categoría. Este desarrollo de la ecología microbiana aparece bien documentado en este libro de 143 artículos, cuidadosamente distribuidos en cinco partes, cada una de las cuales está dividida en subapartados que facilitan el trabajo de referencia y consulta. Siguen la siguiente ordenación: **A. Ecología fisiológica** (40 artículos): condiciones extremas, microorganismos fototróficos, capacidades metabólicas, superficies, gradientes, privación de nutrientes; **B. Interacciones entre poblaciones** (30 artículos): interacciones con animales, interacciones con plantas, interacciones con virus, transferencia del material genético entre microorganismos; **C. Ecología de las comunidades** (31 artículos): estructura de las comunidades, redes alimentarias microbianas, ciclo de los elementos, ecología global; **D. Sistemática y evolución de los microorganismos** (17 artículos): métodos para la identificación *in situ* de microorganismos, estrategias para el aislamiento de nuevos microorganismos, especies microbianas y su evolución; **E. Ecología microbiana aplicada** (25 artículos): virología ambiental, biorrestauración, productividad vegetal, biodeterioro, ecología microbiana médica. Cada una de las partes va precedida de un capítulo a cargo de los investigadores que impartieron conferencias plenarias en el congreso y de una introducción de los directores de libro, donde se ofrece una perspectiva global del desarrollo y del estado actual del tema.

A su utilización como libro de texto y de consulta contribuye el índice de palabras clave, con nueve páginas, y el de autores, con cinco, que facilita la identificación de los 384 autores y coautores.

Trends in Microbial Ecology ofrece una completa información sobre el estado de la ecología microbiana en el año 1992. Esta materia aparece como un campo científico en rápido crecimiento; estudia, en general, todas las formas de vida de este planeta y, debido a que no hay ningún ambiente carente de microorganismos, también sus interacciones mutuas y los factores del ambiente.

Trends in Microbial Ecology deber ocupar un lugar en las bibliotecas de los departamentos e institutos de biología (no sólo de microbiología) y en la biblioteca personal de todo ecólogo (no sólo microbiano). Por la estrecha interacción de la ecología microbiana con las geociencias, es recomendable para geoquímicos y geólogos. Su precio razonable lo hace asequible también a los estudiantes.

Hans G. Trüper
Universidad de Bonn, Alemania

Microbiología

Mecanismo de las enfermedades infecciosas. Enfoque mediante resolución de problemas

M. Schaechter, G. Medoff, B. Eisenstein, H. Guerra.

Editorial Médica Panamericana. 1994. Buenos Aires. 2ª ed. 960 páginas. Con ilustraciones en color y en blanco y negro. ISBN 84-7903-126-3.

La segunda edición de este excelente tratado sobre enfermedades infecciosas mantiene el mismo enfoque de la edición anterior, consistente en la presentación de los temas en un marco biopatológico aplicado a casos clínicos. A este enfoque se debe parte de su éxito y aceptación por los estudiantes. Como novedad, incorpora capítulos sobre biología molecular y enfermedades de reciente aparición, que son imprescindibles en libros de texto de uso actual.

El contenido se divide en tres secciones. La primera, dedicada a los principios básicos, ofrece un panorama de la biología general y molecular de los agentes infecciosos y de las defensas del huésped. La segunda se dedica al estudio de esos agentes infecciosos, en particular bacterias, virus, hongos y parásitos animales, con una introducción general que precede al tratamiento de los grupos de organismos causantes de distintas enfermedades. Hay una amplia exposición de casos clínicos y comentarios de los autores sobre los principios generales de los agentes estudiados, todo lo cual refuerza la importancia que tiene la resolución de problemas prácticos en el aprendizaje. En esta sección se incluyen las patogenias de reciente identificación, como el sida, la enfermedad del legionario (causada por *Legionella pneumophila*) y la enfermedad de Lyme (o borreliosis de Lyme, causada por *Borrelia burgdorferi*). La tercera y última sección se dedica a la fisiopatología de las enfermedades infecciosas y a sus efectos sobre los principales sistemas y aparatos del organismo humano, incluyendo un apartado sobre principios de epidemiología.

En el libro se destacan los aspectos comunes de las propiedades patogénas de los agentes infecciosos, así como las relaciones huésped-parásito. También, diversos agentes y enfermedades que contribuyen a la comprensión de los fenómenos generales de la patogenia, sin pretender hacer un tratamiento exhaustivo, más propio de un libro de consulta. En esa línea, los agentes antimicrobianos y la respuesta del huésped se tratan sólo con el propósito de comprender los mecanismos de tales enfermedades. Contribuyen al valor didáctico del libro los cuestionarios de autoevaluación y los cuadros de revisión, con sus respectivas respuestas. Los temas estudiados incorporan los conocimientos más recientes, que se presentan de manera amena y comprensible. Si algo se echa en falta es la inclusión de bibliografía en el capítulo sobre principios del diagnóstico, que incluye técnicas recientes tales como PCR, y mayor actualización en las referencias sobre los aspectos generales de la genética bacteriana y sobre las bases biológicas de la acción antimicrobiana.

El libro facilita la adquisición de conocimientos mediante un enfoque conceptual y realista que permite predecir la resolución de nuevos casos clínicos. Va dirigido a estudiantes de las carreras de ciencias de la salud: medicina, farmacia, veterinaria, biología y enfermería, y resulta útil en la práctica del diagnóstico clínico. Es igualmente adecuado para que el personal interesado, que no haya cursado especialidades clínicas, conozca el modo de actuación de los agentes causantes de las enfermedades infecciosas. Los profesores de microbiología médica pueden encontrar en él un texto valioso para la preparación de las clases teóricas y prácticas, y multitud de ideas para elaborar diverso material gráfico.

Beatriz S. Méndez
Universidad de Buenos Aires, Argentina

Microbiología médica

Cuaderno de prácticas y demostraciones

Dirigido por G. Prats

Ediciones Doyma S.A. Barcelona, 1993. 114 páginas. ISBN 84-7592-524-3.

En la elaboración de este libro de prácticas han participado un total de nueve autores, todos ellos versados en la docencia teórica y práctica de la microbiología general y clínica. Es precisamente la experiencia adquirida a lo largo de años de ejercicio docente la que se pone de manifiesto en un tratamiento flexible del material de trabajo, en este caso centrado en la organización de sesiones de prácticas de laboratorio. El libro proporciona el material necesario para que alumnos y profesores organicen el trabajo según sus propias pautas. Por ello hay dos introducciones, una dirigida a los alumnos y otra a los profesores, las cuales orientan sobre la utilización del libro, a fin de conseguir el mayor aprovechamiento en el cumplimiento de su función como guía auxiliar en la preparación de prácticas de microbiología médica.

Esta obra va dirigida principalmente a estudiantes de medicina, pero resulta igualmente adecuada en las carreras de farmacia, veterinaria y biología, así como para las enseñanzas relacionadas de formación profesional y de enfermería.

El libro está dividido en dos partes. La primera se dedica a las técnicas básicas de microbiología. Tiene por objeto familiarizar al alumno con la metodología que, posteriormente, va a aplicar en la ejecución de las prácticas propuestas, o con aquellas otras programadas en su asignatura. En esta parte del libro se ofrece un conjunto de siete sesiones prácticas dedicadas a bacteriología general (la parte más extensa), micología, parasitología, virología y pruebas serológicas. Cada sesión incluye los fundamentos teóricos de las prácticas a realizar y de las técnicas correspondientes, así como esquemas detallados de los instrumentos y de las fases de los diferentes procedimientos. Por medio de ellos, el alumno puede comparar su trabajo con el esquema y comprobar si lo está haciendo correctamente. Cada prueba se acompaña de los elementos necesarios para la lectura e interpretación de los resultados.

La segunda parte es una revisión sintetizada de los grandes síndromes infecciosos. Incluye infecciones urinarias, enfermedades de transmisión sexual, gastroenteritis, meningitis, infecciones piógenas y necrotizantes, infecciones respiratorias y sepsis. En cada una de ellas se indica el tipo de muestra que hay que remitir al laboratorio de microbiología, los microorganismos patógenos más frecuentes en cada caso y las técnicas más adecuadas para el diagnóstico. Todo ello facilita la comprensión por parte del estudiante y la aplicación de las técnicas desarrolladas en la primera parte del libro. Los autores introducen aquí orientaciones y sugerencias para la observación y la discusión que, sin duda, ayudarán a completar el resultado del aprendizaje por la práctica.

No se fijan los tiempos de realización de cada una de las siete prácticas propuestas. La idea de los autores es que el cuaderno se utilice como una guía de trabajo por parte de estudiantes y profesores, quienes podrán así adaptarla a las necesidades de su asignatura, al nivel de los grupos y, la disponibilidad de tiempo y medios, entre otras cosas.

Contribuye a la utilidad de esta microbiología médica la inclusión de un apéndice con un completo listado del material y los reactivos necesarios para la realización de las prácticas, y treinta y ocho excelentes fotografías en color.

Núria Martín Casabona
Residencia Sanitaria Valle de Hebrón, Barcelona

Introduction to Diagnostic Microbiology

E.W. Koneman, S. D. Allen,
W. M. Janda, P. C. Schreckenberger,
W.C. Winn Jr.

J. B. Lippincot Co. Philadelphia, 1994. 527 pp. Con ilustraciones en color y en blanco y negro. ISBN 0-397-51215-5.

Cuando se aborda un tema eminentemente práctico, como es el caso de las técnicas para el diagnóstico microbiológico, sin duda hay que hacer una selección entre toda la amplia información disponible. La premisa que ha orientado la labor de los autores es que la gran cantidad de información existente en microbiología diagnóstica excede la capacidad de los cursos que los estudiantes reciben. Se supone, con ello, que su intención no ha sido condensar la materia, sino realizar una prospección de lo que resulta realmente significativo para cada nivel de estudio.

Este texto introductorio se dirige a un público compuesto por licenciados/graduados que siguen programas máster o de preparación de técnicas de laboratorio aplicadas al diagnóstico clínico. Resulta adecuado también como texto preparatorio para diversos tipos de exámenes (MIR, FIR, BIR). Está centrado en la práctica del laboratorio de microbiología clínica y en los métodos necesarios para la recuperación e identificación de los agentes causantes de enfermedades infecciosas. La presentación se completa con la descripción de ensayos de susceptibilidad a agentes antimicrobianos y los nuevos métodos de diagnóstico, basados en reacciones inmunológicas o de hibridación de ácidos nucleicos. Por su contenido y exposición ordenada resulta de utilidad, no sólo para estudiantes, sino también como libro de consulta en laboratorios de análisis clínicos y en los departamentos universitarios.

El libro consta de 19 capítulos, el primero de los cuales es una introducción sobre la recogida y procesamiento de muestras, métodos microbiológicos rutinarios, ensayos presuntivos y normas de seguridad. El tratamiento del aislamiento y diagnóstico de los agentes infecciosos bacterianos se hace considerando los medios de cultivo necesarios y la caracterización por pruebas bioquímicas. También se presentan los métodos de rastreo rápido, los sistemas automáticos y semiautomáticos y los "kits" de identificación. Se describen las principales familias y géneros bacterianos y las especies patógenas más comunes.

Gran parte del libro (capítulos 2 al 11), se dedica a las familias y géneros de bacterias Gram-positivas y Gram-negativas, destacando las especies más importantes de cada grupo.

El objetivo del libro es que el lector pueda formarse una idea clara de las características clave que permitirán identificar los microorganismos más importantes recogidos en las muestras clínicas. A este respecto, son de gran utilidad las tablas y figuras que se incluyen en el texto en relación con los agentes fúngicos y víricos causantes de enfermedades infecciosas. Los autores ya indican que el espacio dedicado a muchos temas es más reducido que el que se puede encontrar en manuales, enciclopedias y monografías. Por ejemplo, sólo se dedica un capítulo a cada uno de los siguientes temas: micoplasmas, hongos, parásitos y virus. Sin embargo, la principal objeción que se puede hacer a los autores es no haber actualizado la bibliografía que se ofrece en cada capítulo, proporcionando así una fuente de consulta mucho más útil.

En conjunto, el texto proporciona a los estudiantes y público interesado lo esencial de la información tradicional y reciente en diagnóstico microbiológico, en un solo volumen de fácil manejo.

Beatriz S. Méndez
Universidad de Buenos Aires, Argentina

Micología médica

J. M. Torres-Rodríguez, A. del Palacio Hernanz, J. Guarro-Artigas, R. Negroni-Briz, M. Pereiro-Miguens

Masson, S. A. 1993. Barcelona. 378 páginas. Con ilustraciones en color y en blanco y negro. ISBN 84-311-0632-8.

Las relaciones entre los hongos y los seres humanos han ampliado considerablemente los límites de la micología clásica. Esta disciplina científica ha pasado, de ser una ciencia descriptiva, a constituir una rama del saber que establece múltiples relaciones, no sólo con otras especialidades, sino con variados aspectos de la cultura. Limitándonos a la medicina, de los lazos que se establecen entre ambas surge una disciplina con nombre propio: la **micología médica**. A ella corresponde el estudio de la microbiología y la toxicología micológicas, de la micotoxinología, de la micotoxicología, de la farmacología micológica (fármacos contra hongos y fármacos producidos a partir de hongos) y de las alergias causadas por hongos.

La micología médica tiene, con esta obra, un excelente compendio escrito originalmente en lengua castellana. Sus autores son investigadores pertenecientes al grupo responsable de la Revista Iberoamericana de Micología, una de las mejores publicaciones en su género. *Micología médica* es una obra muy completa y actualizada, desarrollada con claridad y de amena lectura. Puede decirse que todas las enfermedades y trastornos que los hongos producen en los humanos se analizan en el libro con rigor y claridad. Es un texto integrado, si bien el peso recae fundamentalmente en las infecciones fúngicas, siendo menor el espacio dedicado a las alergias o a las micotoxicosis y micetismos. Pero ello es comprensible —y así lo reconocemos incluso los que tenemos una clara predilección por los temas toxicológicos— por la mayor repercusión de los estados infecciosos en la práctica médica. Paradójicamente, los avances de la medicina han provocado enfermedades iatrogénicas causadas por determinados tratamientos médicos. Los enfermos sometidos a inmunosupresión farmacológica por trasplantes y los que padecen neutropenias severas por tratamientos quimioterápicos enérgicos son susceptibles de sufrir infecciones por hongos, como también las personas afectadas por el síndrome de inmunodeficiencia adquirida.

Micología médica se estructura en tres bloques. El primero se inicia con una introducción sobre taxonomía, distribución geográfica de los hongos patógenos, mecanismos de acción patógena de los hongos y clasificación y nomenclatura de las micosis. A un breve, pero completo, capítulo sobre historia de la micología médica, siguen otros sobre material y equipo necesarios en un laboratorio de micología médica, aspectos inmunológicos de las micosis humanas y fármacos para combatirlas. El segundo y más extenso bloque (capítulos 6 a 33), se dedica a las enfermedades infecciosas humanas causadas por hongos, con apartados sobre definición e historia de las enfermedades, etiología y ecología, epidemiología, clínica, diagnóstico, histopatología, pronóstico y tratamiento. Los últimos capítulos del libro versan sobre micetismos, micotoxicosis, alergias a hongos y hongos contaminantes. Un capítulo final sobre terminología micológica, es muy útil para los no especialistas. Se incluye en cada capítulo una selección actualizada de referencias.

Algo que confiere a *Micología médica* un carácter universal es que se hayan considerado prácticamente todas las enfermedades conocidas que, de una manera u otra, son causadas por hongos de todos los rincones del planeta. Algunos de los capítulos podrían constituir por derecho propio temas de un tratado de medicina tropical.

Cabe destacar la esmerada presentación editorial. *Micología médica* es el primer tratado en castellano de su género dedicado al diagnóstico y control de las enfermedades humanas causadas por hongos, escrito por destacados especialistas en la materia.

Josep Piqueras
Residencia Sanitaria Valle de Hebrón, Barcelona

SCIENCE, CULTURE & COMMUNICATION FOR THE 21st CENTURY

IFSE-8

Eighth International Conference of the International Federation of Science Editors

9 – 13 July 1995, Barcelona, Spain

Organized by:
- International Federation of Science Editors
- Catalan Research Foundation

Venue:
- School of Biology
- University of Barcelona

International Federation of Science Editors

The International Federation of Science Editors (IFSE) will hold its biennial conference and assembly in Barcelona on 9–13 July 1995. The meeting will cover every aspect of science communication: between scientists (science authors, science editors) and to the public (science writers, journalists).

IFSE (formerly IFSEA) was founded at the United Nations Educational, Scientific, and Cultural Organization (UNESCO) in 1978 and is a scientific associate of the International Council of Scientific Unions (ICSU). Among IFSE's aims are the following:

- To improve scientific communication throughout the world and the quality of science editing
- To serve as an international reference point for science editors and their regional associations
- To help form regional associations of science editors and to integrate them into this worldwide network, with particular assistance to organizing groups in developing regions
- To coordinate science publications worldwide and to foster their distribution
- To aid in building curricula for professional excellence in science editing, and to serve as a source of expertise and advice
- To sharpen awareness of the editor's role. IFSE has established the School for the Communication of Science at the Consorzio Mario Negri Sud in Chieti, Italy, where international courses are taught by expert science editors and communicators.

PRE-REGISTRATION FORM

To be returned no later than November 30, 1994

Family name: .....
First name: .....
Institution: .....
Address: .....
.....
.....
Phone: ..... Fax: .....
E-mail: .....

I intend to submit:
( ) an oral presentation ( ) a poster

Tentative title: .....
.....
.....

Abstract forms will be sent with the second announcement

Photocopy and send to: IFSE-8 Secretariat. Apartado 16009. E–08080 Barcelona. Spain

Normas para los autores

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

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

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

La bibliografía será citada en el texto mediante números y se dispondrá numerada y en orden alfabético de acuerdo con los ejemplos que se ofrecen a continuación:

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

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

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

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

Las fotografías, que deberán estar preparadas para su reproducción directa, se limitarán a las estrictamente necesarias para la comprensión del trabajo y serán de calidad suficiente para asegurar una buena reproducción. Deberán estar numeradas al dorso, indicando el apellido del primer autor a lápiz. Los textos de las mismas irán mecanografiados a doble espacio y en hoja aparte. En los artículos en español las figuras incluirán asimismo un texto en inglés. El tamaño de las fotografías no excederá de 13 x 20 cm. Las dimensiones de los rótulos deberán ser las adecuadas para ser legibles en caso de que se reduzca la fotografía. La presentación de dibujos en tinta china y papel vegetal seguirá las mismas normas. No se admitirán fotografías en color.

Las tablas se enviarán en hojas aparte, numeradas independientemente de las figuras, con números arábigos y deberán llevar el correspondiente título explicativo. Los autores deberán indicar a lápiz en el margen del texto del la situación aproximada en donde deben aparecer las tablas y figuras.

NOTAS. Las Notas, que no deberán exceder de seis páginas mecanografiadas incluyendo figuras y tablas, tienen por objeto la presentación de observaciones experimentales, descripción de técnicas o modificaciones metodológicas de interés. Su redacción se efectuará ateniéndose a las Normas previamente descritas para los artículos, pero suprimiendo las divisiones con encabezamiento. Los resúmenes no serán superiores a 50 palabras. Sólo incluirán, como máximo, dos figuras y una tabla, o viceversa.

ARTICULOS DE REVISIÓN. Los artículos de Revisión versarán sobre temas de microbiología de gran interés, y su redacción se solicitará a especialistas. Sin embargo, si algún autor está interesado en publicar artículos de Revisión, éstos tendrán que ser supervisados. Los originales deberán comprender aproximadamente de 12 a 20 páginas (incluidas figuras y tablas), mecanografiadas a doble espacio.

CORRECCIÓN DE PRUEBAS. Los autores recibirán pruebas de imprenta, que deberán estar de vuelta en la redacción en el plazo de una semana. Transcurrido dicho plazo sin devolución de las pruebas, éstas serán publicadas tal como han sido enviadas a los autores. Las correcciones se limitarán a errores tipográficos, gramaticales o de datos incorrectos. Modificaciones más importantes que impliquen recomposición del texto, deberán ser abonadas por los autores. Se enviarán 25 separatas gratuitas por artículo; si se desearan más, deberá indicarse por escrito cuando se devuelvan la pruebas corregidas. Las separatas adicionales serán facturadas a precio de coste.

El artículo, original y dos copias en papel, más una copia en disco de ordenador, se enviará a la siguiente dirección: *Microbiología SEM*. Apartado 16009, 08080 Barcelona, o al miembro del Consejo Editorial de la revista que esté más relacionado con el contenido del artículo.

Guidelines for authors

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

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

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

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

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

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

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

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

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

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

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

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

MINIREVIEWS. Minireviews articles should deal with microbiological subjects of broad interest. Specialists will be called upon to write them. However, if some authors are interested in publishing minireviews, these will be submitted for publication. They should not be longer than approx. 12 to 20 double-spaced typewritten pages, including the space needed for figures and tables.

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

Articles must be submitted, original and two copies in paper, plus a diskette, to the following address: *Microbiología SEM*. Apartado 16009, 08080 Barcelona, Spain, or to one of the members of the Editorial Board according to the discipline represented.

Editorial Board addresses/Direcciones de los miembros del Consejo Editorial

José M. López Pila
Institute for Environmental Hygiene
Corrensplatz 1
D-1000 Berlin 33, FRG

Rubens López García
Centro de Investigaciones Biológicas CSIC
Velázquez, 144
28006 Madrid

Manuel Benjamín Manzanal
Dpto. Interfac. de Microbiología
Facultad de Medicina
Universidad de Oviedo
33071 Oviedo

David A. Mossel
Eijkman Found. for Medical Research
P.O. Box 6025
3503 PA Utrecht
Netherlands

Juan A. Ordóñez
Dpto. Higiene y Microbiol. Alimentos
Facultad de Veterinaria
Universidad Complutense de Madrid
28040 Madrid

José Claudio Pérez Díaz
Servicio de Microbiología
Hospital Ramón y Cajal
28035 Madrid

Manuel de la Rosa
Servicio de Microbiología
Hospital Virgen de las Nieves
Av. Coronel Muñoz, 2
18014 Granada

Moselio Schaechter
Dpt. Molec. Biology and Microbiology
136 Harrison Avenue
Tufts University
Boston, MA 02111, USA

Josep M. Torres-Rodríguez
Unidad de Microbiología
Inst. Municipal de Investigación Médica
UDIMAS, Univ. Autónoma de Barcelona
Pg. Marítim, 25-29
08003 Barcelona

Hans G. Trüper
Institute of Microbiology
University of Bonn
Meckenheimer Allee, 168
D-5300 Bonn 1, FRG

Antonio Ventosa
Departamento de Microbiología
Facultad de Farmacia
Universidad de Sevilla
41012 Sevilla

Miquel Viñas
Dpto. Microbiol. y Parasitol. Sanitarias Facultad de Farmacia
Universidad de Barcelona
08028 Barcelona