Volumen 11, nº 4
Diciembre 1995
ISSN 02 13-4101

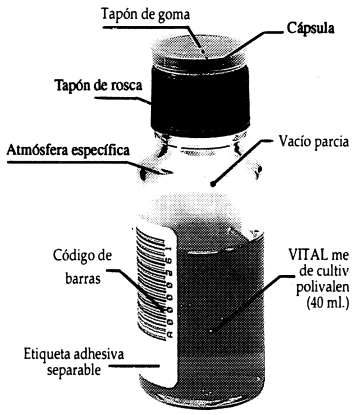
PUBLICACION DE LA SOCIEDAD
ESPAÑOLA DE MICROBIOLOGIA

# Microbiología

SEM

# VITAL

## Sistema automático para hemocultivos




- Tecnología H.F.T.
- Lectura no invasiva:
  - en continuo las 24 horas del día cada 15 minutos.
- Introducción inmediata de frascos nuevos en el ciclo de análisis.
- Detección del crecimiento bacteria mediante:
  - producción $CO_2$.
  - variación del PH.
  - modificación del potencial de oxido-reducción.
- Facilidad de Manejo.
- Interfase windows.
- Sistema modular.
- Conexión bidireccional.

**bioMérieux**

**bioMérieux España, s.a.** Manuel Tovar, 36 / 28034 Madrid / Tel. (91) 358 10 42 / Fax (91) 358 06 29
Delegación: Padilla, 312 / 08025 Barcelona / Tel. (93) 455 03 04 / Fax (93) 347 96 65

BARCELONA 20-25 OCTUBRE 1996

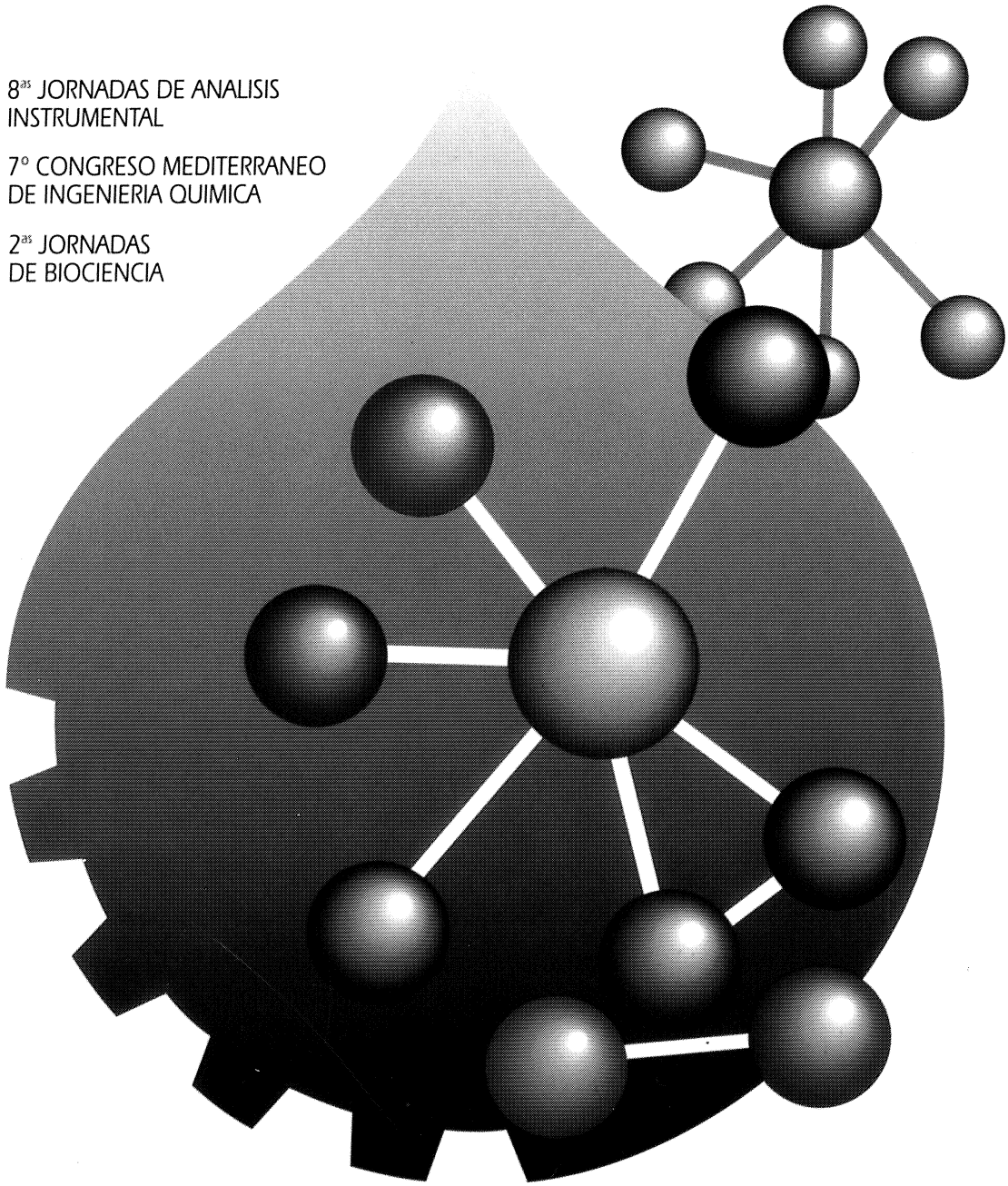
# EXPOQUIMIA'96

## FERIA TECNICA DE LA QUIMICA APLICADA

JAI 8as JORNADAS DE ANALISIS INSTRUMENTAL

7º CONGRESO MEDITERRANEO DE INGENIERIA QUIMICA

JBC 2as JORNADAS DE BIOCIENCIA



Fira de Barcelona

INFORMACION: Avda. Reina Mª Cristina - E-08004 Barcelona - Tel. (93) 233 20 00 - Fax (93) 233 23 11 - (93) 423 63 48 - (93) 233 20 01

Generalitat de Catalunya Departament d'Indústria i Energia

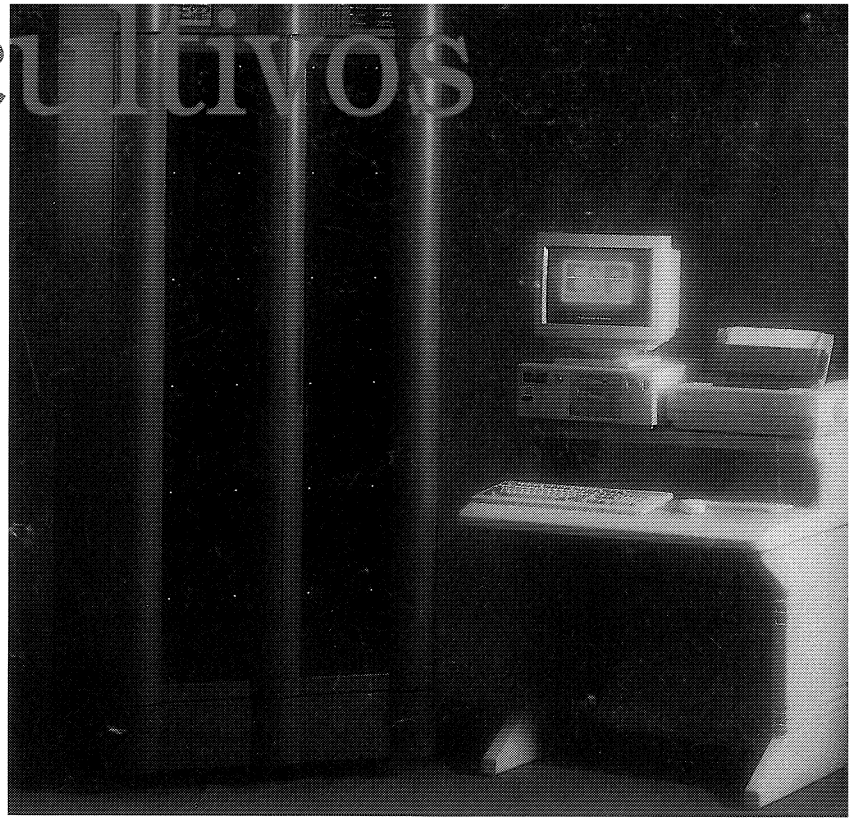
ICEX

IBERIA

# Hemocultivos ESP

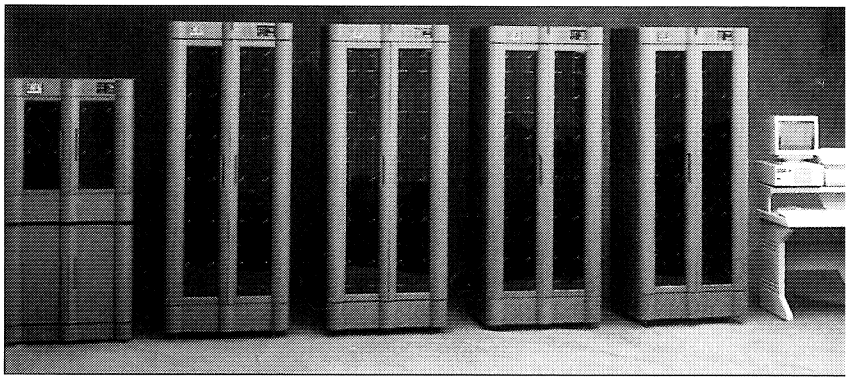
## LA NUEVA TECNOLOGIA CONDUCE A UNA MAYOR RAPIDEZ

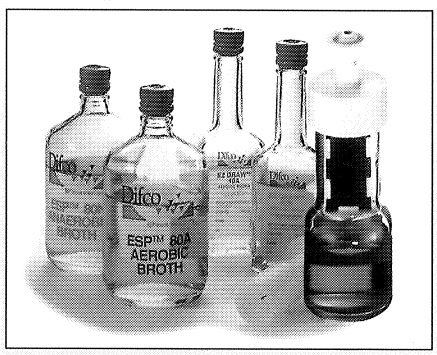
ESP ofrece lectura contínua, no invasiva, de los hemocultivos con automatización total y monitorización tanto de consumo como de producción de cualquier gas.



## Configuraciones

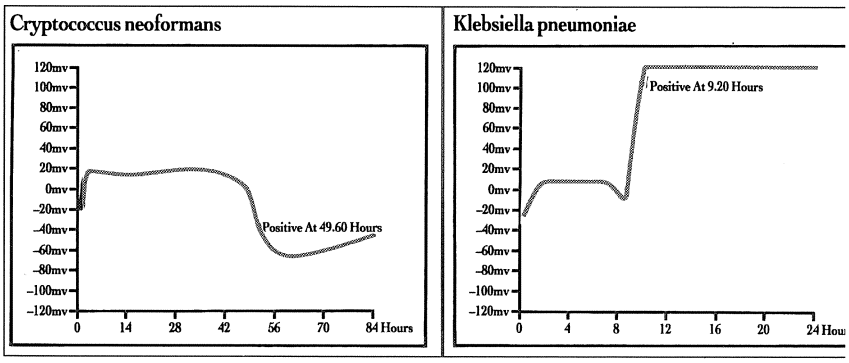
El sistema de Hemocultivos ESP, se ajusta con precisión a su volúmen de trabajo. Los instrumentos están disponibles en dos tamaños, 128 y 384 botellas. El ordenador controla hasta 5 instrumentos de cualquier capacidad.






## 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 producción cualquier gas de forma que los positivos son detectados antes que con sistemas que s 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 6

# MICROBIOLOGÍA SEM

## Publicación de la Sociedad Española de Microbiología





* See addresses in pp. 424 and 523–524.



[MICROBIOLOGÍA SEM 11(4): 421–532 (1995)]

Dirección: Sociedad Española de Microbiología. Serrano, 117.
28006 Madrid (España). Tel. y Fax 561 32 99
Aparecen dos números al año (1995), que se integran en un volumen.
Precio de suscripción anual. Año 1995: España, 12.000 ptas. (IVA incluido);
Internacional 111 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

ATENCION AL CLIENTE
902-21 00 74

APP
ASOCIACION DE PRENSA PROFESIONAL

## Editorial Board/Consejo Editorial*



* See addresses in pp. 424 and 523–524.

[MICROBIOLOGÍA SEM 11(4): 421–532 (1995)]

***Instructions to Authors:***
Information about the Journal, including instructions on the preparation and submission of manuscripts, is published on pp. 521–522 of this issue, and may also be obtained from the Editorial Office.

## Publication Board/Comité de Redacción



**Editorial office**

**Microbiología SEM**
**Apartado 16009**
**E-08080 Barcelona**
**Spain**
**Tel. +34-3-4482373. Fax +34-3-3341079**
**E-mail: guerrero@servicom.es**

**SEM Secretariat address**

**Isabel Perdiguero**
**Sociedad Española de Microbiología**
**Serrano, 117**
**E-28006 Madrid**
**Spain**
**Tel. & Fax: +34-1-5613299**

Preparación y composición de originales:

Ana Fernández de Castillo
Isabel Gallego
Eulàlia Massana

Con la colaboración de Begoña Gómez Miranda

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 11(4): 421–532 (1995)]

Editorial Board addresses/Direcciones de los miembros del Consejo Editorial



*Continúa en p. 523*

# ÍNDICE



ISSN 02 13-4101

CONTENTS



ISSN 02 13-4101

MICROBIOLOGÍA SEM **11** (1995), 427–428

# Editorial

Es práctica habitual en muchas publicaciones comentar, al acabar el año, los principales acontecimientos que puedan tener interés para sus lectores. También es frecuente que muchas de las noticias glosadas sean olvidadas con el tiempo, o recordadas únicamente como anécdotas. La ciencia se desarrolla mediante un flujo continuo de hechos, que son el objeto de la observación, y de ideas, que provienen de la deducción del investigador. Sólo en raras ocasiones nos damos cuenta de que se ha producido una observación revolucionaria, o se asiste a un cambio repentino y trascendental en una idea. Todavía no sabemos por qué descubrimientos científicos será recordado 1995, pero, sin duda, algunos de los miles de artículos científicos publicados en los cientos de revistas especializadas, de todo el mundo, contienen la descripción, explicación o hipótesis de algunos hechos u observaciones que marcarán un hito en el acontecer científico, y seguirán citándose al cabo de mucho tiempo. Volviendo la vista atrás, podemos comprobar que dos de los investigadores que han conseguido este año el premio Nobel de Química (Mario J. Molina y F. S. Rowland), iniciaron la polémica de la destrucción de la capa de ozono con un artículo, ahora famoso, publicado en *Nature* en 1974 (vol. **249**, pp. 810–812): "Stratospheric sink for chlorofluoromethanes: chlorine atomc-atalysed destruction of ozone." (La errata, *atomc-atalysed*, no es nuestra, sino de *Nature*; así aparece, en grandes letras, en el artículo original.)

En 1995 se han publicado por primera vez las secuencias completas de dos células vivas, *Haemophilus influenzae*, que tiene 1.830.137 bases (*Science* **269**: 496–512, del 28 de julio) y *Mycoplasma genitalium,* con 580.070 bases (*Science* **270**: 397–403, del 20 de octubre). *M. genitalium* es una de las células más pequeñas que se conocen y, aunque su cultivo axénico es difícil, no hay duda de que es un organismo de vida libre. Los dos trabajos, en los que han intervenido varios laboratorios, han sido dirigidos por J. Craig Venter, del Institute for Genomic Research, Rockville, Maryland.

Muchos científicos se habrán sorprendido al ver que han sido los DNA de estas dos bacterias los primeros en descifrarse. Sin duda, *Escherichia coli* (4.703.000 bases) es el microorganismo mejor conocido. Y la afirmación sigue siendo cierta aunque le quitemos el micro-. La facilidad de su cultivo la han convertido en el microorganismo de elección para los estudios de fisiología bacteriana, de ingeniería genética y de biología molecular. Aunque, debido al mayor tamaño de su genóforo, ha perdido la carrera de la secuenciación, no pasará mucho tiempo sin que se conozca su genoma completo.

La secuenciación del DNA de un organismo tan sencillo como *M. genitalium* permite hacer algunas deducciones que van más allá del simple conocimiento de esta célula en particular. Llama la atención que un 30% de su DNA esté dedicado a la fabricación de la membrana celular. Tratándose de un organismo muy simple (tiene sólo 482 genes), podemos pensar en el papel destacado que tuvieron las membranas para el establecimiento de los primeros organismos, que habrían sido poco más que un ácido nucleico (probablemente RNA) envuelto por una membrana bicapa, como hace tiempo han indicado algunos estudiosos del origen de la vida.

Pero estas noticias genéticas no han aparecido en la prensa u otros medios de comunicación de masas. En cambio, otras, como la "epidemia" por el virus Ebola, o el cultivo de bacterias "jurásicas", han acaparado la atención de los periódicos durante semanas. Sin duda es bueno que la prensa se ocupe de

la ciencia, pero a veces se corre el peligro de trivializarla. En primer lugar, la importancia de un descubrimiento no se mide con el mismo rasero en los medios de comunicación que en los ámbitos científicos, ni tampoco todas las observaciones se hacen noticia en el momento en que se producen. Descubrimientos u observaciones poco relevantes, pero más espectaculares para el público, pueden ocupar titulares. Es probable que dentro de unos años apenas se recuerde que en 1995 se produjo una epidemia —si así puede considerarse la coincidencia de unos casos de infección— del virus Ebola. En cambio, sí debería recordarse —esperemos que así sea— que ese mismo año se logró por vez primera la secuencia completa del DNA de unas bacterias.

Otro acontecimiento que suscitó innumerables comentarios fue el artículo de Raúl Cano y su colaboradora Monica Borucki (*Science* **268**:1060–1064, del 19 de mayo). Con las propias palabras de los autores: "A partir del contenido abdominal de abejas extintas conservadas durante 25 a 40 millones de años en ámbar enterrado de la República Dominicana, se hizo revivir una espora, que se cultivó y se identificó... Diversas pruebas indican que la bacteria aislada tiene un origen antiguo y no es un contaminante actual. Los característicos perfiles enzimáticos, bioquímicos y del DNA que codifica el RNA 16S indican que esta bacteria antigua está estrechamente relacionada con el actual *Bacillus sphaericus*." La recuperación de estas bacterias "jurásicas" (como se las ha denominado popularmente, aunque, en propiedad, deberían llamarse oligocénicas o miocénicas) es sin duda importante, pero se trata de una de esas noticias que es mejor tomar con cautela. El tiempo dirá si la expectación que provocó su descubrimiento estaba justificada. Cuando otros investigadores hayan conseguido repetir el experimento de Cano, podremos aceptar su validez. Ésta es la grandeza y servidumbre del método científico.

No queremos terminar el año sin un comentario sobre la marcha de *Microbiología SEM*. El equipo de redacción está muy satisfecho del interés de los microbiólogos hacia la revista, que responde al esfuerzo que hace la SEM para publicarla. Este año han aparecido dos números monográficos, uno dedicado a la microbiología de los alimentos (marzo) y otro al origen de la vida (junio). Éste último se envió no sólo a los socios de la SEM y otros subscriptores habituales, sino también a diversos investigadores y centros de Norteamérica y Europa.

En 1995 se han publicado cuatro números, con un total de 532 páginas y 59 artículos, el 75% en inglés. Se han recibido 92 originales, lo que ha representado muchísimas horas de trabajo para el equipo de redacción y para los revisores. Éstos últimos están haciendo una labor intensa y muy eficaz, que ayuda a aumentar la calidad de la revista. Los autores también han contribuido, no sólo enviando artículos, sino siguiendo las indicaciones de los revisores y de la propia revista, lo que ha hecho mejorar la presentación y el contenido. Lógicamente, no han podido publicarse todos los artículos recibidos, pero este hecho también animará a los autores a esforzarse en la preparación de los trabajos. Así tenía que ser, pues las concesiones en detrimento de la calidad no hubieran hecho sino perjudicar a los autores y a la propia revista. En el número de septiembre, que recogía algunas de las ponencias del XV Congreso de la SEM, los organizadores de las diferentes sesiones revisaron también los textos de los ponentes.

Encaramos el año 1996 con ilusión, y con la esperanza de afrontar los retos a los que aludió el presidente de la SEM, Francisco Ruiz Berraquero, durante el reciente Congreso: acceder a los índices internacionales, mantener la periodicidad y potenciar la calidad. Para todo ello, es indispensable seguir contando con la confianza y la colaboración de todos los socios que, desde aquí, agradecemos.

**Ricard Guerrero**
Director-Coordinador

MICROBIOLOGÍA SEM **11** (1995), 429–438

# The social behavior of myxobacteria

José Muñoz-Dorado*, José M. Arias

*Departamento de Microbiología, Facultad de Ciencias, Universidad de Granada, Granada, España*

Received 28 September 1995/Accepted 30 October 1995

**Summary**

Myxobacteria are social microorganisms that undergo a spectacular cell cycle. Under starvation conditions, cells aggregate to certain points originating macroscopic fruiting bodies, inside which cells differentiate into myxospores. To accomplish this developmental cycle, cells must communicate. The signals that cells exchange during development as well as the signal transduction systems used by myxobacteria have been intensively studied during the last years. A family of eukaryotic-like protein serine/threonine kinases has been identified in *Myxococcus xanthus*, indicating that signal transduction systems similar to those used by eukaryotic cells may also function in myxobacteria.

**Key words:** myxobacteria, protein kinases, phosphatases, development, signal transduction

**Resumen**

Las mixobacterias son organismos sociales que llevan a cabo un espectacular ciclo celular. En condiciones de inanición, las células se agregan en ciertos puntos originando cuerpos fructificantes, dentro de los cuales las células se diferencian en mixosporas. Para completar este ciclo de desarrollo, las células se deben comunicar entre sí. Las señales que las células intercambian durante el desarrollo, así como los sistemas de transducción de señales utilizados por las mixobacterias, han sido intensamente estudiados durante los últimos años. En *Myxococcus xanthus* se ha identificado una familia de kinasas de proteinas que fosforilan en serina y treonina parecidas a las de las células eucarióticas, lo que indica que en las mixobacterias pueden también funcionar sistemas de transducción de señales similares a los encontrados en células eucarióticas.

* *Correspondence to*: José Muñoz-Dorado. Departamento de Microbiología. Facultad de Ciencias. Universidad de Granada. Fuentenueva s/n. 18071 Granada. España. Tel.: +34-58-243397. Fax: +34-58-243246. E-mail: jmarias@goliat.ugr.esp

## Introduction

Myxobacteria are rod-shaped Gram-negative bacteria that show several striking and unique features that make them different from other bacteria. They are found in many different types of soils, although they are especially abundant in soils that are rich in organic material such as cultivated soils and rotting wood. They produce an extraordinary extracellular lytic machinery consisting of antibiotics, bacteriocins and enzymes that hydrolyze substrates as different as proteins, lipids, starch, chitin, cellulose and cell walls, not only when they appear as soluble macromolecules, but also when they are a part of entire cells. In fact, myxobacteria can be grown on media containing baker's yeast or *Escherichia coli* cells (44).

Another unusual property of myxobacteria is their ability to glide on the surface of a solid medium or in the interface air-water. The mechanism of this kind of motility is unknown at present, and no organelles have been convincingly identified to be responsible for gliding. Lünsdorf and Reichenbach (28) have found intracellular filaments that might function as the motility apparatus, although conclusive evidence is still presently lacking.

But undoubtedly the most amazing characteristic of myxobacteria is their morphogenetic potential. Myxobacteria are always found as communities of cells known as swarms, preying on other microorganisms or complex macromolecules. Under starvation conditions, cells start to glide to certain points where they pile on top of each other to eventually construct macroscopic structures, the fruiting bodies, which can be observed with the naked eye as colored masses, due to the production of pigments (11). The fruiting bodies may appear just as simple mounds on the soil surface (Fig. 1A), or as sophisticated structures consisting of a stalk and several sporangioles (Fig. 1B). Inside the fruiting bodies the cells differentiate

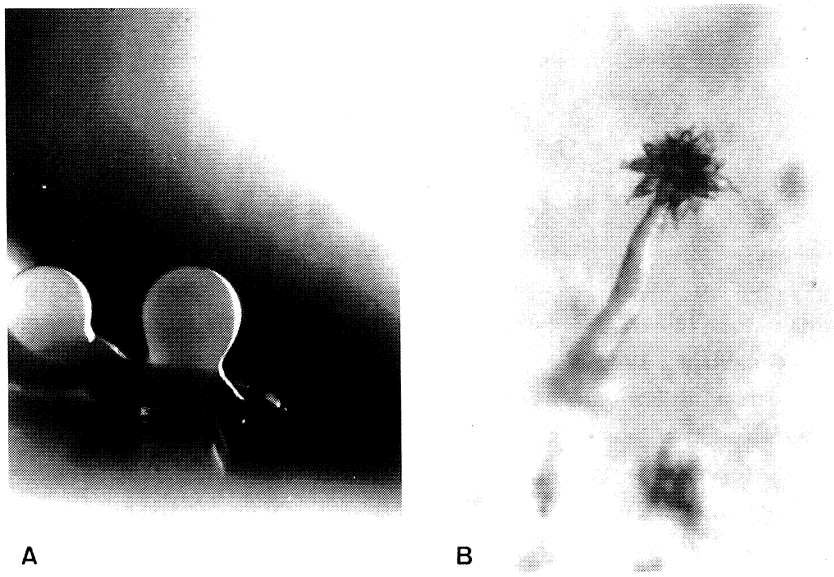



FIG. 1. Fruiting bodies of myxobacteria. A. *Myxococcus fulvus*. B. *Chondromyces apiculatus*. These photographs are a gift from H. Reichenbach.

to originate myxospores, which are resistant to several stress conditions such as desiccation, sonication, and UV irradiation. Myxospores are usually shorter than the vegetative cells and are covered by a thick coat. They appear refringent on the microscope. The developmental cycle of myxobacteria guarantees not only the survival of the cells under stress conditions, but also that a new vegetative cycle is always resumed with a high cell population. Degradation of the complex macromolecules of the habitat where they live will be much more efficient and affordable if the exoenzymes are secreted by many cells instead of by only one.

Since motility is required for the aggregation of the cells, these three characteristics, cooperative feeding, gliding motility and morphogenetic potential, make myxobacteria behave as social microorganisms. In fact, myxobacteria are considered as one of the first attempts at multicellularity during evolution. For this social behavior, cells must somehow communicate with one another and attempts to explain the mechanisms of intercellular communication in myxobacteria are currently being carried out in several laboratories. Two kinds of signal transduction pathways seem to operate in myxobacteria. One controls motility and belongs to the family of the two-component regulatory systems, a typical prokaryotic signal transduction system. The other controls the developmental cycle, and eukaryotic-like protein kinases have been found to be involved.

## Motility

Gliding motility is not unique in myxobacteria, but rather it occurs in many other groups of bacteria. However, a great part of the recent studies on this motility has been performed with *M. xanthus*, the myxobacteria most extensively characterized.

Genetic approaches have revealed that *M. xanthus* possesses two motility systems: A or adventurous, and S or social (20). The A system is responsible for the movement of single cells and the S system for the movement of groups of cells. The latter one requires cell-cell contact. The two systems seem to be genetically independent and therefore mutants $A^+S^-$, $A^-S^+$ and $A^-S^-$ can be obtained. Nine different genes involved in the S motility, and twenty three genes related to the A motility (12, 20) have been found. Recent studies by using Tn*5lac* insertions have identified new genes involved in both motility systems. These studies have shown that the A genes appear grouped into 5 clusters, while the S genes are grouped into 3 clusters (30). Some of the mutations in the loci responsible for S motility result in the loss of polar pili, which may explain why the S motility requires cell-cell contact (21). The role of the genes involved in A and S motility is unclear. It has been proposed that myxobacteria might possess two different gliding motors (31). However, none of the genes of the two systems that have been characterized encode proteins that may function as a part of the gliding apparatus. Further analysis of the other loci will be necessary to elucidate the role of each gene in motility. Both systems are required for the normal development of myxobacteria, and mutations in any of them usually result in poor aggregation.

In addition to the loci of the A and S systems, a locus has been identified that is the only one that completely blocks motility when it is mutated (52). This locus is known as *mgl* and it has been described as containing two open reading frames (*mglA* and *mglB*) (51). Most of the mutations that abolish motility are located in the *mglA* gene. The *mglA* gene encodes a protein of 22-kDa that shows a great similarity to GTP-binding proteins, while *mglB* encodes a protein with similarity to calmodulin (18, 19).

The resemblance of MglA to GTP-binding proteins suggests that this protein might be involved in the regulation of the events that lead to gliding just by switching the states GTP-bound and GDP-bound of this protein (18). On the other hand, MglB regulates the amount of MglA in the cells, indicating that MglB may stabilize MglA or affect its translation (19). The resemblance of MglB to calcium-binding proteins opens new possibilities in the study of gliding motility since calcium is required for A and S motility, and it even affects the expression of the genes involved in motility (14, 60).

Other genes involved in motility in *M. xanthus* are known as *frz* (from frizzy), which control the reversal of movement of the cells (4, 5). The *frz* mutants are therefore motile, but the control of the movement has been lost and when the cells aggregate during the developmental cycle they do not originate fruiting bodies, but frizzy filaments. There are 6 *frz* genes, named as *frzA, B, CD, E, F* and *G*, 5 of which show high similarities to the chemotaxis genes of enteric bacteria (29, 33). *frzA* is homologous to *cheW*, *frzCD* is homologous to the methyl-accepting proteins (MCPs), *frzE* is homologous to both *cheA* and *cheY*, *frzF* is homologous to *cheR*, and *frzG* is homologous to *cheB*. The *frzB* gene, on the contrary, does not show any significant similarity to any other known gene. The similarities between the *frz* and the *che* genes suggest that Frz proteins must function in the same manner as the Che proteins do, controlling the chemotactic behavior of *M. xanthus*. A model for the chemotaxis signal transduction system of *M. xanthus* has been proposed (32). One peculiarity of the *frz* signal transduction pathway is that FrzE shows similarity to both CheA and CheY, members of the two-component regulatory system. In enteric bacteria, CheA is autophosphorylated in a histidine residue and this phosphate is then transferred to an aspartate residue of CheY (34, 35). FrzE contains both the histidine and the aspartate residues and the transfer of the phosphate group from one to the other has to be carried out by an intramolecular reaction. FrzE contains a stretch of 70 residues rich in alanine and proline. This region may function as a hinge that allows the phosphohistidine residue to interact with the aspartate residue (35).

## The developmental cycle

As mentioned in the Introduction, the most amazing and spectacular characteristic of myxobacteria is their developmental cycle (Fig. 2). Myxobacteria follow a vegetative cycle when the nutrients are abundant in the medium. Under these conditions, cells just divide like any other bacteria. However, under nutrient depletion, cells start to aggregate in certain points originating macroscopic fruiting bodies. Inside the fruiting bodies, the cells shorten and cover of a thick wall, originating myxospores. During this process many cells autolyse and only 10 to 20% of the total cells differentiate into myxospores (58, 59). Although initially it was thought that autolysis affected 80 to 90% of the cells in a programmed cell-death fashion, later it has been argued that the lysis observed was only a consequence of technical manipulation and that the cells that did not originate myxospores remained as peripheral rods (41, 42). Nevertheless, further studies where the technical manipulation was completely abolished have shown a correlation between the number of cells autolysed and the number of myxospores originated (45). The higher the number of autolysed cells, the higher the yield of myxospores among the survivors. Although the extent and the role of autolysis during development are still under discussion, five substances, known as autocides, are produced by *M. xanthus* that induce the lysis of the producing and other related

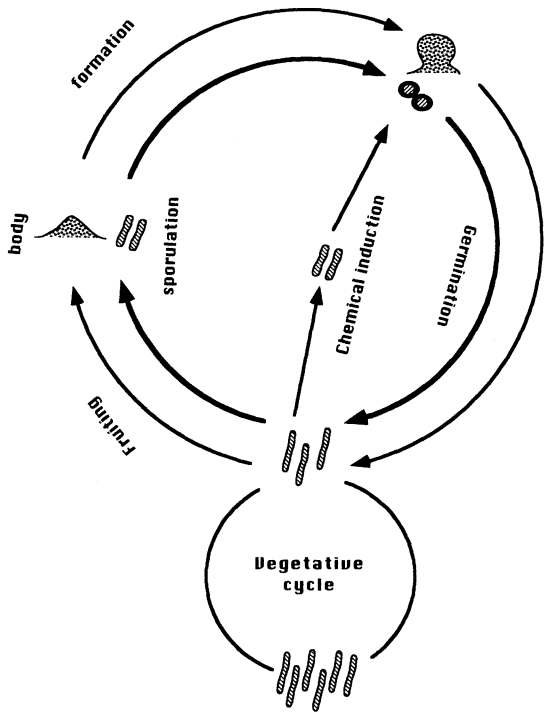



FIG. 2. Schematic diagram of the developmental cycle of myxobacteria.

strains (55). These autocides seem to play some role during the developmental cycle of *M. xanthus* (46, 56). Arias et al. (1) have also shown evidence of a substance responsible for the autolysis in *Myxococcus coralloides*. Nevertheless, the relationship of this substance to autocides has not yet been established. The myxospores are able to germinate and resume the vegetative cycle again when the nutritional conditions are restored. Myxospores can also be induced without previous aggregation and in a rich medium just by adding certain organic compounds, such as glycerol, phenethyl alcohol or dimethylsulfoxide (10). However, glycerol-induced myxospores are different from fruiting-body myxospores. Their coat is much thinner and it does not contain protein S, the major coat protein in fruiting body myxospores (47).

The cooperative feeding and development of myxobacteria require the coordination of many cells. This coordination is possible by the exchange of several extracellular signals. Five different types of signal-defective mutants have been isolated (8, 16). These mutants were unable to sporulate when they were plated alone, but sporulation of the mutants could be rescued just by adding wild-type cells or cells of another complementation group. The mutants have been referred to as *asg*, *bsg*, *csg*, *dsg* and *esg*, and the extracellular signals to which they are dependent on as A, B, C, D and E factors. A and B factors act at the onset of development, while D and C factors act 4 and 6 hours, respectively, after starvation. On the contrary, *esg* is expressed during both vegetative growth and development (8).

A factor was purified and it turned out to consist of two different activities, one heat stable and dialyzable, and the other heat labile and nondialyzable (26, 43). Heat-labile A factor was identified to

be a mixture of proteins with different proteolytic activities, while the heat-stable A factor was identified as a mixture of amino acids and small peptides. These results indicate that the A signal must be the peptides and amino acids released by the activity of the proteases.

B factor has not been purified yet, but *bsgA* gene, the only locus of *bsg* mutants that has been mapped and cloned so far, encodes an ATP-dependent protease, similar to *lon* protease of *E. coli*. This finding indicates that intracellular proteolysis may be involved in the *bsgA*-dependent cell signaling (13).

C factor has been identified as the product of the *csgA* gene (23, 49). C-factor is a membrane-associated protein with a signal-peptide in its N-terminal region similar to that of enteric bacteria (17). C factor is not released to the medium, and therefore requires cell-cell contact to act. On the other hand, C-factor requires motility (25). Nonmotile cells do not express C-factor dependent genes although concentrations of C factor are normal. Investigations about the relationship between motility and C factor have shown that cells have to be aligned in a certain manner. When nonmotile cells were aligned artificially, they could transmit the C factor signal and express the C-factor dependent genes. During normal development, cells move to aggregate in certain points, increasing cell density about 50-fold. In these dense packs, cells align in a regular manner, allowing the C factor to act (24).

D factor has not yet been identified. A *dsg* gene has been cloned and characterized. It encodes the translation initiation factor IF3 of *M. xanthus* (7, 22), and therefore insertions of Tn*5* within this gene are lethal (6). The phenotype of the *dsg* mutants can be explained as an indirect effect caused by a failure in the translation of the gene(s) responsible for the production of the D factor (22).

The *esg* gene has also been cloned. It encodes E1 decarboxylase, which is a component of a multienzyme complex involved in the metabolism of branched-chain amino acids, the branched-chain keto acid dehydrogenase (53). This complex originates branched-chain fatty acids, some of which have been proposed as constituting the E factor (9).

## Protein kinases and phosphatases

Although there is much information about the signals that act during development and the genes whose expression depends on the transmission of these signals, we do not know how the signals are transduced through the membrane to regulate gene expression.

The social behavior of myxobacteria and the similarities of their developmental cycle to that of *Dictyostelium* led Inouye's lab to speculate that myxobacteria could utilize signal transduction systems similar to those reported in eukaryotic cells, consisting of receptor-G protein-effector and protein kinase. In a first attempt to identify G proteins in *M. xanthus*, at least five proteins that bind GTP were found, two of them located in the membrane fraction and the rest in the soluble fraction (37). Characterization of one of the soluble GTP-binding proteins revealed that it is a nucleoside diphosphate kinase (NDP kinase) (38). NDP kinases catalyze the transfer of the γ-phosphate group of nucleoside triphosphate to a nucleoside diphosphate, originating a different nucleoside triphosphate and maintaining cellular concentrations of all nucleoside triphosphate at functional levels. However, NDP kinase of other organisms have been found to be involved in some other functions, such as the development of the wings of *Drosophila* or suppression of metastasis in a human tumor cell line (2, 3, 27, 48, 50). In *M. xanthus*,

on the contrary, no other function of NDP kinase has been found, especially because this protein is essential for cell viability (37). However, the phosphorylation at serine residues found in *M. xanthus* NDP kinase indicates that this protein might also be involved in many other cellular functions (36).

One of the two membrane-associated GTP-binding proteins has also been cloned and characterized, but it turned out to be the α-subunit of a proton-translocating ATPase (40). We do not know whether any other of the five GTP-binding proteins detected in *M. xanthus* is a G protein.

Another attempt to identify a eukaryotic-like signal transduction pathway in *M. xanthus* was focused on the detection of protein kinases. By polymerase chain reaction (PCR) using primers designed according to the conserved regions of eukaryotic protein kinases and the entire *M. xanthus* chromosome as a template, three different products were amplified (39). The sequence of these three products revealed a high similarity to protein kinases. Further hybridization analysis showed that *M. xanthus* may possess 26 different protein kinases (61). Protein kinases with sequences similar to those of eukaryotic cells had not been identified in bacteria previously.

By using the PCR products as probes, the entire genes that encode these protein kinases were cloned. These genes are referred to as *pkn1*, *pkn2* and *pkn3*. The *pkn1* gene encodes a protein of 693 residues (39). The amino terminal portion of this protein contains the eleven subdomains conserved in the eukaryotic protein kinases, while the C-terminal portion showed no similarity to any other known protein. The *pkn1* gene has been expressed in *E. coli* cells and it has been shown that Pkn1 autophosphorylates at both serine and threonine residues, but not at tyrosine. Pkn1 is then the first report of a eukaryotic-style protein serine-threonine kinase. The *pkn1* gene is developmentally regulated to be induced at the onset of sporulation, reaching a maximum at 50 h and staying at a nearly constant level thereafter. To investigate the role of Pkn1, a strain harboring a deletion in the *pkn1* gene was constructed. This strain was able to aggregate and sporulate; however, the fruiting bodies were smaller and less compact than those obtained with the wild-type strain, and the yield of myxospores was only 35%.

The *pkn2* gene encodes a protein of 830 amino acid residues (54). Pkn2 consists of an amino terminal portion with similarities to the catalytic domain of eukaryotic protein kinases and Pkn1, and a C-terminal portion with no similarities to any other known protein. In the middle of this C-terminal portion there is a typical transmembrane domain consisting of 19 hydrophobic residues. Analyses of *phoA* fusions with *pkn2* have shown that Pkn2 is indeed a transmembrane protein, with the kinase domain located in the cytoplasm and the C-terminal domain located outside the cytoplasmic membrane. Pkn2 has been overproduced in *E. coli*. This protein is able to autophosphorylate at both serine and threonine residues. Pkn2 is also able to phosphorylate β-lactamase but only at threonine residues. Phosphorylation of β-lactamase shifted its apparent molecular weight from 29 to 44 kDa, and prevented its secretion to the periplasmic space. The *pkn2* gene is expressed only during vegetative growth, obtaining a peak at the beginning of the stationary phase. Expression then drops sharply to a basal level. Expression also drops to a basal level just after starvation. However, studies with a strain harboring a disruption mutation of *pkn2* have shown that the mutant exhibits no phenotype on rich medium; while during the developmental cycle, fruiting bodies appear loosely packed, and the yield of myxospores is approximately 30 to 50% of that of the wild-type strain.

The finding of eukaryotic-like protein kinases involved in the regulation of the developmental cycle implies the existence of other proteins able to dephosphorylate the substrates phosphorylated by the protein kinases, phosphatases. In myxobacteria several proteins have been reported with phosphatase activities, some of which are induced during the developmental cycle (15,57). However, we do not know

the relationship of these phosphatase activities with the protein kinases. We do not know either the specificity of these phosphatases. Are they eukaryotic-like protein phosphatases or are they similar to unspecific acid, neutral and alkaline phosphatases? To elucidate these questions, we cloned in our laboratory four genes that encode proteins with phosphatase activities (unpublished results). Characterization of these four genes will help to clarify the role that phosphorylation/dephosphorylation plays in the regulation of the developmental cycle of myxobacteria.

The existence of protein kinases in myxobacteria makes it plausible to think that the other components of the signal transduction system used by eukaryotic cells are also present in these bacteria. However, identification of the receptors, G proteins, effectors, cascades of protein kinases and the interactions between all of them, will be necessary to conclude whether this kind of signal transduction system is used by myxobacteria to respond to extracellular signals.

## Acknowledgments

We want to thank Hans Reichenbach (GBF, Braunschweig, Germany) for kindly supplying the pictures of the fruiting bodies shown in Fig. 1. This work was supported by the Dirección General de Investigación Científica y Técnica (DGICYT, PB94-0781).

## References

1. Arias, J. M., Fernández-Vivas, A., Montoya, E. (1983). Evidence for an activating substance related to autolysis in *Myxococcus coralloides* D. Arch. Microbiol. **134**, 164–166.
2. Biggs, J., Herspeger, E., Steeg, P., Liotta, L. A., Shearn, A. (1990). A *Drosophila* gene that is homologous to a mammalian gene associated with tumor metastasis codes for a nucleoside diphosphate kinase. Cell **63**, 933–940.
3. Biggs, J., Tripoulas, N., Hersperger, E., Dearolf, C., Shearn, A. (1988). Analysis of the lethal interactions between the *prune* and killer of *prune* mutations of *Drosophila*. Genes Dev. **2**, 1333–1343.
4. Blackhart, B. D., Zusman, D. R. (1985). Cloning and complementation analysis of the «frizzy genes» of *Myxococcus xanthus*. Mol. Gen. Genet. **198**, 243–254.
5. Blackhart, B. D., Zusman, D. R. (1985). «Frizzy» genes of *Myxococcus xanthus* are involved in control of frequency of reversal of gliding motility. Proc. Natl. Acad. Sci. USA **82**, 8767–8770.
6. Cheng, Y. L., Kaiser, D. (1989). *dsg*, a gene required for *Myxococcus* development, is necessary for cell viability. J. Bacteriol. **171**, 3727–3731.
7. Cheng, Y. L., Kalman, L. V., Kaiser, D. (1994). The *dsg* gene of *Myxococcus xanthus* encodes a protein similar to translation initiation factor IF3. J. Bacteriol. **176**, 1427–1433.
8. Downard, J., Ramaswamy, S. V., Kil, K.-S. (1993). Identification of *esg*, a genetic locus involved in cell-cell signaling during *Myxococcus xanthus* development. J. Bacteriol. **175**, 7762–7770.
9. Downard, J., Toal, D. (1995). Branched-chain fatty acids: the case for a novel form of cell-cell signaling during *Myxococcus xanthus* development. Mol. Microbiol. **16**, 171–175.
10. Dworkin, M., Gibson, S. M. (1964). A system for studying microbial morphogenesis: rapid formation of microcysts in *Myxococcus xanthus*. Science **246**, 243–244.
11. Dworkin, M., Kaiser, D. (1993). Myxobacteria II. American Society for Microbiology, Washington, D.C.
12. Fink, J. M., Zissler, J. F. (1989). Defects in motility and development of *Myxococcus xanthus* lipopolysacharide mutants. J. Bacteriol. **171**, 2042–2048.
13. Gill, R. E., Karlok, M., Benton, D. (1993). *Myxococcus xanthus* encodes an ATP-dependent protease which is required for developmental gene transcription and intercellular signaling. J. Bacteriol. **175**, 4538–4544.

14. Gilmore, D. F., White, D. (1985). Energy-dependent cell cohesion in myxobacteria. J. Bacteriol. **161**, 113–117.
15. González, F., Vargas, A., Arias, J. M., Montoya, E. (1991). Phosphatase activity during development cycle of *Myxococcus xanthus*. Can. J. Microbiol. **37**, 74–77.
16. Hagen, D. C., Bretscher, A. P., Kaiser, D. (1978). Synergism between morphogenetic mutants of *Myxococcus xanthus*. Dev. Biol. **64**, 284–296.
17. Hagen, D. C., Shimkets, L. J. (1990). Nucleotide sequence and transcriptional products of the *csg* locus of *Myxococcus xanthus*. J. Bacteriol. **172**, 15–23.
18. Hartzell, P., Kaiser, D. (1991). Function of MglA, a 22-kilodalton protein essential for gliding in *Myxococcus xanthus*. J. Bacteriol. **173**, 7615–7624.
19. Hartzell, P., Kaiser, D. (1991). Upstream gene of the *mgl* operon controls the level of MglA protein in *Myxococcus xanthus*. J. Bacteriol. **173**, 7625–7635.
20. Hodgkin, J., Kaiser, D. (1979). Genetics of gliding motility in *Myxococcus xanthus*: two gene systems control movement. Mol. Gen. Genet. **171**, 177–191.
21. Kaiser, D. (1979). Social gliding is correlated with the presence of pili in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **76**, 5952–5956.
22. Kalman, L. V., Cheng, Y. L., Kaiser, D. (1994). The *Myxococcus xanthus dsg* gene product performs functions of translation initiation factor IF3 in vivo. J. Bacteriol. **176**, 1434–1442.
23. Kim, S. K., Kaiser, D. (1990). C-factor: a cell-cell signaling protein required for fruiting body morphogenesis of *Myxococcus xanthus*. Cell **61**, 19–26.
24. Kim, S. K., Kaiser, D. (1990). Cell alignment required in differentiation of *Myxococcus xanthus*. Science **249**, 926–928.
25. Kim, S. K., Kaiser, D. (1990). Cell motility is required for the transmission of C-factor, an intercellular signal that coordinates fruiting body morphogenesis of *Myxococcus xanthus*. Genes Dev. **4**, 896–905.
26. Kuspa, A., Plamann, L., Kaiser, D. (1992). Identification of heat-stable A-factor from *Myxococcus xanthus*. J. Bacteriol. **174**, 3319–3326.
27. Leone, A., Flatow, U., King, C. R., Sandeen, M. A., Margulies, I. M. K., Liotta, L. A., Steeg, P. S. (1991). Reduced tumor incidence, metastatic potential and cytokine responsiveness of nm-23 transfected melanoma cells. Cell **65**, 225–235.
28. Lünsdorf, H., Reichenbach, H. (1989). Ultrastructural details of the apparatus of gliding motility of *Myxococcus fulvus*. J. Gen. Microbiol. **135**, 1633–1641.
29. MacNab, R. M. (1987). Motility and chemotaxis. *In* Neidhart, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., Umbarger, H. E. (ed.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, vol. 1, pp. 732–759. American Society for Microbiology, Washington, D.C.
30. MacNeil, S. D., Calara, F., Hartzell, P. L. (1994). New clusters of genes required for gliding motility in *Myxococcus xanthus*. Mol. Microbiol. **14**, 61–71.
31. McBride, M. J., Hartzell, P., Zusman, D. R. (1993). Motility and tactic behavior of *Myxococcus xanthus*. *In* Dworkin, M., Kaiser, D. (ed.), Myxobacteria II, pp. 285–332. American Society for Microbiology, Washington, D.C.
32. McBride, M. J., Kohler, T., Zusman, D. R. (1992). Methylation of FrzCD, a methyl-accepting taxis protein of *Myxococcus xanthus*, is correlated with factors affecting cell behavior. J. Bacteriol. **174**, 4246–4257.
33. McBride, M. J., Weinberg, R. A., Zusman, D. R. (1989). «Frizzy» aggregation genes of the gliding bacterium *Myxococcus xanthus* show sequence similarities to the chemotaxis genes of enteric bacteria. Proc. Natl. Acad. Sci. USA **86**, 424–428.
34. McCleary, W. R., Zusman, D. R. (1990). FrzE of *Myxococcus xanthus* is homologous to both CheA and CheY of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **87**, 5898–5902.
35. McCleary, W. R., Zusman, D. R. (1990). Purification and characterization of the *Myxococcus xanthus* FrzE protein shows that it has autophosphorylation activity. J. Bacteriol. **172**, 6661–6668.
36. Muñoz-Dorado, J., Almaula, N., Inouye, S., Inouye, M. (1993). Autophosphorylation of nucleoside diphosphate kinase from *Myxococcus xanthus*. J. Bacteriol. **175**, 1176–1181.

37. Muñoz-Dorado, J., Inouye, M., Inouye, S. (1990). Nucleoside diphosphate kinase from *Myxococcus xanthus*: I. Cloning and sequencing of the gene. J. Biol. Chem. **265**, 2702–2706.
38. Muñoz-Dorado, J., Inouye, S., Inouye, M. (1990). Nucleoside diphosphate kinase from *Myxococcus xanthus*: II. Biochemical characterization. J. Biol. Chem. **265**, 2707–2712.
39. Muñoz-Dorado, J., Inouye, S., Inouye, M. (1991). A gene encoding a protein serine/threonine kinase is required for normal development of *Myxococcus xanthus*, a Gram-negative bacterium. Cell **67**, 995–1006.
40. Muñoz-Dorado, J., Inouye, S., Inouye, M. (1994). Identification of the *Myxococcus xanthus* 59-kDa membrane-associated GTP-binding protein as a proton-translocating ATPase. Gene **138**, 133–137.
41. O'Connor, K., Zusman, D. R. (1988). Reexamination of the role of autolysis in the development of *Myxococcus xanthus*. J. Bacteriol. **170**, 4103–4112.
42. O'Connor, K., Zusmand, D. R. (1991). Development in *Myxococcus xanthus* involves differentiation into two cell types, peripheral rods and spores. J. Bacteriol. **173**, 3318–3333.
43. Plamann, L., Kuspa, A., Kaiser, D. (1992). Proteins that rescue A-signal-defective mutants of *Myxococcus xanthus*. J. Bacteriol. **174**, 3311–3318.
44. Reichenbach, H. (1993). Biology of the myxobacteria: ecology and taxonomy. *In* Dworkin, M., Kaiser, D. (ed.), Myxobacteria II, pp. 13–62. American Society for Microbiology, Washington, D.C.
45. Rosenbluh, A., Nir, R., Sahar, E., Rosenberg, E. (1989). Cell-density-dependent lysis and sporulation of *Myxococcus xanthus* in agarose beads. J. Bacteriol. **171**, 4923–4929.
46. Rosenbluh, A., Rosenberg, E. (1989). Autocide AMI rescues development in *dsg* mutants of *Myxococcus xanthus*. J. Bacteriol. **171**, 1513–1518.
47. Rosenbluh, A., Rosenberg, E. (1989). Sporulation of *Myxococcus xanthus* in liquid shake flask cultures. J. Bacteriol. **171**, 4521–4524.
48. Rosengard, A. M., Krutzsch, H. C., Shearn, A., Biggs, J. R., Barker, E., Margulies, I. M. K., King, C. R., Liotta, L. A., Steeg, P. S. (1989). Reduced Nm23/Awd protein in tumor metastasis and aberrant *Drosophila* development. Nature (London) **342**, 177–180.
49. Shimkets, L. J., Rafiee, H. (1990). CsgA, an extracellular protein essential for *Myxococcus xanthus* development. J. Bacteriol. **172**, 5299–5306.
50. Stahl, J. A., Leone, A., Rosengard, A. M., Porter, L., King, C. R., Steeg, P. S. (1991). Identification of a second human nm23 gene, nm23H2. Cancer Res. **51**, 445–449.
51. Stephens, K., Hartzell, P., Kaiser, D. (1989). Gliding motility in *Myxococcus xanthus*: *mgl* locus, RNA, and predicted protein products. J. Bacteriol. **171**, 819–830.
52. Stephens, K., Kaiser, D. (1987). Genetics of gliding motility in *Myxococcus xanthus*: molecular cloning of the *mgl* locus. Mol. Gen. Genet. **207**, 256–266.
53. Toal, D. R., Clifton, S. W., Roe, B. A., Downard, J. (1995). The *esg* locus of *Myxococcus xanthus* encodes the E1α and E1β subunits of a branched-chain keto acid dehydrogenase. Mol. Microbiol. **16**, 177–189.
54. Udo, H., Muñoz-Dorado, J., Inouye, M., Inouye, S. (1995). *Myxococcus xanthus*, a Gram-negative bacterium, contains a transmembrane protein serine/threonine kinase that blocks the secretion of β-lactamase by phosphorylation. Genes Dev. **9**, 972–983.
55. Varon, M., Cohen, S., Rosenberg, E. (1984). Autocides produced by *Myxococcus xanthus*. J. Bacteriol. **160**, 1146–1150.
56. Varon, M., Tietz, A., Rosenberg, E. (1986). *Myxococcus xanthus* autocide AMI. J. Bacteriol. **167**, 356–361.
57. Weinberg, R. A., Zusman, D. R. (1990). Alkaline, acid and neutral phosphatase activities are induced during development in *Myxococcus xanthus*. J. Bacteriol. **172**, 2294–2302.
58. Wireman, J. W., Dworkin, M. (1975). Morphogenesis and developmental interactions in myxobacteria. Science **189**, 516–523.
59. Wireman, J. W., Dworkin, M. (1977). Developmentally induced autolysis during fruiting body formation by *Myxococcus xanthus*. J. Bacteriol. **129**, 796–802.
60. Womack, B. J., Gilmore, D. F., White, D. (1989). Calcium requirement for gliding motility in myxobacteria. J. Bacteriol. **171**, 6093–6096.
61. Zhang, W., Muñoz-Dorado, J., Inouye, M., Inouye, S. (1992). Identification of a putative eukaryotic-like protein kinase family in the developmental bacterium *Myxococcus xanthus*. J. Bacteriol. **174**, 5450–5453.

MICROBIOLOGÍA SEM **11** (1995), 439–446

# Role of extracellular products in the pathogenicity of *Vibrio* strains on cultured gilt-head seabream (*Sparus aurata*)

M. Carmen Balebona, Miguel A. Moriñigo, Juan J. Borrego*

*Departamento de Microbiología, Facultad de Ciencias, Universidad de Málaga, Málaga, España*

Received 16 June 1995/Accepted 15 September 1995

**Summary**

The enzymatic activities of extracellular products (ECP) from different pathogenic *Vibrio* strains and their virulence for fish (cultured gilt-head seabream, *Sparus aurata* L.) were tested. ECP were obtained by growing the bacteria on cellophane overlays. Cytotoxic effects of ECP on fish cell lines were rapid and strong, provoking mortality of fish after 24 to 72 h of the ECP inoculation. A close relationship between proteolytic activities (gelatinase and caseinase) of the ECP and the presence of lytic effects in the muscular tissue at the injection site was observed. Therefore, these activities may be considered responsible for the fish tissue damage.

**Key words**: *Vibrio*, extracellular products (ECP), *Sparus aurata* (gilt-head seabream/dorada), fish pathogens, cytotoxicity

**Resumen**

Se ha estudiado la relación de las actividades enzimáticas contenidas en los productos extracelulares (ECP) de diferentes cepas de *Vibrio* con la virulencia para peces (dorada de cultivo, *Sparus aurata* L.). Los ECP se obtuvieron por el método de crecimiento en láminas de celofán. Los efectos citotóxicos de los ECP sobre líneas celulares fueron rápidos e intensos; además, provocaron la muerte de los peces entre las 24 y las 72 h después de la inoculación de ECP. Se ha observado una relación directa y estrecha entre las actividades proteolíticas (gelatinasa y caseinasa) de los ECP y la producción de efectos líticos tras inoculación intramuscular. Por consiguiente, estas actividades enzimáticas pueden considerarse responsables de daños tisulares en los peces.

* *Correspondence to*: Juan J. Borrego. Departamento de Microbiología. Facultad de Ciencias. Universidad de Málaga. Campus Universitario Teatinos. 29071 Málaga. España. Tel.: +34-5-2131893. Fax: +34-5-2132000. E-mail: borrego@ccuma.uma.es

## Introduction

Strains belonging to the genus *Vibrio* are involved in diseases of a wide range of cultured and wild finfish and shellfish, although little is known about the mecanisms related to the pathogenesis of these diseases (1, 2, 10, 12, 16).

Several studies have demonstrated that extracellular products (ECP) are one of the main factors responsible for the toxic effects of the pathogenic microorganisms, since they allow the bacteria to survive and grow within the host tissues (8). ECP of the microorganisms may contain different substances considered as virulence factors, such as protease, cytolysins, haemolysins, siderophore, RNAase, esterases, phospholipases, to name a few. Such ECP may exert two different effects on the hosts: toxicity and ability to digest their tissues (5).

In the present study, the enzymatic activities of ECP isolated from different *Vibrio* strains pathogenic for gilt-head seabream (*Sparus aurata* L.), as well as their virulence for this fish species were tested.

## Materials and methods

**Microorganisms.** *Vibrio* strains were isolated from cultured *Sparus aurata* specimens affected with different pathologies following the methodology specified by the authors (3). The strains used, their taxonomic identification, source of isolation and virulence for fish expressed as $LD_{50}$ (50% mean lethal dose) are given in Table 1. Stock cultures were stored in basal broth (peptone, 4 g/l; yeast extract 1 g/l, NaCl 10 g/l) supplemented with 20% glycerol (v/v) at –80°C. The strains were cultured on tryptone soya agar (TSA) or broth (TSB) (Oxoid, Unipath Ltd., Basingstoke, Hampshire, UK), supplemented with 1.5% NaCl at 22°C for 24 to 48 h.

**Obtainment of extracellular products.** Bacterial ECP were obtained basically following the technique described by Liu (13). Briefly, tubes containing 5 ml of TSB were inoculated with one swab of a 24 h bacterial growth on TSA and incubated for 18 h at 22°C. A volume of 0.2 ml of the culture was spread on a cellophane overlaying TSA plate and incubated at 22°C for 48 h. Bacterial cells were harvested in saline solution pH 7 and the cell suspensions centrifuged at 13,000 × *g* (20 min at 4°C). The supernantants were filtered through 0.45 μm pore size membrane filters and used as crude ECP.

**Virulence assays for fish.** *S. aurata* specimens weighing between 5 and 10 g were inoculated (5 per dose) intramuscularly (above the lateral line in the region below the dorsal fin) and intraperitoneally with 0.1 ml of serial dilutions of the ECP. Fish were maintained in 40 l aquaria with filtered and circulating seawater at 20°C, fed daily and observed for the presence of pathological symptoms.

**Quantifications of the proteins, carbohydrates and fatty acids of the extracellular products.** Total protein was measured in ECP samples following the method described by Bradford (4) using bovine serum albumin as standard (Sigma Chemical Co., St. Louis, MO, USA). Carbohydrate content

TABLE 1. Source and virulence of the strains used

| Strain | *Vibrio* spp.[a] | Source | Associated pathology | $LD_{50}$[b] |
|---|---|---|---|---|
| P3-H-2 | *V. nereis* | Liver | Exophthalmy | $>10^7$ |
| 127 | *V. nereis* | Liver | Skin ulcers | $3.9 \times 10^7$ |
| V3 | *V. nereis* | Liver | Swollen abdomen | $1.3 \times 10^7$ |
| 141 | *V. nereis* | Kidney | Skin ulcers | $>10^8$ |
| 140 | *V. fischeri* | Spleen | Skin ulcers | $>10^8$ |
| P3-H-3 | *V. fischeri* | Liver | Exophthalmy | $1 \times 10^7$ |
| P5-O-3 | *V. fischeri* | Eye | Exophthalmy | $1 \times 10^7$ |
| P8-H-1 | *V. fischeri* | Liver | Exophthalmy | $1.3 \times 10^7$ |
| DC7-R-1 | *V. anguillarum* | Kidney | Haemorrhages | $7 \times 10^6$ |
| DC12-R-8 | *V. anguillarum* | Kidney | Swollen abdomen | $3.4 \times 10^5$ |
| DC12-R-7 | *V. anguillarum* | Kidney | Swollen abdomen | $4.6 \times 10^6$ |
| 128 | *V. aestuarianus* | Liver | Swollen spleen | $8 \times 10^6$ |
| DC10-R-4 | *V. aestuarianus* | Kidney | Swollen abdomen | $9.2 \times 10^7$ |
| CAN | *V. alginolyticus* | Kidney | Haemorrhagic fins | $2.7 \times 10^5$ |
| AO35 | *V. alginolyticus* | Kidney | Haemorrhagic fins | $<10^5$ |
| 28 | *V. harveyi* | Liver | Swollen abdomen | $1 \times 10^6$ |
| DP1-U-3 | *V. harveyi* | Ulcer | Ulcers, haemorrhages | $4.6 \times 10^6$ |
| DP2-HE-6 | *V. harveyi* | Ulcer | Ulcers, haemorrhages | $5.5 \times 10^6$ |
| 122 | *V. campbellii* | Larvae | Swollen abdomen | $1.3 \times 10^7$ |
| P4-R-2 | *Vibrio* spp. | Kidney | Exophthalmy | $2 \times 10^5$ |
| AO28 | *Vibrio* spp. | Kidney | Haemorrhagic fins | $1 \times 10^6$ |
| DC11-R-2 | *Vibrio* spp. | Kidney | Swollen abdomen | $2.5 \times 10^5$ |

[a] Taxonomic identification was performed according to Balebona (2).

[b] Virulence of the live cells measured as lethal dose 50, expressed as colony forming units/g fish.

in ECP was determined using the phenol-sulphuric technique (6). The method described by Duncombe (7) was used for the quantification of fatty acids in ECP.

**Detection of exoenzymatic and haemolytic activities.** Global enzymatic activities of ECP were evaluated by the use of the API ZYM system (BioMerieux Ibérica, Madrid, Spain). Proteolytic, lipolytic, phospholipolytic, elastolytic, amylolytic and haemolytic activities were evaluated on agarose plates (0.8% agarose in Phosphate Buffered Saline 0.1 M, pH 7) containing one of the following substrates: 1% (w/v) gelatin, 2% (w/v) skimmed milk, 1% (v/v) Tween 80, 1% (v/v) egg yolk, 1% (w/v) elastin, 0.4% (w/v) starch and 5% (v/v) gilt-head erythrocytes. A 20-µl volume of each ECP sample was inoculated in 2- to 3-mm diameter wells perforated in the plates.

**Cytotoxicity tests.** Cytotoxicity of extracellular products was tested using three fish cell lines: Chinook Salmon Embryo (CHSE-214), Fathead Minnow peduncle (FHM) and Epithelioma Papullosum

of Carp (ETPC). Cells were grown as monolayers in 24-well culture plates (Nunc) at 18°C using MEM medium (Gibco, Life Technologies Ltd., Paisley, UK) supplemented with 10% foetal calf serum (Sigma). For the toxicity test, the different cell lines were inoculated with 0.1 ml serial dilutions of ECP sample. Wells inoculated with saline solution (0.85%) were used as negative controls. Plates were incubated at 18°C and the effects of ECP on the monolayers were observed after 24 and 48 h.

## Results

The ECP of the strains tested possessed moderate levels of proteins (between 167.5 and 1196 μg/ml) and carbohydrates (between 137 and 875.8 mg/ml), but the concentration of long chain fatty acids (between 50 and 806.6 μM/l) was high.

In the ECP tested, eight different exoenzymatic activity patterns were found, which were only discriminated on the basis of the following enzymes: valyl arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, phosphohydrolase and β-glucuronidase. All the ECP exhibited the following enzymatic activities: alkaline- and acid- phosphatase, esterase, lipase-esterase, leucine arylamidase and N-acetyl-β-glucosaminidase. On the contrary, all the ECP tested were negative for lipase, α- and β-galactosidase, α- and β-glucosidase, α-mannosidase and β-fucosidase activities. In Table 2 other exoenzymatic and haemolytic activities of the ECP are shown, caseinase and gelatinase being the most frequently detected hydrolytic activities.

Fish mortality occurred 72 h after inoculation of the ECP (Table 3). Haemorrhagic and necrosis signals at the site of the injection were frequently observed in the affected fish. Intramuscular inoculation produced symptoms earlier (6 h after injection), mainly if the ECP effects were lysis of the tissues. However, $LD_{50}$ of the ECP were, in most cases, lower for intraperitoneal (ranging between 4.3 and 20 μg protein/g fish) than for intramuscular inoculation (ranging between 5.8 and 25.9 μg protein/g fish) (Table 3).

Most of the strains tested (higher than 77%) were cytotoxic for the three fish cell lines used. However, the fish cell line FHM was the most susceptible, being positive for 90.9% of the strains (Table 3). Cytotoxic effects could be observed within 3 to 6 h after inoculation of the ECP. However, results were recorded after 24 h in order to avoid discarding some late positive effects. Fig. 1 shows the morphological changes detected in one of the cell lines tested (CHSE). A high number of vesicles were observed in affected FHM cells, while dendritic formations were present in EPC and CHSE cells. In all the cell lines tested, ECP produced rounding, shrinking, detaching and finally monolayer destruction.

## Discussion

Although the exoenzymatic synthesis by *Vibrio anguillarum* and *V. vulnificus* is relatively well documented (10, 16, 21), a comparison of extracellular enzyme production and its toxic activities among different *Vibrio* species has not previously been performed.

In the present study, we tested strains belonging to several species of *Vibrio* which were isolated from cultured *S. aurata* showing several pathological symptoms (Table 1). Thus, this can be considered

TABLE 2. Exoenzymatic and haemolytic activities of the extracellular products (ECP)

| Strain | CAS | GEL | AMYL | P-LIP | TW-80 | ELST | HMOL |
|---|---|---|---|---|---|---|---|
| P3-H-2 | – | – | – | – | – | – | – |
| 127 | – | – | – | – | – | – | – |
| V3 | – | – | – | – | – | – | – |
| 141 | – | – | ++ | – | – | – | – |
| 140 | – | – | – | – | – | – | – |
| P3-H-3 | – | – | – | – | – | – | – |
| P5-O-3 | – | – | – | – | – | – | – |
| P8-H-1 | – | – | – | – | – | – | – |
| DC7-R-1 | – | + | – | – | – | – | – |
| DC12-R-8 | +++ | +++ | – | + | + | +++ | + |
| DC12-R-7 | +++ | +++ | + | + | + | +++ | + |
| 128 | ++ | ++ | – | – | – | – | – |
| DC10-R-4 | ++ | +++ | – | – | – | – | + |
| CAN | + | ++ | ++ | + | ++ | – | – |
| AO35 | ++ | +++ | ++ | + | + | – | + |
| 28 | ++ | ++ | ++ | + | ++ | – | – |
| DP1-U-3 | ++ | +++ | ++ | + | + | – | + |
| DP2-HE-6 | ++ | ++ | +++ | + | ++ | – | ++ |
| 122 | + | ++ | ++ | + | + | – | – |
| P4-R-2 | ++ | ++ | ++ | – | – | – | – |
| AO28 | ++ | +++ | ++ | – | +++ | – | ++ |
| DC11-R-2 | +++ | +++ | – | – | – | ++ | + |

Abbreviations: CAS, caseinase; GEL, gelatinase; AMYL, amylase; P-LIP, phospholipase; TW-80, hydrolysis of Tween-80; ELST, hydrolysis of elastin; HMOL, lysis of gilt-head sea bream erythrocytes.

Symbols: +, diameter of the activity halo ≤ 10 mm; ++, diameter of the activity halo between 11 and 20 mm; +++, diameter of the activity halo ≥ 21 mm; –, no activity halo.

as the first study on the pathogenic activities of ECP from *Vibrio* strains involved in epizootic outbreaks of cultured *S. aurata*.

A high variability in the chemical components of ECP (proteins, carbohydrates and fatty acids) from the different strains of *Vibrio* tested was found. This variability occurred even within strains of the same species, which suggests an intraspecific heterogeneity in the *Vibrio* genus, similar to that reported for other fish pathogens, such as *Aeromonas* (1, 11, 17). For the strains studied no significant correlations between the protein and carbohydrate contents of their ECP and their virulence was observed. However, the fatty acid content of ECP shows a significant correlation ($\alpha = 0.05$, Kendall correlation coefficient) with their virulence for *S. aurata*.

Proteolytic activities of different bacterial ECP have been related to the pathogenicity degree by several authors (8, 10, 15). The presence of caseinase and gelatinase activities was the most frequently

TABLE 3. Cytotoxic activities and virulence for fish in ECP from *Vibrio* strains

| | Cell lines | | | Death percentages | | $LD_{50}$[a] | |
|---|---|---|---|---|---|---|---|
| Strain | CHSE | ETPC | FHM | IM[b] | IP[c] | IM | IP |
| P3-H-2 | + | – | + | 0 | 0 | – | – |
| 127 | ++ | ++ | ++ | 0 | 66 | – | 19.3 |
| V3 | – | – | ++ | 0 | 33 | – | ND |
| 141 | + | + | + | 0 | 0 | – | – |
| 140 | – | – | – | 0 | 0 | – | – |
| P3-H-3 | + | + | + | 0 | 0 | – | – |
| P5-O-3 | – | ++ | ++ | 30 | 0 | ND | – |
| P8-H-1 | + | + | + | 0 | 0 | – | – |
| DC7-R-1 | + | – | + | 0 | 0 | – | – |
| DC12-R-8 | ++ | ++ | ++ | 30 | 100 | 25.9 | 12.5 |
| DC12-R-7 | ++ | ++ | ++ | 100 | 100 | 11.4 | 4.4 |
| 128 | + | + | + | 0 | 0 | – | – |
| DC10-R-4 | + | + | + | 0 | 0 | – | – |
| CAN | ++ | ++ | ++ | 30 | 20 | 15.6 | 20.0 |
| AO35 | ++ | ++ | ++ | 66 | 66 | 5.8 | 4.3 |
| 28 | + | + | + | 0 | 0 | – | – |
| DP1-U-3 | – | – | – | 0 | 0 | – | – |
| DP2-HE-6 | + | + | + | 0 | 0 | – | – |
| 122 | + | + | + | 0 | 0 | – | – |
| P4-R-2 | + | + | + | 0 | 0 | – | – |
| AO28 | ++ | ++ | ++ | 66 | 30 | 17.4 | 17.4 |
| DC11-R-2 | + | + | + | 0 | 0 | – | – |

[a] µg protein/g fish.

[b] Intramuscular inoculation.

[c] Intraperitoneal inoculation.

Abbreviations: CHSE, Chinook Salmon Embryo; ETPC, Epithelioma Papullosum of Carp; FHM, Fat Head Minnow Peduncle.

Symbols: +, cytotoxic effects in the crude ECP; ++, cytotoxic effects in the 1:10 dilution; –, no citotoxic effects; ND, not determined.

detected, although a lack of the exoenzymatic activities tested was found in seven strains studied. A close relationship between exoenzymatic activities and the presence of lytic effects in the muscular tissue at the injection site was observed. Thus, ECP showing positive gelatinase and caseinase activities always caused destruction of muscular tissue, this damage sign also being especially important in the positive elastolytic activity ECP. Haemolytic activity of ECP was always correlated with the presence of visible haemolytic lesions in the fish.

However, a low percentage of the strains which produced cytotoxicity in cell lines also caused death of fish. These results have also been reported by several authors, who found no significant correlation between cytotoxicity of ECP from different *Vibrio* species and the virulence of the strains

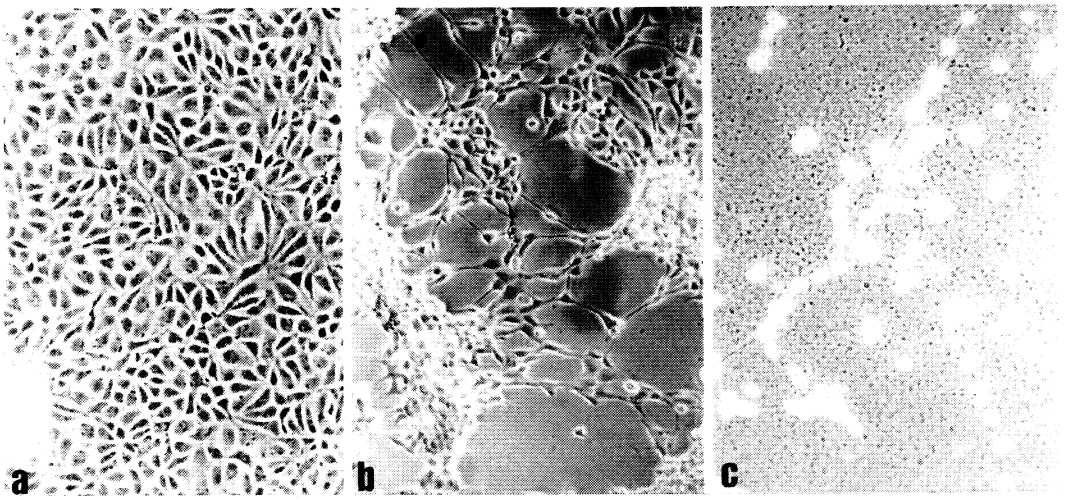



FIG. 1. Cytotoxic effects in CHSE cells. Control (a), inoculated cells showing vesicles (b) and glomerular formations (c).

expressed as $LD_{50}$ for fish (9, 20). Santos et al. (19) reported that ECP from *Aeromonas* spp. caused cytotoxic effects in fish cell lines regardless of the virulence capabilities of the strains. However, we observed that those strains, the diluted 1:10 ECP of which caused cytotoxic effects in the three fish cell lines, had $LD_{50}$ lower than 30 μg/g fish.

Protease activity can be an important virulence factor of *Vibrio* strains related to diseases of *S. aurata*, since these factors cause massive tissue damage in the host, which enables the bacteria to enter the host cell (12, 18).

Finally, although the degree of bacterial pathogenicity often depends on the production of tissue damaging hydrolytic enzymes, this is not the only virulence factor and we noticed that for some strains there is no correlation between the $LD_{50}$ values obtained for the whole bacterial cells and for the isolated ECP. The fact that some strains with low $LD_{50}$ for cells do not produce death when their ECP are injected in fish suggests that either not all the toxins are produced in laboratory conditions or that the presence of virulence mechanisms in the cells different from the toxins is necessary for the pathogenicity.

The results obtained in this study indicate that, according to several studies in other species (14), the mechanism of pathogenicity must be multifactorial, the hydrolytic activities of the different *Vibrio* species being an important factor in the virulence for *S. aurata*, a fish in which most of the reported diseases have been associated with adverse culture conditions. These conditions could affect fish defences and facilitate the entry of bacteria due to their hydrolytic activities. Therefore, further studies must be carried out in order to determine cellular virulence factors of these strains.

## Acknowledgements

We thank Dr. A. E. Toranzo and Dr. J. L. Barja from Universidad de Santiago (Spain) for supplying some fish cell lines, and M. J. Navarrete for her assistance in the English revision of the manuscript.

## References


1. Amaro, C., Biosca, E. G., Esteve, C., Fouz, B., Toranzo, A. E. (1992). Comparative study of phenotypic and virulence properties in *Vibrio vulnificus* biotypes 1 and 2 obtained from a European eel farm experiencing mortalities. Dis. Aquat. Org. **13**, 29–35.
2. Balebona, M. C. (1994). Caracterización y mecanismos de virulencia de cepas del género *Vibrio* aisladas de patologías de doradas (*Sparus aurata* L.) cultivadas. Tesis Doctoral, Universidad de Málaga.
3. Balebona, M. C., Moriñigo, M. A., Faris, A., Krovacek, K., Mansson, I., Borrego, J. J. (1995). Influence of salinity and pH on the adhesion of pathogenic *Vibrio* strains to *Sparus aurata* skin mucus. Aquaculture **132**, 113–120.
4. Bradford, N. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Ann. Biochem. **72**, 248–254.
5. Campbell, C. M., Duncan, D., Price, N. C., Stevens, L. (1990). The secretion of amylase, phospholipase and protease from *Aeromonas salmonicida*, and the correlation with membrane-associated ribosomes. J. Fish Dis. **13**, 463–474.
6. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F. (1956). Colorimetric method for determination of sugar related substances. Ann. Chem. **28**, 350–356.
7. Duncombe, W. G. (1963). The colorimetric micro-determination of long-chain fatty acids. Biochem. J. **88**, 7–10.
8. Ellis, A. E. (1991). An appraisal of the extracellular toxins of *Aeromonas salmonicida* ssp. *salmonicida*. J. Fish Dis. **14**, 265–277.
9. Fouz, B., Barja, J. L., Amaro, C., Rivas, C., Toranzo, A. E. (1993). Toxicity of the extracellular products of *Vibrio damsela* isolated from diseased fish. Curr. Microbiol. **27**, 341–347.
10. Inamura, H., Muroga, K., Nakai, T. (1984). Toxicity of extracellular products of *Vibrio anguillarum*. Fish Pathol. **19**, 89–96.
11. Kawahara, E., Oshima, S., Nomura, S. (1990). Toxicity and immunogenecity of *Aeromonas salmonicida* extracellular products to salmonids. J. Fish Dis. **13**, 495–503.
12. Kothary, M. H., Kreger, A. S. (1985). Production and partial characterization of an elastolytic protease of *Vibrio vulnificus*. Infect. Immun. **50**, 534–540.
13. Liu, P. V. (1957). Survey of hemolysin production among species of *Pseudomonads*. J. Bacteriol. **74**, 718–727.
14. McGarey, D. J., Milanesi, L., Foley, D. P., Reyes Jr., B., Frye, L. C., Lim, D. V. (1991). The role of motile aeromonads in the fish disease, Ulcerative Disease Syndrome (UDS). Experientia **47**, 441–444.
15. Milton, D. L., Norqvist, A., Wolf-Watz, H. (1992). Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum*. J. Bacteriol. **174**, 7235–7244.
16. Miyoshi, N., Shimizu, C., Miyoshi, S. I. (1987). Purification and characterization of *Vibrio vulnificus* protease. Microbiol. Immun. **31**, 13–25.
17. Nieto, T. P., Ellis, A. E. (1991). Heterogeneity of extracellular proteases produced by different isolates of *Aeromonas hydrophila* and *A. sobria* pathogenic for fish. J. Fish Dis. **14**, 229–235.
18. Nishina, Y., Miyoshi, S. I., Nagase, A., Shinoda, S. (1992). Significant role of an extracellular protease in utilization of heme by *Vibrio vulnificus*. Infect. Immun. **60**, 2128–2132.
19. Santos, Y., Toranzo, A. E., Dopazo, C. P., Nieto, T. P., Barja, J. L. (1987). Relationship among virulence for fish, enterotoxigenicity, and phenotypic characteristics of motile *Aeromonas*. Aquaculture **67**, 29–39.
20. Toranzo, A. E., Barja, J. L., Colwell, R. R., Hetrick, F. M., Crosa, J. H. (1983). Haemagglutinating haemolytic and cytotoxic activities of *Vibrio anguillarum* and related vibrios isolated from striped bass on the Atlantic coast. FEMS Microbiol. Lett. **18**, 257–262.
21. Toranzo, A. E., Santos, Y., Lemos, M. L., Ledo, A., Bolinches, J. (1987). Homology of *Vibrio anguillarum* strains causing epizootics in turbot, salmon and trout reared on the Atlantic coast of Spain. Aquaculture **67**, 41–52.

MICROBIOLOGÍA SEM **11** (1995), 447–454

# Study of a hydrocarbon-utilizing and emulsifier-producing *Acinetobacter calcoaceticus* strain isolated from heating oil

M. Mercedes Marín, Ana M. Pedregosa, M. Luisa Ortiz, Fernando Laborda*

*Departamento de Microbiología y Parasitología, Universidad de Alcalá de Henares, Alcalá de Henares, España*

Received 17 July 1995/Accepted 6 October 1995

**Summary**

Twenty bacterial strains were isolated from a sample of contaminated heating oil and screened for their ability to use petroleum and several common fuels as the sole source of carbon and energy. One of the isolates, named MM5, was able to grow on petroleum derivatives and brought about an emulsification of those compounds. Gas chromatography studies showed that strain MM5 was able to degrade hydrocarbons of heating oil. MM5 has been tentatively identified as a strain of *Acinetobacter calcoaceticus*. The fine structure of MM5 was examined by transmission electron microscopy. Incubation in the presence of hydrocarbon substrates resulted in the development of intracellular electron-transparent inclusions. These structures were absent in the non-hydrocarbon cultures studied.

**Key words**: petroleum microbiology, *Acinetobacter calcoaceticus*, heating oil biodegradation, bioremediation, bioemulsifier

**Resumen**

Se aislaron veinte cepas de bacterias aeróbicas a partir de una muestra de gasóleo de calefacción alterado, determinándose su capacidad de utilizar petróleo y distintos carburantes como única fuente de carbono y energía. Una de las cepas aisladas, denominada MM5, mostró un crecimiento significativo

* *Correspondence to*: Fernando Laborda. Departamento de Microbiología y Parasitología. Universidad de Alcalá de Henares. Carretera de Madrid-Barcelona, km 33. 28871 Alcalá de Henares. España. Tel.: +34-1-8854621. Fax: +34-1-8854621. E-mail: mplaborda@alcala.es

sobre derivados petrolíferos, siendo además capaz de producir una emulsificación de dichos productos en el medio. Estudios realizados mediante cromatografía de gases mostraron que la cepa MM5 es capaz de degradar en gran medida los hidrocarburos presentes en gasóleo de calefacción. MM5 fue identificada como una cepa de *Acinetobacter calcoaceticus*. Durante el crecimiento del microorganismo en presencia de hidrocarburos se observó, mediante microscopía electrónica de transmisión, la aparición de inclusiones intracelulares transparentes a los electrones, que no se detectaron durante la incubación de la bacteria sobre substratos solubles en agua.

## Introduction

Different microorganisms, isolated from several sources, use hydrocarbons as the sole source of carbon and energy (16). Several stimuli have prompted the study of numerous aspects of the hydrocarbon microbiology. Problems of biodeterioration of petroleum products, such as fuels, lubricating and oil emulsions appear to be increasing, with obvious economic implications (17). Besides, the increase of contamination in soil and water by petroleum and its derivates has stressed the need for suitable remedial measures. Biodegradation of hydrocarbons by microorganisms represents one of the primary mechanisms by which these pollutants could be eliminated from the environment (13). Restoration of oil-polluted areas by microbial degradative activities (bioremediation) is slow and has its limitations, mainly in open systems such as the sea. However, this process is usually a less costly cleanup alternative, as well as a less destructive one from an environmental point of view, mostly in closed systems, e.g. soil and storage tanks (2).

In addition, considerable attention must be focused on the possibility of exploiting the ability of certain microorganisms to produce biotechnological products related to hydrocarbon use or biotransformation. Many hydrocarbon-degrading microorganisms produce emulsifying agents (bioemulsifiers) that make the use of hydrocarbons easy (9). In some cases these products have proven to be useful additives in the field of bioremediation (6). Other potential applications of bioemulsifiers include their use in industry, agriculture, medicine and cosmetics to replace synthetic surfactants, and to enhance the recovery of oil from reservoir rock pools (18).

The isolation and identification of a new bacterial strain that grows on petroleum derivatives and emulsifies hydrocarbons has been carried out. We also report the ultrastructural peculiarities of this microorganism when growing on hydrocarbon substrates.

## Materials and methods

**Isolation of microorganisms from heating oil.** The strains of bacteria used in the present study were isolated from samples of altered heating oil that were collected in a storage tank. Two phases were distinguished in the samples: an upper hydrocarbon layer and a lower aqueous phase, containing a dark sediment. Pure cultures of microorganisms were obtained by the modified procedure of Herbert et al. (10). Aliquots of the two phases of the sample were filtered through sterile 0.22 μm pore size membranes (Millipore). Filters from the oily phase were washed with pentane and 0.9% NaCl

and those from the aqueous phase were washed with the saline solution and both were placed on the surface of solid media.

Isolation of microorganisms was carried out on nutrient agar (NA) or malt extract, yeast extract, peptone, glucose agar (MYPGA). Plates were incubated for 7 days at 28°C. The bacterial isolates were named as MM. Stock cultures were maintained on NA.

**Selection of hydrocarbon degrading bacteria**. Screening of the bacterial isolates for growth on hydrocarbons was carried out in a liquid mineral medium (4) (BH medium), supplemented with 0.01% yeast extract as organic nitrogen source and containing 1% of one of the following carbon sources: crude oil (constituted by a complex mixture of hydrocarbons, with a wide range of chain lengths), and several commercial fuels: gasoline and unleaded gasoline (mainly constituted by linear hydrocarbons with 4 to 10 carbon atoms), jet fuel ($C_{10}$ to $C_{14}$) and diesel oil, heating oil and heating oil without additives (with hydrocarbons ranging in general from $C_{14}$ to $C_{20}$). A medium containing heating oil without additives and glucose was used to determine the tolerance of microorganisms to hydrocarbons. Bacteria were also grown on glucose for comparative purposes. Tubes with 10 ml of those media were inoculated with the bacteria and incubated at 28°C for 20 days with occasional agitation. Microbial growth in each medium was monitored by visual comparison with an uninoculated medium and with a medium without any carbon source but inoculated with the microorganism, which served as controls. The emulsification of the hydrocarbons was determined by examining the presence of droplets of emulsion in the cultures.

**Heating oil degradation by bacteria**. The bacterial isolate MM5 was selected for further studies. To have a comparative estimate of the potential of this microorganism to degrade hydrocarbons, the strain MM5, a mixture of the bacterial strains isolated from heating oil or a mixture of bacterial isolates except strain MM5 were incubated on BH medium. Heating oil without additives (0.5%) was used as carbon source. An uninoculated medium served as a control. Incubations were carried out in 500 ml Erlenmeyer flasks containing 100 ml of the medium, at 28°C in an orbital shaker operating at 200 rpm. Samples were taken after 20 days of incubation.

The residual heating oil was recovered by extracting with hexane (5). After volatilizing the solvent, the heating oil was redissolved in hexane (1:9). Then, 1 µl of the solution was analysed by gas chromatography (GC) using a Hewlett Packard 5890 instrument equipped with a flame ionization detector. An Ultra 1 capillary column (25 m long × 0.2 mm diameter) was used. Operational temperature ranged from 80 to 280°C, raising the temperature 8°C $min^{-1}$. Helium was used as a carrier gas.

**Taxonomic study of the bacterial strain MM5**. Taxonomic characterization of the strain MM5 was carried out following the procedures recommended by Juni (11), Bouvet and Grimont (3) and Towner (22). Tests were also done by using commercial API 20 NE galleries (API System, BioMérieux).

**Microscopical study of the bacterial strain MM5**. For transmission electron microscopy studies, strain MM5 was grown in the liquid BH medium containing 1% of one of the following carbon sources: crude petroleum, heating oil without additives and tetradecane. Bacteria incubated in BH medium with 1% yeast extract or on NA were used for comparative purposes. Bacterial cells were prepared for TEM as previously reported (14).

TABLE 1. Growth of the bacterial strains isolated from altered heating oil on fuels and crude oil

| Strain | Carbon source | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | GWP | GAS | JET | HWA | HEO | DIO | CRO | HPG | GLU |
| MM1 | ± | ± | +E | ± | ± | + | + | ++E | ++ |
| MM2 | – | – | – | – | – | – | – | ++ | ++ |
| MM3 | – | – | +E | ± | – | ± | ± | ++E | ++ |
| MM4 | – | – | – | – | – | – | ± | ++E | ++ |
| MM5 | – | – | ± | ++E | ++E | ++E | ++E | ++E | – |
| MM6 | – | – | – | – | – | ± | + | ++E | ++ |
| MM7 | ± | ± | + | – | – | ± | + | ++ | ++ |
| MM8 | ± | ± | +E | ± | ± | ± | + | ++E | ++ |
| MM9 | ± | ± | + | ± | ± | – | + | ++ | ++ |
| MM10 | – | – | – | – | – | – | – | + | + |
| MM11 | – | – | ± | – | – | – | – | ++ | ++ |
| MM12 | ± | ± | – | – | – | – | – | ++ | ++ |
| MM13 | – | – | – | – | – | – | – | ++ | ++ |
| MM14 | – | – | – | – | – | – | – | ++ | ++ |
| MM15 | – | – | – | – | – | – | – | ++ | ++ |
| MM16 | ± | – | + | – | – | ± | – | ++ | ++ |
| MM17 | ± | ± | + | – | – | – | ± | ++ | ++ |
| MM18 | – | – | – | – | – | – | – | ± | + |
| MM19 | – | ± | – | – | – | – | – | ++ | ++ |
| MM20 | ± | ± | ± | ± | ± | ± | ± | ++ | ++ |

Symbols: –, negative; ±, weak; +, positive; ++, strongly positive.

Abbreviations: E, emulsification of the hydrocarbons in the culture medium; GWP, unleaded gasoline; GAS, gasoline; JET, jet fuel; HWA, heating oil without additives; HEO, heating oil; DIO, diesel oil; CRO, crude oil; HPG, heating oil plus glucose; GLU, glucose.

## Results

**Isolation of microorganisms from heating oil.** Fifty microbial strains were isolated from samples of altered heating oil that contained an upper oily phase and a lower aqueous phase. After a microscopical examination they corresponded to: bacteria, 20; filamentous fungi, 8; and yeast, 22. Only one fungal isolate was obtained from the hydrocarbon layer, whereas the aqueous phase contained all the microorganisms isolated. The strains of bacteria, named MM1 to MM20 were used for further studies.

**Selection of hydrocarbon-utilizing and emulsifier-producing bacteria.** Bacterial isolates were tested in liquid cultures to establish what particular organisms were able to use hydrocarbons as the sole

carbon source. The results obtained are shown in Table 1. A medium with glucose was used for comparative purposes. Only MM5 isolate failed to grow on this sugar. All the bacteria tolerated the presence of hydrocarbons as they were able to grow in a medium with glucose and diesel oil without additives. When gasoline was used as the sole carbon source, a slight growth of only a few bacteria was found. Although the bacterial strain MM5 failed to grow on gasoline, it was able to grow significantly on diesel oil, heating oil and crude oil, and to carry out a strong emulsification of these products in the culture medium. This strain was selected for further studies.

**Heating oil degradation by bacteria.** Gas chromatography was used to determine the capability of the bacterial strains isolated from altered heating oil to degrade hydrocarbons. GC studies showed that strain MM5 was able to degrade hydrocarbons (mainly n-alkanes) of heating oil (Fig. 1). MM5 was the main hydrocarbon-degrading isolate. Thus, the chromatographic profiles of the heating oil incubated for 20 days with a mixture of isolated bacteria except MM5 were similar to the sterile controls shown in Fig. 1A. In the same way, gas chromatograms of heating oil were similar both when the strain MM5 was incubated alone (Fig. 1B) and mixed with the other bacterial isolates.

**Characteristics of the bacterial isolate MM5.** Colonies on NA were circular, convex, smooth, brilliant, cream-white and with slightly irregular margins. Cells were Gram-negative coccobacilli, strictly aerobic, oxidase negative, catalase positive, gelatinase negative, non motile, and did not form spores. This strain did not use the following carbohydrates: glucose, arabinose, mannose and maltose as carbon and energy sources. However, it used DL-β-OH-butyrate, 2,3-butanediol, DL-lactate, citrate, aspartate and histidine. Furthermore, bacterial growth was resistant to 100 U $ml^{-1}$ penicillin-G.

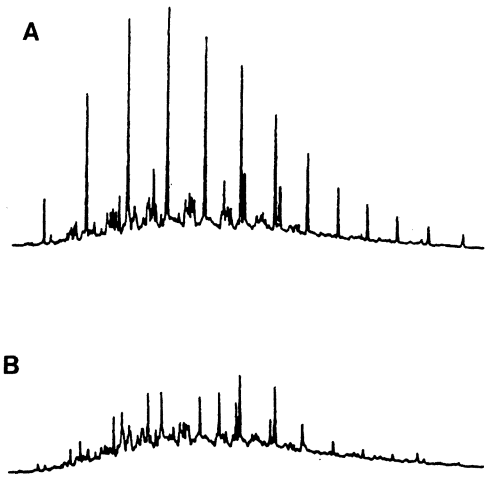



FIG. 1. Gas chromatograms of heating oil without additives: (A) GC of uninoculated control; (B) GC after 20 days incubation with the bacterial strain MM5.

**Ultrastructure of the bacterial isolate MM5.** Cells grown on hydrocarbons as the sole energy and carbon source contained electron transparent inclusions. Numerous inclusions, which were predominantly located around the periphery of the cell, were evident during the growth of strain MM5 on heating oil without additives (Fig. 2A) or crude oil (Fig. 2B). In contrast, when strain MM5 was incubated on pure hydrocarbons, such as tetradecane, the microorganisms contained fewer large inclusions (Fig. 2C). These inclusions were not evident in bacteria when grown on nutrient agar or in a salt medium with yeast extract as carbon source (Fig. 2D).

## Discussion

Bacteria and fungi were isolated from an altered heating oil, which contained an aqueous layer under the hydrocarbon phase. Microbial contamination in fuels is a known problem. Microorganisms are usually present in stored fuels. When the product comes into contact with water, microbial biomass develops (7) and can cause problems, such as a subsequent blockage of the fuel distribution lines, and corrosion and changes in fuel characteristics, which may affect its combustion properties (21). An

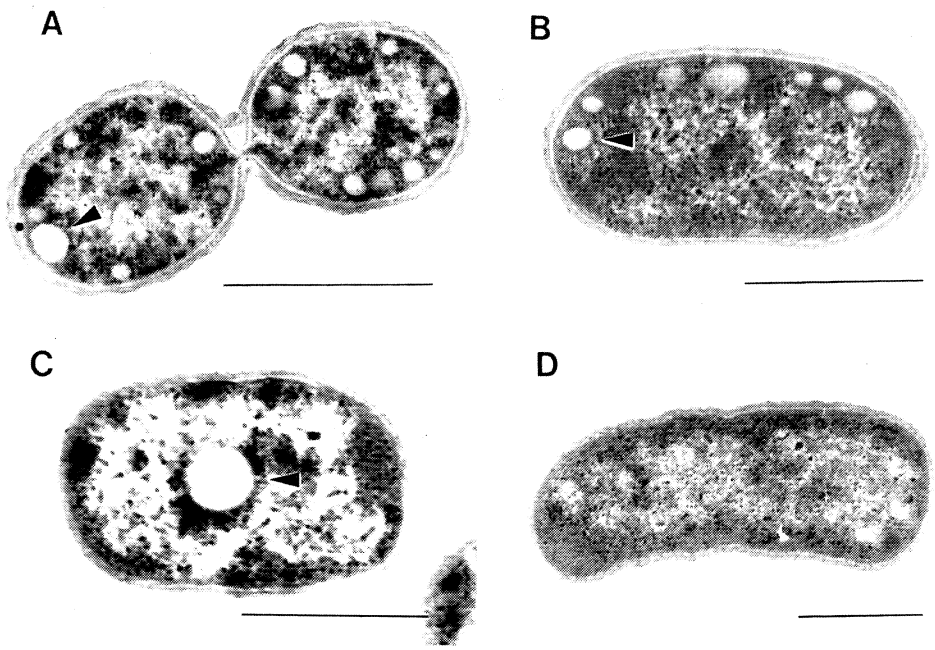



FIG. 2. Thin section of the bacterial strain MM5 grown on diesel oil without additives (A), crude oil (B), tetradecane (C) or nutrient agar (D). The arrowheads in A, B and C show the electron transparent inclusions observed during growth on hydrocarbons. Scale bars = 0.5 μm.

increase of bacteria as fuel contaminants has been reported, in contrast to the previously predominant fungi (21). Studies were conducted to select bacteria capable of using hydrocarbons. All the bacteria isolated from heating oil tolerated the presence of hydrocarbons. The strains that were not capable of using petroleum derivatives as the only carbon and energy source could grow in the altered fuel as contaminants on the organic matter produced by the hydrocarbon degraders or, eventually, on the grit that becomes entrapped when biomass accumulates. One of the bacterial isolates, named MM5, grew significantly on petroleum and several common fuels. During the growth on heating oil, strain MM5 was able to degrade hydrocarbons (mainly n-alkanes) of this fuel, as was assessed by gas chromatography. Gaseous and liquid hydrocarbons are the most readily degraded by microorganisms, but liquids of lower molecular weight are in general inhibitory to hydrocarbon-utilizing microorganisms, by virtue of their solvent effect (1). This explained why the MM5 strain was not able to grow on gasoline. The growth of MM5 on petroleum derivatives was usually accompanied by their emulsification. The characterization of an extracellular emulsifier produced by the strain MM5 and a microscopical study of the emulsions formed during the growth on hydrocarbons are reported elsewhere (14). The production of emulsifiers has been reported for other hydrocarbon utilizing microorganisms. Since the most significant property of hydrocarbons with respect to their utilization as metabolic substrates is their low solubility in water, the production of emulsifiers renders such molecules more accessible to cells (15, 19).

Based on the taxonomic properties described and according to Bergey's Manual (11), the bacterial isolate MM5 has been considered to be a strain of *Acinetobacter calcoaceticus*, and could be included in the phenotypic group A2 of this species. According to further revisions of the taxonomy of the genus *Acinetobacter* (3, 8, 22) the phenotypic characteristics studied suggest that MM5 is a member of the genospecies 11. Several strains of *Acinetobacter calcoaceticus* have been isolated from natural environments and shown to grow on hydrocarbons and to produce extracellular emulsifying activity (19).

Transmission electron microscopy has shown that the growth of MM5 at the expense of hydrocarbon substrates was characterized by the presence of intracellular electron transparent inclusions. Similar structures have been illustrated in several hydrocarbon-oxidizing microorganisms (20) and have been documented as hydrocarbon pools in a strain of *Acinetobacter* (12). However, further studies need to be made to establish a functional correlation between internal electron transparent structures observed in the bacterial isolate MM5 and the oxidation of hydrocarbons.

Results of the present work shown the ability of *A. calcoaceticus* MM5 to degrade hydrocarbons. Since bioremediation of petroleum pollution is currently an important practical application of petroleum biotechnology, experiments using the potential biodegradative characteristics of this microorganism in the treatment of hydrocarbon wastes are in progress in our laboratory.

## References

1. Asperger, O., Kleber, H. P. (1991). Metabolism of alkanes by *Acinetobacter*. *In* Towner, K. J., Bergogne-Bérézin, E., Fewson, C. A. (ed.), The Biology of *Acinetobacter*, pp. 323–350. Plenum Press, New York, NY.
2. Atlas, R. M., Bartha, R. (1992). Hydrocarbon biodegradation and oil spill bioremediation. *In* Marshall, K. C. (ed.), Advances in Microbial Ecology, vol. 12, pp. 287–338. Plenum Press, New York, NY.

3. Bouvet, P. J. M., Grimont, P. A. D. (1986). Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. Int. J. Syst. Bacteriol. **36**, 228–240.
4. Bushnell, L. D., Haas, H. F. (1941). The utilization of certain hydrocarbons by microorganisms. J. Bacteriol. **41**, 653–673.
5. Dott, W., Feidieker, D., Kämpfer, P., Scleibinger, H., Strechel, S. (1989). Comparison of autochthonous bacteria and commercially available cultures with respect to their effectiveness in fuel oil degradation. J. Indust. Microbiol. **4**, 365–367.
6. Finnerty, W. R. (1994). Biosurfactants in environmental biotechnology. Curr. Opin. Biotechnol. **5**, 291–295.
7. Genner, C., Hill, E. C. (1981). Fuels and oils. *In* Rose, A. H. (ed.), Microbial Biodeterioration, pp. 260–306. Academic Press, London.
8. Grimont, P. A. D., Bouvet, P. J. M. (1991). Taxonomy of *Acinetobacter*. *In* Towner, K. J., Bergogne-Bérézin, E., Fewson, C. A. (ed.), The Biology of *Acinetobacter*, pp. 25–36. Plenum Press, New York, NY.
9. Gutnick, D. L., Shabtai, Y. (1987). Exopolysaccharide bioemulsifiers. *In* Kosaric, N., Cairns, W. L., Gray, N. C. C. (ed.), Biosurfactants and Biotechnology, pp. 211–246. Marcel Dekker, New York, NY.
10. Herbert, B. N., Hill, E. C., Oates, P. D., Powell, D., Shennan, J. L., Whittle, R. (1987). A method for testing light distillated fuels. *In* Hopton, A., Hill, E. C. (ed.), Industrial Microbiological Testing, pp. 215–219. Blackwell, Oxford.
11. Juni, E. (1984). Genus III. *Acinetobacter* Brisou et Prévot 1954. *In* Krieg, N. R., Holt, J. G. (ed.), Bergey's Manual of Systematic Bacteriology, vol. 1, pp. 303–307. Williams & Wilkins, Baltimore, MD.
12. Kennedy, R. S., Finnerty, W. R., Sudarsanan, K., Young, R. A. (1975). Microbial assimilation of hydrocarbons, I. The fine structure of a hydrocarbon oxidizing *Acinetobacter* sp. Arch. Microbiol. **102**, 75–83.
13. Leahy, J. G., Colwell, R. R. (1990). Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. **54**, 305–315.
14. Marín, M., Pedregosa, A., Laborda, F. (1995). Emulsifier production and microscopical study of emulsions and biofilms formed by the hydrocarbon-utilizing bacteria *Acinetobacter calcoaceticus* MM5. Appl. Microbiol. Biotechnol., in press.
15. Ratledge, C. (1988). Products of hydrocarbon-microorganisms interaction. *In* Houghton, D. R., Smith, R. N., Eggins, H. O. W. (ed.), Biodeterioration 7, pp. 219–236. Elsevier, London.
16. Rosenberg, E. (1992). The hydrocarbon-oxidizing bacteria. *In* Balows, A., Trüper, H. G., Dworkin, M., Harder, W., Schleifer, H. K. (ed.), The Prokaryotes: A Handbook on the Biology of Bacteria, vol I, pp. 446–459. Springer-Verlag, New York, NY.
17. Rosenberg, E., Legmann, R., Kushmaro, A., Taube, R., Adler, E., Ron, E. Z. (1992). Petroleum bioremediation-a multiphase problem. Biodegradation **3**, 337–350.
18. Rosenberg, E. (1993). Exploiting microbial growth on hydrocarbons-new markets. Trends Biotechnol. **11**, 419–424.
19. Sar, N., Rosenberg, E. (1983). Emulsifier production by *Acinetobacter calcoaceticus* strains. Curr. Microbiol. **9**, 309–314.
20. Scott, C. C. L., Finnerty, W. R. (1976). Characterization of intracytoplasmic hydrocarbon inclusions from the hydrocarbon-oxidizing *Acinetobacter* species HO1-N. J. Bacteriol. **127**, 481–489.
21. Shennan, J. L. (1988). Control of microbial contamination of fuels in storage. *In* Houghton, D. R., Smith, R. N., Eggins, H. O. W. (ed.), Biodeterioration 7, pp. 248–255. Elsevier, London.
22. Towner, K. J. (1992). The genus *Acinetobacter*. *In* Balows, A., Trüper, H. G., Dworkin, M., Harder, W., Schleifer, K. M. (ed.), The Prokaryotes: A Handbook on the Biology of Bacteria, vol. IV, pp. 3137–3143. Springer-Verlag, New York, NY.

MICROBIOLOGÍA SEM **11** (1995), 455–460

# Flow cytometry determination of acute physiological changes in a marine diatom stressed by copper

Ángeles Cid, Pablo Fidalgo, Concepción Herrero, Julio Abalde*

*Laboratorio de Microbiología, Departamento de Biología Celular y Molecular, Facultad de Ciencias, Universidad de La Coruña, La Coruña, España*

Received 27 March 1995/Accepted 9 October 1995

**Summary**

Flow cytometry (FCM) was used to determine changes in cellular volume, transmembrane potential, mitochondrial membrane potential and intracellular pH in the marine microalga *Phaeodactylum tricornutum* immediately (5 to 10 seconds) after the addition of selected concentrations of copper. An acute increase in the forward scatter signal of this diatom was detected after the addition of 10 mg $l^{-1}$ of copper. Stress produced by the copper addition resulted in various physiological alterations that can be easily and quickly detected by FCM: (i) the hyperpolarization of the cell membrane, as a result of an immediate increase in the cytoplasmic membrane potential, (ii) the increase of the mitochondrial membrane potential, being maximum at the higher copper concentration assayed, and (iii) the increase in the intracellular pH with the highest copper concentration assayed (10 mg $l^{-1}$).

**Key words**: *Phaeodactylum tricornutum*, flow cytometry, forward scatter, membrane potential, intracellular pH

**Resumen**

Se ha utilizado la citometría de flujo (CMF) para determinar cambios en el volumen celular, potenciales de membrana citoplasmática y mitocondrial y pH intracelular de la diatomea marina *Phaeodactylum tricornutum*, inmediatamente después de la adición de determinadas concentraciones

* *Correspondence to*: Julio Abalde. Laboratorio de Microbiología. Departamento de Biología Celular y Molecular. Facultad de Ciencias. Universidad de La Coruña. Campus da Zapateira s/n. 15071 La Coruña. España. Tel.: +34-81-280788. Fax: +34-81-105641. E-mail: bmjabald@udc.es

de cobre (5 a 10 segundos). Se ha detectado por este método un inmediato aumento de la señal de "forward scatter" de esta diatomea después de la adición de 10 mg $l^{-1}$ de cobre. El estrés producido por la adición de cobre provocó varias alteraciones fisiológicas que se detectaron fácil y rápidamente por CMF: (i) la hiperpolarización de la membrana celular, como resultado de un incremento inmediato del potencial de membrana citoplasmática, (ii) el aumento del potencial de membrana mitocondrial, siendo máximo con las concentraciones de cobre más altas de las ensayadas, y (iii) el aumento del pH intracelular con la mayor concentración de cobre ensayada (10 mg $l^{-1}$).

## Introduction

Flow cytometry (FCM) is now being put to extensive use in the study of microalgae and has been introduced as an alternative to more traditional techniques of analyzing cells in culture and from natural populations (16).

The FCM determination of cellular constituents has been in use for many years, e.g. for DNA distributions, cellular antigen contents or natural pigment analysis, like chlorophylls or phycobiliproteins (4). Biophysical measurements of electrical or optical cell volume (forward scatter, FSC) and optical granularity (side scatter, SSC) are used in addition for cell characterization. Although very useful in many cases, such measurements do not make possible to follow functional changes, like e.g. changes of intracellular pH, of cellular excitation or energy production. It is important to realize that in many cases cells react by the alteration of functions and not by alterations of constituents. Therefore, the knowledge of constituents does not exhaustively describe cells (12).

The determination of cell function or physiology has been difficult and limited in the past because microelectrodes had to be introduced in single cells, e.g. for transmembrane potential or pH-measurements (10). Cell functions of freshwater and marine microorganisms can quickly be determined by flow cytometry with a multitude of biochemically specific, non toxic and fluorescent indicator molecules at conditions close to the "in vivo" state without prolonged exposure to unusual light levels.

In the present work, some acute changes of the marine diatom *Phaeodactylum tricornutum* stressed by the presence of copper were studied, using the flow cytometric functional analysis to study different aspects of cell physiology. The parameters evaluated by flow cytometry were: forward scatter signal (related to cellular volume), transmembrane potential, mitochondrial membrane potential and intracellular pH.

## Materials and methods

The marine microalga *Phaeodactylum tricornutum* Bohlin (Bacillariophyceae) was cultured in batch conditions, previously described (2). Stress was produced by copper addition and changes in forward scatter, cytoplasmic and mitochondrial membrane potentials and intracellular pH were determined using flow cytometric protocols. Cellular volume was studied in function of the intensity of laser light scattered in the forward direction, FSC in flow cytometric terms (12).

The examination of the remaining parameters required the use of different specific fluorochromes, with a stable fluorescent emission in seawater. After blue light excitation, all the fluorochromes assayed showed a green fluorescent emission. Staining protocols were adapted to *P. tricornutum* cells. The variations in cytoplasmic membrane potential were measured using 3,3-dihexyl-oxacarbocyanine (abbreviated $DiOC_6(3)$; Sigma Chemical Co., St. Louis, MO, USA) (9), dissolved in ethanol and diluted in dimethyl-formamide (DMF; Sigma Chemical Co.), at a final concentration in culture of 0.35 µM.

Changes in mitochondrial membrane potential were measured using a mitochondrial specific fluorochrome, rhodamine 123 (Rh123; Sigma Chemical Co.) (3), dissolved in DMF; final concentration in culture was 10 µg $ml^{-1}$.

The flow cytometric protocol used for the determination of variations in the intracellular pH is based on the pH-dependent emission intensity of a fluorescein derivate, 2'-7'-dichlorofluorescein (DCF) (15); final DCF concentration achieved was 20 µM.

Flow cytometry determinations were carried out before and immediately after the addition of different copper concentrations. Aliquots of microalgal cultures in logarithmic phases were collected and, after staining with the corresponding fluorochrome, 2500 cells were analyzed in a FACScan flow cytometer (Becton Dickinson Instruments, San Jose, CA, USA), equipped with an argon-ion laser (blue light, 488 nm); these cells were used as control in each case. Different stock solutions of copper were prepared by dissolving $CuCl_2$ in redistilled and sterilized water; a constant volume (5 µl) of the different stock copper solutions was added to 500 µl of microalgal culture to obtain final copper concentrations of 2.5, 5 and 10 mg Cu $l^{-1}$. Immediately (5 to 10 seconds) after copper addition, at least 5000 cells were analyzed in the flow cytometer. Another control analysis was carried out adding 5 µl of redistilled water to 500 µl of culture medium in order to verify that the variations observed were due to the effect of copper and not to water.

Experiments were carried out in triplicate and data obtained by flow cytometry were statistically analyzed by the instrument software (LYSIS II program; Becton Dickinson Instruments).

## Results and discussion

The addition of 10 mg $l^{-1}$ of copper to the cultures provoked an increase in the forward scatter signal (related to cell volume) of the marine diatom *Phaeodactylum tricornutum* (Fig. 1), while the addition of 2.5 and 5 mg $l^{-1}$ did not provoke significant differences. The mean forward scatter expressed as the mean channel number was 237 in control cells without copper, increasing to 289 after the addition of 10 mg $l^{-1}$ of copper (Fig. 1). Slower increases in cell volume as a response to heavy metal stress have been reported for some other microalgae, but these changes were observed using optic or electron microscopy (1, 6, 14).

Flow cytometry analysis performed showed physiological changes provoked by copper stress. This copper stress, at all the concentrations assayed, produced the hiperpolarization of the cell membrane in *P. tricornutum* as a result of an increase of the cytoplasmic membrane potential, observed by the increase of the $DiOC_6(3)$ fluorescent emission (Fig. 2). Histograms show that 2.5 and 5 mg $l^{-1}$ of copper produced a lower increase of the transmembrane potential than that produced by 10 mg $l^{-1}$ of copper. The mean channel number of green fluorescence was 8 in control cells without copper, and after addition of

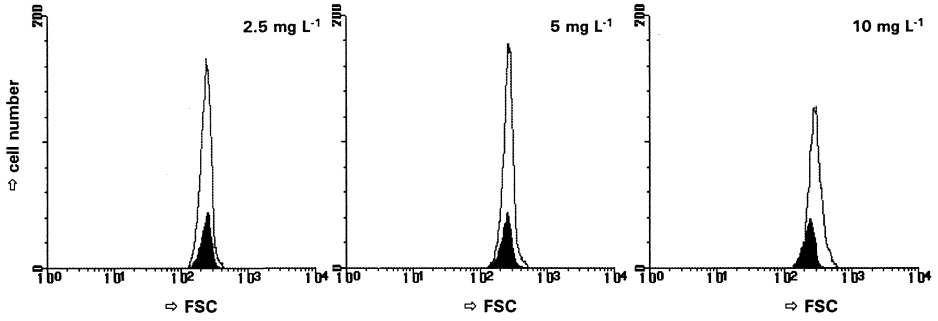



FIG. 1. Forward light scatter histograms (related to cell volume) of *Phaeodactylum tricornutum* cells without copper (■) and immediately after addition of different copper concentrations (❑).

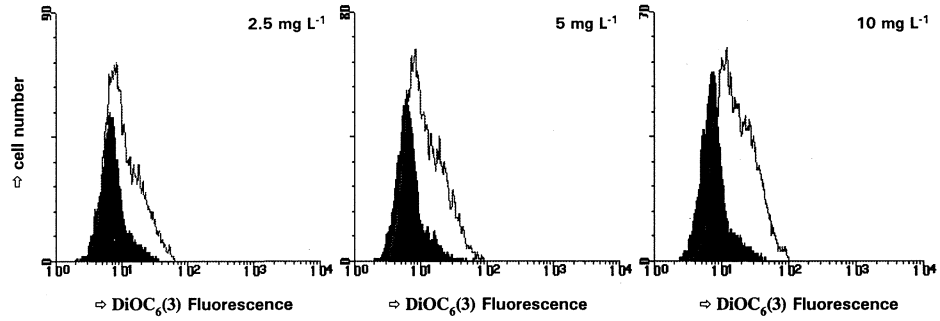



FIG. 2. $DiOC_6$ (3) fluorescence histograms (related to cytoplasmic membrane potential) of *Phaeodactylum tricornutum* cells without copper (■) and immediately after addition of different copper concentrations (❑).

copper was 13, 14 and 20 for 2.5, 5 and 10 mg $l^{-1}$, respectively (Fig. 2). Unicellular microorganisms are directly in contact with the medium, only separated by the cell membrane and the cell wall; therefore, any alteration produced in their medium provokes a first response in the microorganism at the cellular membrane level (8), that can easily be detected by flow cytometry.

The mitochondrial membrane potential increased after copper stress for all the concentrations assayed, being maximum at the higher concentration (Fig. 3). Rh123-stained cells have been used in flow cytometric studies to analyse mitochondrial activity, since the preferential accumulation of the dye in mitochondria depends on the mitochondrial membrane potential (11, 13). Therefore, *P. tricornutum* cells increase their mitochondrial activity a few seconds after addition of the different copper concentrations assayed.

The highest copper concentration assayed (10 mg $l^{-1}$) provoked an acute increase of the intracellular pH in *Phaeodactylum tricornutum* cells evidenced by the increase of fluorescent emission of DCF (Fig. 4). The mean channel number of green fluorescence was 740 in control cells while after addition

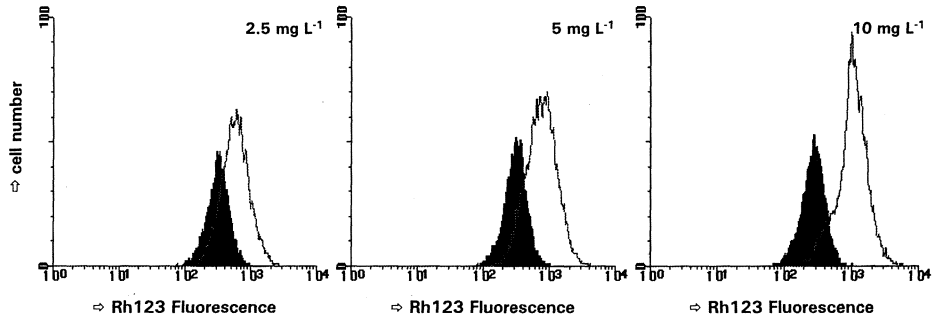



FIG. 3. Rhodamine 123 fluorescence histograms (related to mitochondrial membrane potential) of *Phaeodactylum tricornutum* cells without copper (■) and immediately after addition of different copper concentrations (❑).

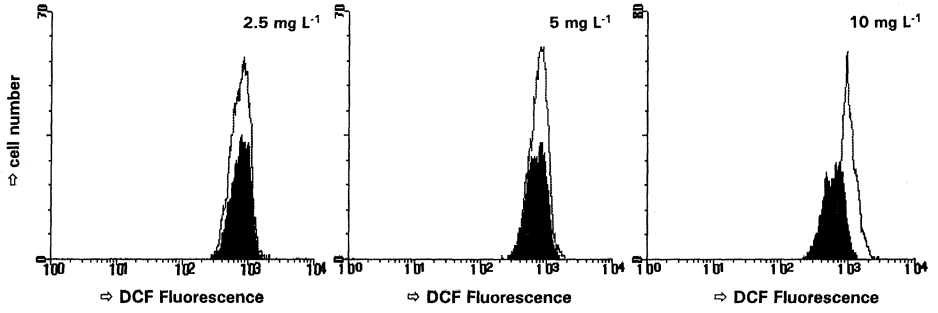



FIG. 4. Dichlorofluorescein fluorescence histograms (related to intracellular pH) of *Phaeodactylum tricornutum* cells without copper (■) and immediately after addition of different copper concentrations (❑).

of 10 mg $l^{-1}$ of copper was 1084. Cells exposed to 2.5 and 5 mg $l^{-1}$ did not show significant variations of their intracellular pH (Fig. 4). Proteins are sensitive to modifications of the proton concentration in their environment, and pH regulates the activity of key enzymes and metabolic steps (10). It seems that changes in cytoplasmic pH play an important role as second messenger in animal systems (7), but in vegetal cells the information is poor (5).

Results obtained show that flow cytometry is a very useful tool in microalgal functional studies, allowing the fast determination of cellular parameters difficult to determine by other techniques.

## Acknowledgements

This work was supported in part by a grant from Consellería de Educación e Ordenación Universitaria, Xunta de Galicia, Spain. Part of this work was supported by a grant to A. Cid from Xunta de Galicia.

## References


1. Bolaños, L., García-González, M., Mateo, P., Bonilla, I. (1992). Differential toxicological response to cadmium in *Anabaena* strain PCC7119 grown with $NO_3^-$ and $NH_4^+$ as nitrogen source. J. Plant Physiol. **140**, 345–349.
2. Cid, A., Herrero, C., Torres, E., Abalde, J. (1995). Copper toxicity on the marine microalga *Phaeodactylum tricornutum*: effects on photosynthesis and related parameters. Aquat. Toxicol. **31**, 165–174.
3. Collins, J. M., Foster, K. A. (1983). Differentiation of promyelocytic (HL-60) cells into mature granulocytes: mitochondrial-specific Rhodamine-123 fluorescence. J. Cell Biol. **96**, 94–99.
4. Cunningham, A. (1993). Analysis of microalgae and cyanobacteria by flow cytometry. *In* Lloyd, D. (ed.), Flow Cytometry in Microbiology, pp. 131–142. Springer-Verlag, Berlin.
5. Felle, H. (1989). pH as a second messenger in plants. *In* Boss, W. F., Morré, D. J. (ed.), Second Messengers in Plant Growth and Development, pp. 145–166. Alan Liss Inc., New York, NY.
6. Fisher, N. S., Jones, G. J., Nelson, D. M. (1981). Effects of copper and zinc on growth, morphology, and metabolism of *Asterionella japonica* (Cleve). J. Exp. Mar. Biol. Ecol. **51**, 37–56.
7. Frelin, C., Vigne, P., Ladoux, A., Lazdunski, M. (1988). The regulation of the intracellular pH in cells from vertebrates. Eur. J. Biochem. **174**, 3–14.
8. Gadd, G. M., Griffiths, A. J. (1978). Microorganisms and heavy metal toxicity. Microbial Ecol. **4**, 303–317.
9. Hasmann, M., Valet, G. K., Tapiero, H., Trevorrow, K., Lampidis, T. (1989). Membrane potential differences between adriamycin-sensitive and -resistant cells as measured by flow cytometry. Biochem. Pharmacol. **38**, 305–312.
10. Kurkdjian, A., Guern, J. (1989). Intracellular pH: measurement and importance in cell activity. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**, 271–303.
11. Ronot, X., Benel, L., Adolphe, M., Mounolou, J. C. (1986). Mitochondrial analysis in living cells: the use of Rhodamine 123 and flow cytometry. Biol. Cell. **57**, 1–8.
12. Shapiro, H. M. (1995). Practical Flow Cytometry, 3rd. ed. Wiley-Liss Inc., New York, NY.
13. Skowronek, P., Krummeck, G., Haferkamp, O., Rödel, G. (1990). Flow cytometry as a tool to discriminate respiratory-competent and respiratory-deficient yeast cells. Curr. Genet. **18**, 265–267.
14. Stauber, J. L., Florence, T. M. (1987). Mechanism of toxicity of ionic copper and copper complexes to algae. Mar. Biol. **94**, 511–519.
15. Vissier, J. W. M., Jongeling, A. A. M., Tanke, H. J. (1979). Intracellular pH determination by fluorescence measurements. J. Histochem. Cytochem. **27**, 32–35.
16. Yentsch, C. M., Horan, P. K. (1989). Cytometry in aquatic sciences. Cytometry **10**, 497–499.

MICROBIOLOGÍA SEM **11** (1995), 461–470

# Study of a viral-dual infection in rainbow trout (*Oncorhynchus mykiss*) by seroneutralization, Western blot and polymerase chain reaction assays

Sylvia Rodríguez, M. Pilar Vilas, Marta Alonso, Sara I. Pérez*

*Departamento de Microbiología Molecular, Centro de Investigaciones Biológicas, CSIC, Madrid, España*

Received 10 September 1995/Accepted 30 October 1995

**Summary**

Viral-dual infections in fish are of interest to aquaculture practices but they are rarely described and studied. Several methods were applied in this work to demonstrate a case of coinfection in a reared rainbow trout (*Oncorhynchus mykiss*) population. Inoculation in cell cultures and cross-neutralization tests were the standard procedures that made it possible to isolate and identify a birnavirus, the infectious pancreatic necrosis virus (IPNV), and suspect of a second virus. Western blotting with both polyclonal and monoclonal antibodies, and reverse transcriptional-polymerase chain reaction (RT-PCR) demonstrate coexistence of both, IPNV and a rhabdovirus.

**Key words**: viral coinfections, infections pancreatic necrosis virus (IPNV), fish pathogens, *Oncorhynchus mykiss* (rainbow trout/trucha), aquaculture

**Resumen**

Las infecciones dobles en peces tienen importancia para la acuicultura, pero no existen muchas descripciones, ni están bien estudiadas. En este trabajo se aplicaron diversos métodos para demostrar un caso de coinfección en una población de trucha arco iris (*Oncorhyncus mykiss*) cultivada. Los procedimientos estándar (cultivos celulares y seroneutralización), permitieron aislar e identificar un

* *Correspondence to*: Sara I. Pérez. Departamento de Microbiología Molecular. Centro de Investigaciones Biológicas. CSIC. Velázquez, 144. 28006 Madrid. España. Tel.: +34-1-5611800. Fax: +34-1-5627518. E-mail: CIBRS2L@cc.csic.es

birnavirus, el virus de la necrosis pancreática infecciosa (IPNV), y sospechar que existía un segundo virus implicado. La técnica de "Western blot" utilizando antisueros y anticuerpos monoclonales, así como la técnica de la transcriptasa inversa-reacción en cadena de la polimerasa (RT-PCR), demostraron la coexistencia de dos virus, el IPNV y un rhabdovirus.

## Introduction

In January 1993 a virological survey was conducted in three cultured rainbow trout (*Oncorhynchus mykiss*, Walbaum) populations (less than 6 months old) that exhibited above normal mortalities for at least 30 days. Mortality in one population increased to 60%. Two viruses associated with the epizootic were detected. This included a birnavirus, infectious pancreatic necrosis virus (IPNV), and a rhabdovirus serologically related to infectious hematopoietic necrosis virus (IHNV) (18). Official confirmation of this type species was not obtained, so viral coinfection and the IPNV-rhabdovirus relationship warranted additional research.

## Materials and methods

**Cell lines.** Four cell lines were used for the isolation and identification of virus in this study. The BF-2 from Bluegill fry, *Lepomis macrochirus*, American Type Culture Collection (ATCC) CCL 91, the CHSE-214 line (ATCC CRL 1681) from embryos of Chinook salmon, *Oncorhynchus tshawytscha* (10), the epithelioma papulosum cyprini (EPC) line from common carp, *Cyprinus carpio* (6), and the rainbow trout gonad (RTG-2) cell line (ATCC CCL 55).

The cells were propagated in Eagle's minimum essential medium (MEM, Flow, Scotland) supplemented with 10% foetal bovine serum (FBS, Flow, Scotland), L-glutamine (10 mM), penicillin (100 IU $ml^{-1}$) and streptomycin (100 µg $ml^{-1}$) (P+S) and buffered with 0.15 mM Hepes (Flow). In open cell culture systems, Leibowitz (L-15) medium with 10% FBS and P+S was used.

**Viruses.** The strains used as reference viruses included IPNV strain VR 299 (ATCC), strain Ab (ATCC VR 1319), strain Sp (ATCC VR 1318); viral haemorrhagic septicemia virus (VHSV) strain D1 from J. L. Barja, Santiago de Compostela, Spain; IHNV ATCC VR 714. VHSV and IHNV were propagated in EPC cells and IPNV in CHSE-214 cells. Virus titrations were performed by the 50% tissue culture infectious dose ($TCID_{50}$ $ml^{-1}$) procedure, using six wells per dilution.

**Sera.** Serological identification of virus isolates was conducted using antisera raised in female New Zealand rabbit infected with purified virus strain according to Hill (7). A polyvalent anti-IPNV serum was provided by Dr. de Kinkelin, (INRA, Jouy-en-Josas, France) and a polyclonal anti-IHNV rabbit serum was provided by Dr. McAllister (U.S. National Fish Health Research Laboratory, Kearneysville, West Virginia). The commercial monoclonal antibodies anti-IPNV and anti-IHNV (Bio-X, Dilbek, Belgium) were also used.

**Case study**. In December 1992, samples of fingerlings 3 to 4 cm in length were received from a group of rainbow trout exhibiting continuous low levels of mortality. Fish with typical signs of infectious pancreatic necrosis (IPN) from this farm had been diagnosed with IPNV previously. Water temperature was 12°C.

One month later the mortality of the same population increased. Fish were treated with chemotherapeutants because a mixobacterial infection had been diagnosed. Water temperature decreased to 9°C and handling of the fish increased the mortality to 60%. Moribund fish submitted for virus isolation showed darkened pigmentation, exophthalmia, abdominal swelling with ascites, edematous kidney and were anemic. Rainbow trout in adjacent ponds also began exhibiting similar symptoms. Additional disease signs included excessive mucus and fecal casts. These included different strains of rainbow trout that were 3 to 5 months old and exhibited varying mortality rates. Specimens were designated S46-S48 and all samples were examined for virus according to standard protocols (1) as previously described (15, 16). Briefly, 4 pools of 10 fish from each population were eviscerated, homogenized with sterile seasand, diluted 1:5 in cell culture medium, centrifuged at 300 × *g* for 10 min at 4°C, and incubated with antibiotics for 2 h at 4°C. The samples were then clarified by syringe filtration (Millipore filter 0.45 μm) and inoculated onto cell cultures at final dilutions of 1:100, 1:200 and 1:1000 (six to nine wells by dilution in 24-well tissue culture plates). Aliquots of the homogeneous dilutions were also neutralized with 1:1000 (v:v) diluted polyvalent anti-IPNV serum for 1 h and inoculated onto the cell cultures. Plates were incubated at 15°C and examined daily for cytopathic effect (CPE).

Identification of the viruses implicated in the outbreak was performed by means of neutralization, immunofluorescence stain, immunoblot (Western blotting), and polymerase chain reaction.

**Neutralization**. It was accomplished by standard procedures (1) using specific rabbit antisera (anti-IPNV, anti-VHSV and anti-IHNV) as previously described (16).

**Western blotting**. Virus suspensions were combined with an equal volume of double-strength sample buffer (9), boiled for 2 min and applied to the preparative well of a 12% SDS-PAGE gel overlayed with a 5% stacking gel in a miniature protein electrophoresis unit (Minicell, Linus, Cultek, Spain). Electrophoresis was performed at 110 V for 90 min. The standard molecular weight marker proteins (BioRad, Richmond, CA) were prepared as described by the manufacturer.

The proteins from the gel were transferred to nitrocellulose filters as described by Towbin et al. (17) in an electroblot apparatus (Linus, Cultek). The blot was washed with TBS (20 mM Tris, 500 mM NaCl, pH 7.5), and blocked with a 3% gelatin solution in TBS for 1 h. The blot was incubated at room temperature for 1 h with 1:1000 dilution of rabbit anti-IHNV serum or monoclonal antibody diluted 1:10 in TBS. Following 3 rinses in TTBS (TBS containing 0.05% Tween 20, BioRad 170-6464 kit [Richmond, CA]) bound antibodies were visualized by horseradish peroxidase reaction. Goat anti-rabbit or anti-mouse antibody coupled to horseradish peroxidase was added (1:3000 dilution) and incubated for 30 min at room temperature. The blot was washed twice with TTBS before color development with chemiluminiscent Western blotting detection system (Amersham, Little Chalfont, Buckinghamshire, UK).

**Extraction of genomic RNA.** RNA was extracted from infected CHSE-214 cells by the use of the guanidinium thiocyanate method (4). The RNA pellets were washed with 70% ethanol, dried and resuspended in diethyl pyrocarbonate (DEPC)-treated distilled water.

**Reverse transcription (RT) and polymerase chain reaction (PCR).** For reverse transcription 5 µg of each dRNA was heated at 99°C for 5 min and incubated at 42°C for 1 h in 20 µl of PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl) containing 20 U of avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer Mannheim, Barcelona, Spain), 50 U of ribonuclease inhibitor 2.5 µM of the hexamer primer, 1 mM of each of the four deoxynucleotide triphosphates, dNTP (Boehringer Mannheim), 0.001% gelatin and 5 mM of $MgCl_2$. cDNA amplifications were performed according to the protocol supplied with the Gene Amp™ PCR Reagent kit (Perkin Elmer, Norwalk, CT). Briefly, 50 pmoles of each of the primers (sense and antisense) and 2.5 U of amply *Taq* DNA polymerase, were added to a final volume of 100 µl. The reaction mixtures were overlaid with 50 µl of light mineral oil (Sigma, St. Louis, MO). PCR amplification was for 30 cycles in a programmable thermal cycler (Perkin Elmer Cetus) as follows: 1 min at 94°C, 1 min 10 s at 55°C and 1 min at 72°C. An additional 7 min at 72°C was used for the last cycle.

**Primers.** The oligonucleotides used in this study were selected according to McAllister et al. (13) and synthesized at the Laboratorio de Química de Proteínas, Centro de Investigaciones Biológicas (CSIC), Spain. The following primers were used:

| | | |
|---|---|---|
| IHNV: | 5'-GAACGTTACCTTCGCAGATCCC-3' | (sense) (product length - 522 bp) |
| | 5'-ACACCGTACTTTGCTGCTACCC-3' | (antisense) |
| IPNV: | 5'-AAAGCCATAGCCGCCCATGAAC-3' | (sense) (product length - 339 bp) |
| | 5'-ATCCTCCTTTGACCACTCATAC-3' | (antisense) |

## Results

The first specimens were examined when daily mortality was approximately 20%. A virus was isolated and identified as IPNV. However, rhabdovirus-like CPE appeared in a portion of the wells inoculated with the IPNV neutralized samples. The results of a cross-neutralization test suggested the presence of a second virus. New samples (S46, S47 and S48) were obtained. The procedure was repeated by two different people using two different cell stocks. The same results were obtained. Aliquots of diluted samples that were neutralized with a polyvalent anti-IPNV serum for 1 h at 15°C to identify one of the suspected virus and to promote the replication of other different viruses exhibited different types of CPE. In CHSE-214 cell line, the CPE was partially neutralized with anti-IPNV serum ($1 \times 10^{-3}$ diluted) at 3 days post-inoculation and a different type of CPE became more apparent at 7 days post-inoculation. In the EPC cell line, the CPE appeared as rhabdovirus-like foci and many cells became

TABLE 1. Neutralization indices of S46, S47 and S48 isolants by antisera to IHNV, in EPC cells

| Virus | Anti-IHNV (1:500)[a] | Anti-IHNV (1:300)[b] | Anti-VHSV (1:500)[a] |
|---|---|---|---|
| IHNV VR 714 | 4[c] | 3 | 1 |
| VHSV | 1 | ND[d] | 3 |
| Isolant S46 | 4 | 3 | 1 |
| Isolant S47 | 3 | 2 | 1 |
| Isolant S48 | 4 | 3 | 1 |

[a] From the author's laboratory.

[b] From McAllister.

[c] Results are expressed as $\log_{10}$ neutralization index (log N1) calculated by the formula:
log [($TCID_{50}$) of virus control – ($TCID_{50}$) of tested antibody neutralized]

[d] ND, not determined.

rounded and detached after 7 days post-inoculation. Samples that were inoculated into the cell cultures without a previous anti-IPN neutralization, exhibited CPE characteristic of IPNV that was different from that produced by the neutralized samples.

Reinoculation of the cell culture supernatants following the same schedule, confirmed the results of previous test. The neutralization titers of immune sera against reference viruses and the isolants S46, S47 and S48, were then determined. High neutralization titers were obtained with IPNV antiserum (100,000 for reference IPNV, more than 20,480 for S46, S47 and S48 isolates) and IHNV antiserum (5,120 for all S46, S47, S48 isolates and reference IHNV), while serum raised against VHSV reacted weakly with any of the viral isolates. These results suggested a dual infection of IPNV and a rhabdovirus. The rhabdovirus-like isolant that became apparent after the second passage in presence of anti-IPNV serum, was collected and assayed to calculate the neutralization index ($\log_{10}$) of each isolant and reference viruses with anti-VHS and anti-IHN sera. The results indicate a 3 to 4 log reduction for samples S46, S47, S48 and IHNV (Table 1). This suggests a serological relationship with IHNV and the rhabdovirus-like isolant.

Blotting patterns corresponding to samples S46, S47 and S48 (Fig. 1A, lanes 5, 6 and 7 respectively) clearly showed a band situated in the position of IPN viral protein VP3. The band was not present in the blotting patterns of the samples, after they had been neutralized against IPNV, prior to their inoculation onto the cell cultures. (Fig. 1A, lanes 2, 3 and 4). The blot in Fig. 1B illustrates the result obtained when the polyclonal anti-IHNV serum was used as first antibody. The major IHN viral proteins G, N, $M_1$ and $M_2$ are recognized by the anti-IHNV serum in S46, S47 and S48 isolants (lanes 2, 3, 4). Analysis of blots A and B had diagnostic applications because it allowed determination that two different viruses were present. Because the rabbit antisera produced strong reaction with the virus controls, masking the individual recognition of some viral protein bands, other immunoblots were performed using commercial monoclonal antibodies (Mab), anti IPNV and anti IHNV (Bio-X). IPN viral protein VP2 was recognized by the specific Mab in all the problem samples (Fig. 2A, lanes 1–3) as well as in other Spanish isolates previously identified as IPN Sp virus (lanes 4, 5 and 6 in Fig. 2A) (16) and the reference virus that was

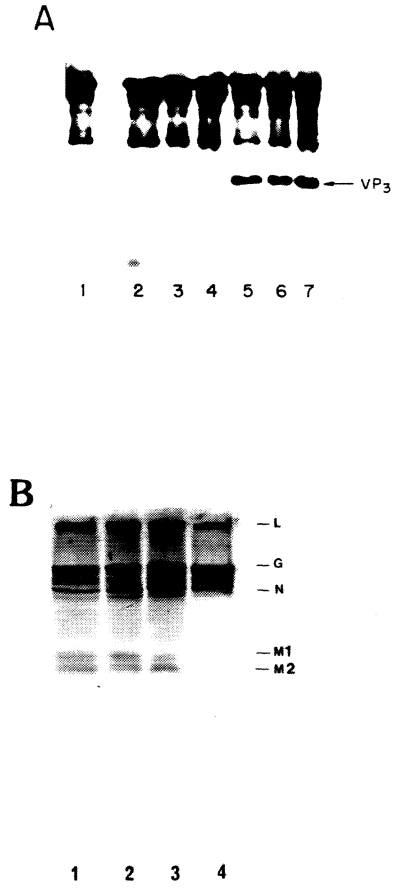



FIG. 1. Western-blot analyses of supernatant obtained from S46, S47 and S48 samples inoculated onto CHSE-214 cell monolayers. Fig. 1A. Rabbit polyvalent anti-IPNV serum (1:1000) was used as primary antibody. Fig. 1B. Rabbit anti-IHNV serum (1:1000) was used as primary antibody. (Both A and B blots developed were performed with a commercial rabbit IgG anti-horseradish peroxidase (1:3000, Sigma) as second antiserum (ARPN 2106, Amersham). In profile A, lane 1 contains cell control, lanes 2, 3, 4, samples S46, S47 and S48 respectively, which have been neutralized against IPNV before their first inoculation onto cell cultures. Lanes 5, 6, 7, samples S46, S47 and S48 (not neutralized). In profile B, lane 1 contains IHNV VR714, lanes 2, 3, 4, samples S46, S47 and S48 respectively. Positions of the significant viral proteins, $VP_3$ for IPNV and L, G, N, $M_1$ and $M_2$ for IHNV, are indicated on the right of the figure.

used as control (lane 7). Fig 2B shows the blot in which IHN viral protein N is recognized by the anti IHNV-Mab in the samples S46, S47 and S48 (lanes 1, 2 and 3 respectively) and in the control IHNV R714 (lane 5).

Polymerase chain reaction assays were carried out with supernatants of CHSE-214 cell cultures that had been infected with anti-IPNV neutralized samples. After amplification using the IHNV primers of RNA extracted from samples S46 and S47 (S48 not done), a 522 bp PCR product was detected on agarose gels (Fig. 3, lanes 2 and 3, and Fig. 4, lanes 5, 6). Stronger amplification of the fragment of 522 bp resulted in the IHNV control (VR-714) (Fig. 3, lanes 1 and 4, and Fig. 4, lane 4). The band corresponded to the

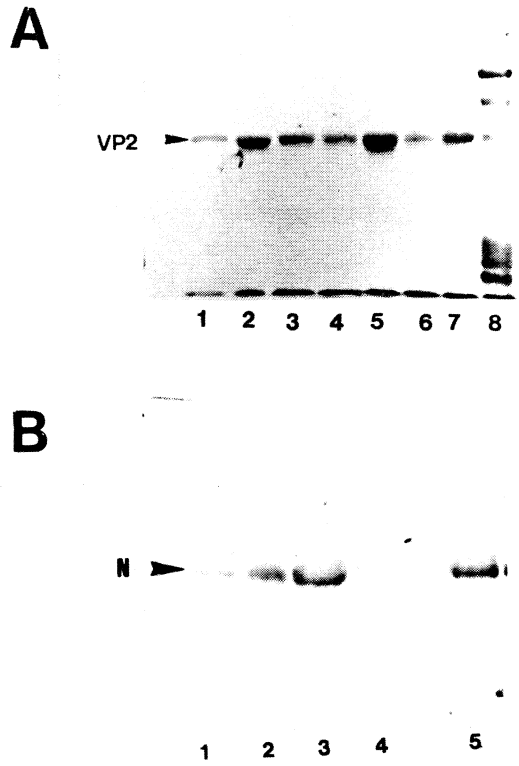



FIG. 2. Western-blot analysis of proteins from CHSE-214 cultures infected with samples S46, S47 and S48. In profile A, the blot was incubated with anti-IPNV monoclonal antibody (1:10). In profile B the blot was incubated with anti-IHNV monoclonal antibody (1:10) (Bio-X). The second antibody in both cases was a commercial goat anti-mouse immunoglobulin g horseradish peroxidase conjugate (Sigma). In both profiles lanes 1, 2, 3, contain samples S46, S47 and S48 respectively. In profile A, lanes 4, 5, 6 contain other IPNV Spanish isolates previously identified as IPNV Sp, lane 7 contains IPNV Sp, and lane 8 the molecular markers. In profile B, lanes 4 and 5 contain IPNV Sp and IHNV VR714, respectively.

expected size when the supernatants contained IHNV sequences, as described by McAllister et al. (13). No product was obtained from RNA extracts from VHSV infected cells (Fig. 4, lane 2), or noninfected cells (Fig. 4, lane 3).

The PCR assay was also performed using the IPNV primers for amplification of RNA extracted from duplicates of the same samples, as well as from IPN Sp virus control. In this case the primers were able to generate a PCR product of the expected size (399 bp) in both S46 and S47 samples (Fig. 3, lanes 5 and 6). Stronger amplification of the fragment resulted with the IPN control virus (lane 8).

## Discussion

Inoculation of samples into two cell lines suggested the presence of two viruses. When the inoculum had been neutralized with anti-IPNV serum, rhabdovirus-like CPE in EPC cells was observed.

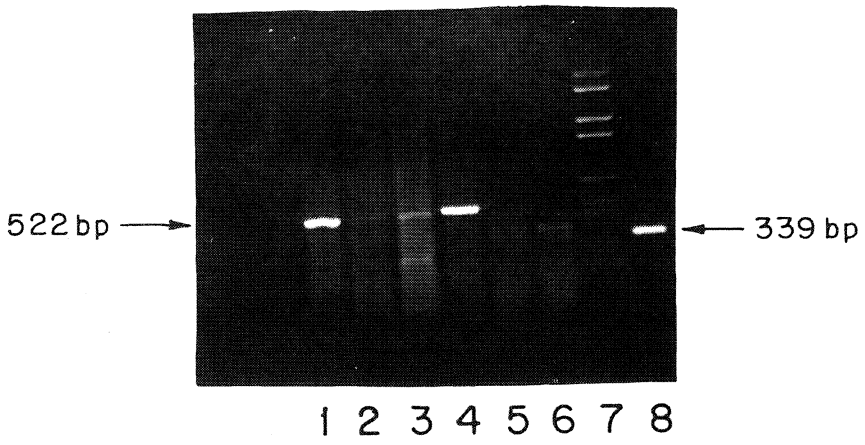



FIG. 3. Agarose gel electrophoresis of products from reverse transcription followed by PCR amplification of total RNA from harvest medium of infected CHSE-214 cells. Lanes 1 and 4, virus control (IHN VR 714). Lane 2, sample S46, lane 3, sample S47 (lanes 1 to 4 with IHNV primers). Lane 5 and 6, samples S46 and S47 respectively, with IPNV primers. Lane 7, DNA marker ladder (DNA MVI Boehringer-Manheim). Lane 8, virus control IPN strain Sp (with IPNV primer).

Neutralization titers suggested there was a close serological relationship with IHNV but not whit VHSV. Rhabdoviruses have not been routinely detected in Spain. Between 1984 and 1986, epizootics of VHSV occurred (8) that were efficaciously eradicated and the virus has not been reisolated. Infectious haematopoietic necrosis virus has been described in several countries (2, 3, 5) but it has not previously been detected in Spain. In our laboratory, from 1988 to 1991, a virological survey was conducted at the culture facilities of rainbow trout farmers who voluntarily participated. Four cell lines were parallely used to favour detection of any type of virus and 94 isolates of IPNV were obtained (16) that caused mortalities from 10% to 90%. Our conclusion was that IPNV was highly dispersed in many river basins around the country, but also that it was the only virus isolated in more than 300 samplings. Several other studies in different Spanish regions agreed with these results (12, 14).

In the case described herein, clinical signs at the beginning of the epizootic were not definitive; however, the farm had a clinical history of IPNV. In the second outbreak, 30 days after the first, disease signs resembled those described for a disease caused by a rhabdovirus. Two viruses isolated from the three samples (S46, S47 and S48) showed different infective titers and the CPE produced by each one, was clearer at the second passage in both CHSE-214 and EPC cells, after the cross-neutralization assay with anti IPNV and anti-IHNV sera.

Viral-dual infections have been described in other countries. LaPatra et al. (11) studied a case of coinfection IHNV-IPNV in two rainbow trout production lots (mean weight 1.5 g) in Idaho. Those authors observed a high IHNV prevalence and concentration while IPNV was detected only at low concentrations in the group suffering high daily mortality. In the second group, daily mortality was very low, but dead fish were consistently observed and both IPNV and IHNV were detected in almost all samples tested. The second description is similar to the Spanish case, but mortality peaked (60%) one month later after the first appearance of symptoms of the disease.

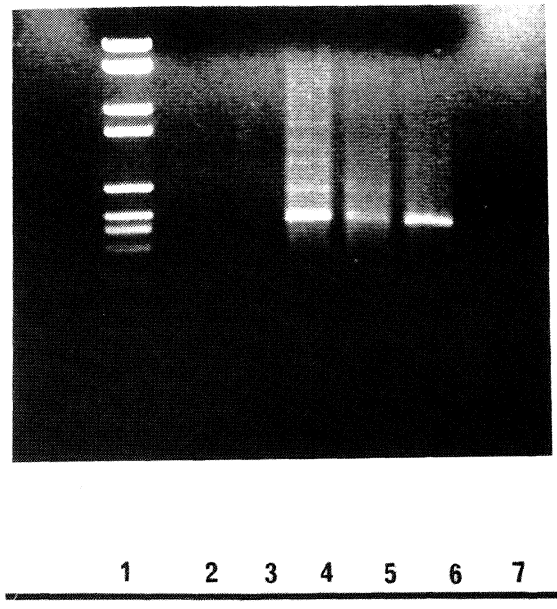



FIG. 4. Agarose gel electrophoresis of products from RT-PCR amplification of total RNA harvest medium of infected CHSE-214 cells, using IHNV primers. Lane 1, DNA marker ladder. Lane 2, VHS virus. Lane 3, uninfected CHSE-214 cells. Lane 4, IHN virus control (VR 714). Lane 5, sample S46. Lane 6, sample S47 and lane 7, IPNV. The band is in the position of the expected 522 bp product obtained for IHN viruses.

Our results obtained using PCR technology confirmed the great utility that new methods offer to find and study coinfections. No other than our case has been reported in Spain. We do not yet know how the temperature could influence the survival of one or both viruses in a fish coinfected population, and this knowledge may be relevant in countries such as Spain, where water temperature could rise from 4°C in winter to 22°C in summer in some geographic areas, while in others could only fluctuate from 12 to 16°C, or stay at 12°C all year long. We are currently involved in the study of IPNV and the rhabdovirus multiplication and viral antigen detection at different temperatures, as well as the direct sequencing of PCR products, to determine possible differences with other reference viruses.

## Acknowledgments

We would like to thank Dr. S. E. LaPatra for his valuable suggestions and critical review of the manuscript. This work was in part supported by the Spanish Comisión Interministerial de Ciencia y Tecnología (Grants 91/0365 and 95/016).

## References

1. Amos, K. H. (1985). Procedures for the Detection and Identification of Certain Fish Pathogens, 3rd ed. Fish Health Section, American Fisheries Society, Bethesda, MD.

2. Baudin-Laurencin, F. (1987). Infectious haematopoietic necrosis in France. Bull. Eur. Ass. Fish Pathol. **7**, 104.
3. Bovo, G., Giorgetti, G., Jorgensen, P., Olesen, N. (1987). Infectious haematopoietic necrosis: First detection in Italy. Bull. Eur. Ass. Fish Pathol. **7**, 124.
4. Chomczynski, P., Sachi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. Anal. Biochem. **162**, 156–159.
5. Enzmann, P. J., Dangschat, H., Feneis, B., Schmit, D., Wizigmann, G., Schlotfeldt, H. (1992). Demonstration of IHN virus in Germany. Bull. Eur. Ass. Fish Pathol. **12**, 185.
6. Fijan, N. (1983). Some properties of the epithelioma papulosum cyprini (EPC) cell line from common carp *Cyprinus carpio*. Ann. Virologie **134E**, 207–220.
7. Hill, B. J., Williams, R. F., Finlay, J. (1981). Preparation of antisera against fish virus disease agents. Dev. Biol. Stand. **49**, 209–218.
8. Jimenez de la Fuente, J., Marcotegui, M., San Juan, M., Basurco, B. (1988). Diagnosis of viral diseases in salmonid farms in Spain. Bull. Eur. Ass. Fish Pathol. **8**, 3–5.
9. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680–685.
10. Lannan, C. N., Winton, J. R., Fryer, J. L. (1984). Fish cell lines: establishment and characterization of nine cell lines from salmonids. In Vitro **20**, 671–676.
11. LaPatra, S. E., Lauda, K. A., Wooley, M. J., Armstrong, R. (1993). Detection of a naturally occurring coinfection of IHNV and IPNV in rainbow trout. FHS/AFS Newsletter **21**, 9–10.
12. Ledó, A., Lupiani, B., Dopazo, C. P., Toranzo, A. E., Barja, J. L. (1990). Fish viral infections in northwest of Spain. Microbiología SEM **6**, 21–29.
13. McAllister, P. E., Schill, W. B., Owens, W. J., Hodge, D. L. (1991). Infectious pancreatic necrosis virus: a comparison of methods used to detect and identify virus in fluids and in tissues of fish. Proc. Sec. Inter. Symp. on Viruses of Lower Vertebrates, 191–201.
14. Novoa, B., Toranzo, A. E., Dopazo, C. P., Barja, J. L., Figueras, A. (1993). Isolation of IPN virus serotype VR 299 from turbot in Europe. Dis. Aquat. Org. **17**, 61–65.
15. Rodríguez, S., Vilas, M. P., Pérez, S. I. (1993). A viral diagnostic survey of Spanish rainbow trout farms. I. Sensitivity of four cell lines to wild IPNV isolates. Bull. Eur. Ass. Fish Pathol. **13**, 119–122.
16. Rodríguez, S., Vilas, M. P., Pérez, S. I. (1994). Prevalence of infectious pancreatic necrosis virus in salmonid fish farmed in Spain. J. Aquat. Animal Health **6**, 138–143.
17. Towbin, H., Staehelin, T., Gordon. J. (1979). Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**, 4350–4354.
18. Vilas, M. P., Rodríguez, S., Pérez, S. I. (1994). A case of coinfection of IPN and IHN virus in farmed rainbow trout in Spain. Bull. Eur. Ass. Fish Pathol. **14**, 47–50.

MICROBIOLOGÍA SEM **11** (1995), 471–484

# Modelo cinético del crecimiento de microorganismos: el caso de *Xanthomonas campestris*

Félix García-Ochoa,* Cristina Delgado, Victoria E. Santos

*Departamento de Ingeniería Química, Facultad de Ciencias Químicas, Universidad Complutense de Madrid, Madrid, España*

Recibido 15 enero 1995/Aceptado 25 septiembre 1995

**Summary**

Microbial growth is studied and kinetic models to describe the process rate useful in the scale-up are proposed. The growth of *Xanthomonas campestris* NRRL B-1459, a bacterium producing xanthan, a major industrial gum, is studied. Experimental data are arranged by means of different methods, and linear and non-linear regression techniques are applied in several ways (i.e. fixing or not fixing the values of certain parameters) and they are compared. To obtain parameter values with statistical meaning, two parameters must be calculated (namely, the maximum specific growth rate and the maximum biomass concentration available) by means of a non-linear regression technique employing the logistic equation. The maximum specific growth rate is related to temperature by means of different equations, but that of Ratkowsky et al. is the most suitable for *X. campestris* growth. Studied variables present no tendency to error and the reproduction of experimental data is very good.

**Key words**: kinetic model of growth, bacterial growth, *Xanthomonas campestris*, linear regression, non-linear regression

**Resumen**

Se estudia el crecimiento microbiano proponiendo modelos cinéticos para la descripción de la velocidad del proceso, utilizables en el cambio de escala. Se aplica al caso de *Xanthomonas campestris* NRRL B-1459, una bacteria que produce xantano, polisacárido de amplio uso. Se ajustan los datos

* *Correspondencia:* Félix García-Ochoa. Departamento de Ingeniería Química. Facultad de Ciencias Químicas. Universidad Complutense de Madrid. 28040 Madrid. España. Tel.: +34-1-3944176. Fax: +34-1-3944114. E-mail: chapu@eucmax.sim.ucm.es

experimentales por diversos métodos, comparando la técnica lineal y la no lineal, y distintas posibilidades de parámetros a calcular (fijando en ciertos casos algunos y dejando flotar otros). Un ajuste de datos experimentales con sentido estadístico debe realizarse utilizando la ecuación logística, por técnica no lineal y calculando dos parámetros (la velocidad específica de crecimiento y la concentración de biomasa máxima alcanzable). Estos parámetros son relacionables con la temperatura por diversas ecuaciones, de las que la más adecuada resulta la propuesta por Ratkowsky et al. La reproducción de los datos experimentales conseguida es muy buena, sin tendencia en el error con ninguna de las variables estudiadas.

## Introducción

El desarrollo de procesos biotecnológicos a escala industrial es uno de los problemas que hay que resolver en biotecnología. Este tipo de procesos son difíciles de describir, porque son multifásicos: gas–líquido–sólido (el aire, el medio y el microorganismo). Tienen un cierto carácter autocatalítico, porque el microorganismo, que puede entenderse como un catalizador (crece o se multiplica, a veces por división y separación celular, o a veces por micelios). Y las relaciones estequiométricas no son evidentes, ya que el substrato no se invierte únicamente en formar el producto, sino que sirve también para el crecimiento y el mantenimiento microbiano. La complejidad del sistema a describir es, en realidad, mucho mayor, ya que cualquier bacteria es una fábrica donde se producen, al menos, decenas de reacciones químicas, bioquímicas en su mayoría, entre decenas de especies químicas, normalmente aminoácidos y proteínas. A lo largo de este siglo se han ido utilizando distintos grados de complejidad en dicha descripción, desde planteamientos elementales a situaciones ciertamente muy complejas. Esto ha llevado a la formulación de lo que hoy se denominan "modelos cinéticos".

## Modelos cinéticos

Los modelos cinéticos para sistemas que emplean microorganismos se suelen agrupar según dos conceptos: estructura y segregación (Tabla 1). Un modelo es "no estructurado" cuando considera al microorganismo como un reactante más en el sistema, utilizando una sola variable para su descripción, generalmente la concentración de biomasa (X o $C_X$). Un modelo es considerado "estructurado" cuando describe al microorganismo teniendo en cuenta que está formado por multitud de componentes internos que reaccionan entre sí (DNA, RNA, proteínas, compartimentos, etc.). Un modelo es "segregado" cuando considera que una población microbiana está formada por individuos diferentes, mientras que un modelo "no segregado" considera que todos los microorganismos de la población son iguales a aquél que se toma como individuo medio.

Los modelos más sencillos (modelos no estructurados-no segregados) son una gran simplificación del problema real. Además, suelen ser útiles para ser usados con fines tecnológicos, ya que proporcionan ecuaciones sencillas con sentido físico, en las que se trata al microorganismo como una especie reactante sencilla.

TABLA 1. Tipos de modelos cinéticos en transformaciones microbianas

| Modelos | No estructurado | Estructurado |
|---|---|---|
| No segregado | Población celular tratada como un conjunto, individuo medio y con un solo componente | Descripción de una célula media multicomponente, con variación de sus componentes con el tiempo |
| Segregado | Población celular con distribución en alguna propiedad, un único componente | Descripción multicomponente de una población celular, heterogeneidad célula a célula |

Los modelos no estructurados-no segregados más sencillos describen únicamente el crecimiento del microorganismo como una función de la cantidad de biomasa presente en el cultivo. La ley de Malthus es el más simple de este tipo de modelos:

$$\frac{dC_x}{dt} = \mu \cdot C_x \qquad [1]$$

Otra ecuación ampliamente utilizada (9, 14), debido originariamente a que la forma de la función matemática es la misma que la que presenta un cultivo de microorganismos unicelulares creciendo en cultivo discontinuo, es la conocida como "ecuación logística", propuesta por Verhurst en 1844 y Pearl y Reed en 1920, según comentan Bailey y Ollis (1):

$$\frac{dC_x}{dt} = \mu \cdot C_x \cdot \left[1 - \frac{C_x}{C_{x_m}}\right] \qquad [2]$$

Existe otro grupo de modelos clasificables dentro del mismo tipo, en el que se incluye un substrato considerado como limitante del crecimiento, de forma que la velocidad específica de crecimiento ($\mu$) depende de la cantidad del citado nutriente limitante, según la expresión general:

$$\frac{dC_x}{dt} = \mu\,(C_s) \cdot C_x \qquad [3]$$

El primer modelo de este tipo fue el propuesto por Blackmann en 1905, que está formado por las siguientes ecuaciones:

$$\mu\,(C_s) = \mu_m \therefore C_s > \mu_m \cdot B \qquad [4]$$

$$\mu\,(C_s) = \frac{C_s}{B} \therefore C_s \leq \mu_m \cdot B$$

siendo B una constante, conocida como constante de Blackmann.

Después, M'Kendrick y Pai en 1910 propusieron, para describir el crecimiento, la expresión:

$$\frac{dC_x}{dt} = \mu_m \cdot C_s \cdot C_x \qquad [5]$$

siendo estos autores los primeros que señalan la existencia de una relación entre la velocidad de consumo del substrato y la de producción de biomasa:

$$\frac{dC_s}{dt} = -\frac{1}{Y_{xs}} \cdot \frac{dC_x}{dt} \qquad [6]$$

Tiessier, en 1942, propuso otra expresión perteneciente al grupo de ecuaciones que presentan la forma general de la ecuación [3], en este caso:

$$\frac{dC_x}{dt} = \mu_m \cdot \left[1 - \exp\left(-\frac{C_s}{K}\right)\right] \cdot C_x \qquad [7]$$

Es a finales de la década de los años 40 cuando Monod (7, 8) propone un modelo para la descripción del crecimiento de microorganismos unicelulares. El citado modelo está formado por la ecuación [6] y la siguiente expresión:

$$\frac{dC_x}{dt} = \frac{\mu_m \cdot C_s}{K_s + C_s} \cdot C_x \qquad [8]$$

El modelo de Monod (7, 8) se puede simplificar en los siguientes casos particulares:

$$K_s << C_s \therefore \frac{dC_x}{dt} = \mu_m \cdot C_x \qquad \text{(ley de Malthus)} \qquad [9]$$

$$K_s >> C_s \therefore \frac{dC_x}{dt} = \frac{\mu_m}{K_s} \cdot C_s \cdot C_x \qquad \text{(M'Kendrick y Pai, 1910)}$$

Estas mismas condiciones particulares se corresponden con el modelo propuesto por Blackmann (ecuación [4]). Así mismo, a partir de la simplificación del modelo de Monod que se corresponde con el propuesto por M'Kendrick y Pai, es deducible la ecuación logística, tomando la forma:

$$\frac{dC_x}{dt} = \frac{\mu_m}{K_s} \cdot \left(\frac{C_{x_0}}{Y_{xs}} + C_{s_0}\right) \cdot C_x \cdot \left[1 - \frac{C_x}{C_{x_0} + Y_{xs} \cdot C_{s_0}}\right] \qquad [10]$$

La ecuación logística deducida a partir de las ecuaciones [6] y [9] se cumple únicamente cuando el nutriente considerado como limitante del crecimiento del microorganismo se emplea sólo para crecer, es decir, de acuerdo a la ecuación [6].

Pirt (10) propuso una expresión para tener en cuenta que el citado substrato se puede emplear tanto para el crecimiento como para el mantenimiento de los microorganismos en estado viable:

$$\frac{dC_s}{dt} = -\frac{1}{Y_{xs}} \cdot \frac{dC_x}{dt} - m_s \cdot C_x \qquad [11]$$

Cuando el citado substrato se utiliza también para la producción, se suele definir un nuevo "rendimiento macroscópico" de substrato en producto ($Y_{ps}$), incorporándose el término de consumo del substrato para la producción, de la siguiente forma:

$$\frac{dC_s}{dt} = -\frac{1}{Y_{xs}} \cdot \frac{dC_x}{dt} - \frac{1}{Y_{ps}} \cdot \frac{dC_p}{dt} - m_s \cdot C_x \qquad [12]$$

En los modelos no estructurados-no segregados se ha intentado incorporar la influencia de la temperatura, especialmente en la velocidad específica máxima de crecimiento, según diferentes ecuaciones. Así, Esener et al. (2) y Bailey y Ollis (1) expresan la citada variación de la siguiente forma:

$$\mu_m(T) = \frac{k_{0_1} \cdot \exp\left(-\frac{\Delta H_1}{RT}\right)}{1 + k_{0_2} \cdot \exp\left(-\frac{\Delta H_2}{RT}\right)} \qquad [13]$$

mientras que Sinclair (13) y Shu y Yang (12), plantean:

$$\mu_m(T) = k_{0_1} \cdot \exp\left(-\frac{E_1}{RT}\right) - k_{0_2} \cdot \exp\left(\frac{-E_2}{RT}\right) \qquad [14]$$

y, por último, Ratkowsky et al. (11) y de nuevo Shu y Yang (12), emplean la expresión:

$$\mu_m(T) = \left\{C_1 \cdot (T - T_{min}) \cdot \left[1 - \exp\left(C_2 \cdot (T - T_{max})\right)\right]\right\}^2 \qquad [15]$$

En cuanto a la influencia de la citada variable en la concentración de biomasa alcanzable en la fase estacionaria del crecimiento, únicamente Shu y Yang (12) proponen una expresión, como la siguiente:

$$C_{x_m} = \frac{X_1 + X_2 \cdot \exp(T_0 - T)}{1 + \exp(T_0 - T)} \qquad [16]$$

El objeto del presente trabajo es aplicar los modelos más sencillos, no estructurados-no segregados, al crecimiento de una bacteria, *Xanthomonas campestris*, útil industrialmente para obtener un polisacárido, el xantano, ampliamente utilizado como espesante en la industria alimentaria, cosmética, farmacéutica y en la recuperación forzada de petróleo. Se pretenden mostrar diferentes técnicas de interpretación de datos experimentales, proponiendo el método estadísticamente más correcto para determinar el modelo cinético del crecimiento y el valor óptimo de los parámetros de dicho modelo.

## Materiales y métodos

La bacteria empleada ha sido *Xanthomonas campestris* NRRL B-1459, proporcionada por el Nothern Regional Research Laboratory of the U.S. Department of Agriculture (Peoria, Illinois). La cepa se mantuvo a 4°C en tubos con agar YM y fue transferida cada 14 días. El método experimental y las técnicas de análisis empleadas son las utilizadas en trabajos anteriores (5).

## Resultados y discusión

Para determinar el modelo cinético del crecimiento de *X. campestris* se consideraron las variables cuya influencia en los modelos no estructurados-no segregados son capaces de tener en cuenta: temperatura y concentración inicial de biomasa. Por tanto, los experimentos llevados a cabo se recogen en la Tabla 2.

El cálculo de los parámetros de las ecuaciones cinéticas, para obtener el modelo cinético del crecimiento de microorganismos, se puede abordar de diferentes formas. En primer lugar, se puede emplear el método diferencial (3) o el método integral (4); el uso de uno u otro método depende principalmente del tipo de datos cinéticos obtenidos. Debido a que los datos generalmente son integrales ($C_x$ vs. t), el método de cálculo de los parámetros debe ser integral, para evitar la derivación de los datos experimentales, lo que siempre lleva consigo un gran error (3, 4). En el caso de que la ecuación diferencial que representa la velocidad de crecimiento de la biomasa no sea integrable analíticamente, es necesario el empleo del algoritmo de Runge-Kutta acoplado al correspondiente de la optimación para el cálculo de los parámetros cinéticos, realizando de esta manera el cálculo en forma integral. Esto es lo que sucede en el caso del modelo de Monod (7, 8).

En la experimentación realizada en el crecimiento de *X. campestris* se ha observado que dicho crecimiento cesa cuando se agota el substrato nitrogenado (Santos, V. E., 1993, Tesis doctoral, Universidad Complutense de Madrid). Por tanto, debido a que el xantano (producido por la bacteria en presencia de azúcar) no incorpora nitrógeno en su molécula, se puede emplear la expresión [6] para

TABLA 2. Experimentos llevados a cabo para el modelado del crecimiento de *X. campestris*

| Experimento | T (°C) | $C_{X0}$ (g/l) |
|---|---|---|
| 1 | 28 | 0,01 |
| 2 | 28 | 0,02 |
| 3 | 28 | 0,04 |
| 4 | 22 | 0,01 |
| 5 | 25 | 0,01 |
| 6 | 31 | 0,01 |
| 7 | 34 | 0,01 |

explicar la evolución del citado substrato. Para la modelización del crecimiento de *X. campestris* se va a emplear la ecuación logística, ecuación [10], ya que otras ecuaciones, como la de Malthus, no son capaces de describir toda la curva de crecimiento. Dicha ecuación [10] es integrable analíticamente, obteniéndose:

$$C_X = \frac{C_{X_0} \cdot \exp(\mu \cdot t)}{1 - \frac{C_{X_0}}{C_{X_m}} \cdot (1 - \exp(\mu \cdot t))} \qquad [17]$$

Esta última ecuación es linealizable, llegándose a la expresión:

$$\ln\left[\frac{(C_{X_m} - C_{X_0}) \cdot C_X}{(C_{X_m} - C_X) \cdot C_{X_0}}\right] = \mu \cdot t \qquad [18]$$

El empleo de la ecuación [18], para la obtención de los parámetros, permite el cálculo de $\mu$ únicamente, siendo necesario emplear los valores experimentales de la concentración de biomasa inicial y la obtenida en la fase estacionaria del crecimiento.

En el caso de emplear la expresión [17], se hace necesario utilizar una regresión no lineal para llevar a cabo el cálculo de parámetros, ya que la ecuación logística en forma integrada, teniendo en cuenta las expresiones para $\mu$ y $C_{X_m}$, obtenidas a partir de la ecuación de Monod simplificada, toma la siguiente forma:

$$C_X = \frac{C_{X_0} \cdot \exp\left[k_X\left(\frac{C_{X_0}}{Y_{XN}} + C_{N_0}\right) \cdot t\right]}{1 - \frac{C_{X_0}}{C_{X_0} + Y_{XN} \cdot C_{N_0}} \cdot \left\{1 - \exp\left[k_X\left(\frac{C_{X_0}}{Y_{XN}} + C_{N_0}\right) \cdot t\right]\right\}} \qquad [19]$$

No tiene sentido la aplicación de una regresión lineal a la ecuación [19], ya que para ello habría que fijar a priori el valor del parámetro $Y_{XN}$, y éste se desconoce. Por ello, se deben emplear únicamente regresiones tipo no lineal (6). De esta forma, se pueden calcular los tres parámetros de la curva de crecimiento ($C_{X0}$, $Y_{XN}$ y $k_X$) a partir de los datos experimentales obtenidos.

El cálculo de parámetros se puede llevar a cabo de dos formas:

(i) **Ajuste con dos parámetros**: se obtienen por regresión no lineal los valores de los parámetros $k_X$ e $Y_{XN}$, fijando el valor de la concentración de biomasa inicial en el empleado experimentalmente.

(ii) **Ajuste con tres parámetros**: se obtienen por regresión no lineal los tres parámetros de la ecuación [19], dejando flotar también $C_{X0}$.

Los resultados obtenidos a partir de los citados ajustes se recogen en la Tabla 3. En la citada tabla se presentan, además de los valores de los parámetros de la ecuación logística, los valores de los parámetros estadísticos obtenidos a partir de las regresiones realizadas (t de Student, F de Fischer, intervalo

TABLA 3. Valores de los parámetros de la ecuación [19] y los parámetros estadísticos obtenidos por regresión no lineal de los datos de los experimentos de la Tabla 2

| Exp. | Parám. | Valor ópt. | Valor mín. | Valor máx. | $t_s$ | F | SRC/N | $t_{stab95\%}$ | $F_{tab95\%}$ |
|---|---|---|---|---|---|---|---|---|---|
| | $k_X$ | 1,496 | 1,459 | 1,533 | 82,93 | 3328 | 0,0074 | 2,052 | 3,35 |
| | $Y_{XN}$ | 7,304 | 7,049 | 7,558 | 58,83 | | | | |
| 1 | $k_X$ | 1,619 | 1,390 | 1,849 | 4,52 | | | | |
| | $Y_{XN}$ | 7,233 | 6,959 | 7,508 | 54,12 | 2248 | 0,0070 | 2,056 | 2,98 |
| | $C_{X0}$ | $6,5\cdot10^{-3}$ | $1,4\cdot10^{-3}$ | $1,2\cdot10^{-3}$ | 2,620 | | | | |
| | $k_X$ | 1,369 | 1,324 | 1,412 | 66,68 | 2008 | 0,0096 | 2,131 | 3,68 |
| | $Y_{XN}$ | 9,311 | 8,859 | 9,764 | 43,87 | | | | |
| 2 | $k_X$ | 1,104 | 0,940 | 1,269 | 14,38 | | | | |
| | $Y_{XN}$ | 9,691 | 9,160 | 10,22 | 39,39 | 2237 | 0,0053 | 2,145 | 3,34 |
| | $C_{X0}$ | 0,050 | 0,023 | 0,070 | 4,24 | | | | |
| | $k_X$ | 1,352 | 1,312 | 1,391 | 68,48 | 4703 | 0,0210 | 2,021 | 3,23 |
| | $Y_{XN}$ | 9,568 | 9,287 | 9,849 | 68,83 | | | | |
| 3 | $k_X$ | 1,116 | 0,931 | 1,301 | 12,18 | | | | |
| | $Y_{XN}$ | 9,695 | 9,374 | 10,02 | 61,02 | 3558 | 0,0181 | 2,021 | 2,841 |
| | $C_{X0}$ | 0,081 | 0,037 | 0,125 | 3,780 | | | | |
| | $k_X$ | 1,120 | 1,060 | 1,179 | 40,95 | 732 | 0,0133 | 2,179 | 3,88 |
| | $Y_{XN}$ | 6,942 | 6,146 | 7,738 | 19,01 | | | | |
| 4 | $k_X$ | 1,754 | 1,369 | 2,140 | 10,02 | | | | |
| | $Y_{XN}$ | 6,213 | 5,798 | 6,628 | 32,97 | 1183 | 0,0052 | 2,201 | 3,59 |
| | $C_{X0}$ | $6,2\cdot10^{-4}$ | $-4,0\cdot10^{-4}$ | $1,7\cdot10^{-3}$ | 1,305 | | | | |
| | $k_X$ | 1,359 | 1,336 | 1,383 | 121,5 | 3421 | 0,0018 | 2,093 | 3,52 |
| | $Y_{XN}$ | 6,764 | 6,472 | 7,057 | 48,39 | | | | |
| 5 | $k_X$ | 1,461 | 1,342 | 1,598 | 22,46 | | | | |
| | $Y_{XN}$ | 6,635 | 6,321 | 6,949 | 44,41 | 2456 | 0,0016 | 2,101 | 3,16 |
| | $C_{X0}$ | $7,1\cdot10^{-3}$ | $3,8\cdot10^{-3}$ | 0,0103 | 4,59 | | | | |
| | $k_X$ | 1,402 | 1,361 | 1,444 | 70,22 | 2830 | 0,0059 | 2,080 | 3,47 |
| | $Y_{XN}$ | 6,870 | 6,604 | 7,137 | 53,58 | | | | |
| 6 | $k_X$ | 1,547 | 1,289 | 1,805 | 12,59 | | | | |
| | $Y_{XN}$ | 6,788 | 6,501 | 7,076 | 49,19 | 1925 | 0,0056 | 2,086 | 3,10 |
| | $C_{X0}$ | 0,0058 | $3,9\cdot10^{-4}$ | 0,011 | 2,236 | | | | |
| | $k_X$ | 0,796 | 0,769 | 0,824 | 65,61 | 2440 | 0,0022 | 2,262 | 4,26 |
| | $Y_{XN}$ | 6,856 | 6,586 | 7,126 | 57,40 | | | | |
| 7 | $k_X$ | 0,832 | 0,626 | 1,038 | 9,304 | | | | |
| | $Y_{XN}$ | 6,815 | 6,456 | 7,175 | 43,71 | 1477 | 0,0021 | 2,306 | 4,07 |
| | $C_{X0}$ | 0,0083 | $-9\cdot10^{-4}$ | 0,017 | 2,075 | | | | |

de confianza de los parámetros y suma de los residuos al cuadrado, SRC). Los valores de los citados parámetros estadísticos proporcionan una idea sobre la bondad del ajuste realizado y la fiabilidad de cada uno de los parámetros de la ecuación ajustada. Como es de esperar, a mayor número de parámetros la reproducción de los datos experimentales mejora (SRC disminuye), pero la calidad del ajuste no siempre mejora (la F de Fischer a veces disminuye al aumentar el número de parámetros).

Cuando se emplea el ajuste con tres parámetros, se está obteniendo por regresión un parámetro conocido, ya que ha sido fijada la concentración inicial de biomasa en cada experimento. Por ello, la regresión realizada con tres parámetros lleva algunas veces a valores absurdos de $C_{X0}$ e incluso, a veces, el citado parámetro incluye el cero en su intervalo de confianza, no siendo, por tanto, significativo. Sin embargo, en el caso de los ajustes con dos parámetros, en todos los experimentos los valores obtenidos tienen sentido estadístico, superando los valores tabulados de t y F para el 95% de confianza. Por ello, se debe emplear una regresión no lineal con dos parámetros ($k_X$ e $Y_{XN}$) para determinar el modelo cinético con la ecuación logística.

A partir de los resultados con dos parámetros obtenidos (Tabla 3), se observa que en el parámetro $Y_{XN}$ la temperatura no presenta influencia (en el caso de $k_X$ la variación con la temperatura es muy pronunciada). Por tanto, a la vista de los resultados obtenidos, no parece tener sentido la expresión propuesta por Shu y Yang (12) para la variación de la concentración de biomasa en la fase estacionaria con la temperatura, ya que experimentalmente no se observa influencia (ecuación [16]). En el caso de la influencia de la concentración inicial de biomasa, se observa que el parámetro $Y_{XN}$ se ve afectado por la cantidad de biomasa inicial empleada en el experimento. Sin embargo, esta influencia no se puede explicar con el modelo cinético elegido, ya que la citada influencia se debe a factores no considerados en el modelo no estructurado-no segregado empleado.

El cálculo de los parámetros que reproducen los datos obtenidos a diferentes temperaturas se realizó a partir de las tres expresiones comentadas anteriormente (ecuaciones [13] a [15]), intentando discriminar cuál de ellas es la más apropiada en este caso. Los ajustes empleados para la determinación de los parámetros de las ecuaciones citadas fueron, en todos los casos, regresiones no lineales, siendo aplicadas de dos formas para cada ecuación:

(i) En primer lugar, para disponer de valores iniciales de los parámetros de las diferentes expresiones, se realizó un ajuste de los valores de $k_X$ obtenidos a partir de las regresiones con dos parámetros. En este ajuste se trata de obtener cuatro parámetros a partir de cinco datos, lo que, en todos los casos, llevó a intervalos de confianza que incluían el cero.

(ii) En segundo lugar, y partiendo de los valores obtenidos en el caso anterior, se realizó la regresión a partir de los datos experimentales biomasa–tiempo, teniendo, por tanto, 98 datos y dos variables independientes (tiempo y temperatura), a partir de los cuales se calculan cuatro parámetros. El valor del parámetro $Y_{XN}$ se fijó en el valor medio de los cinco obtenidos por regresión no lineal con dos parámetros. Es decir, la expresión empleada para llevar a cabo la regresión comentada es la ecuación [19], sustituyéndose en ella $k_X(T)$ por las ecuaciones [13] a [15], según la expresión empleada de la velocidad específica de crecimiento en función de la temperatura.

Los valores de los parámetros y la calidad de los ajustes obtenidos (parámetros estadísticos t y F, intervalo de confianza y SRC) se recogen en la Tabla 4. En la citada tabla se observa que, tanto en el caso de la ecuación [13] como en el de la expresión [14], los parámetros obtenidos no tienen ninguna fiabilidad

TABLA 4. Resultados obtenidos del ajuste a la ecuación [19], empleando diferentes expresiones para la función $k_X(T)$. ($t_{stab95\%} = 12,70$; $F_{tab95\%} = 225$)

| Ecuación | Parám. | Valor ópt. | Valor mín. | Valor máx. | $t_s$ | F | SRC |
|---|---|---|---|---|---|---|---|
| [13] | $k_{01}$ | 25,10 | $-5,9\cdot10^{5}$ | $5,9\cdot10^{5}$ | 0,001 | 7,55 | 0,267 |
| | $\Delta H_1/R$ | 790,8 | $-6,8\cdot10^{6}$ | $6,8\cdot10^{6}$ | 0,001 | | |
| | $k_{02}$ | $5,8\cdot10^{9}$ | $-9,6\cdot10^{13}$ | $9,6\cdot10^{13}$ | 0,001 | | |
| | $\Delta H_2/R$ | 7029,8 | $-9,4\cdot10^{6}$ | $9,4\cdot10^{6}$ | 0,001 | | |
| [14] | $k_{01}$ | 4271 | $-2,1\cdot10^{7}$ | $2,1\cdot10^{7}$ | 0,000 | 8,05 | 0,244 |
| | $E_1/R$ | 1519 | $-1,3\cdot10^{7}$ | $1,3\cdot10^{7}$ | 0,001 | | |
| | $k_{02}$ | $2,1\cdot10^{6}$ | $-1,2\cdot10^{11}$ | $1,2\cdot10^{11}$ | 0,000 | | |
| | $E_2/R$ | 4272 | $-2,3\cdot10^{7}$ | $2,3\cdot10^{7}$ | 0,002 | | |
| [15] | $C_1$ | $4,5\cdot10^{-2}$ | $8,1\cdot10^{-3}$ | $8,1\cdot10^{-2}$ | 15,51 | 31578 | $0,62\cdot10^{-4}$ |
| | $T_{min}$ | −2,080 | 21,09 | 16,92 | −1,393 | | |
| | $C_2$ | 0,266 | $2,7\cdot10^{-2}$ | 0,500 | 14,13 | | |
| | $T_{max}$ | 37,01 | 35,49 | 38,54 | 308,5 | | |
| [15] | $C_1$ | $4,92\cdot10^{-2}$ | $4,85\cdot10^{-2}$ | $4,99\cdot10^{-2}$ | 137,8 | 6347 | 0,685 |
| | $C_2$ | $2,46\cdot10^{-1}$ | 0,224 | 0,267 | 22,46 | | |
| | $T_{max}$ | 37,09 | 36,80 | 37,38 | 262,8 | | |
| [15] y [19] | $C_1$ | $4,91\cdot10^{-2}$ | $4,84\cdot10^{-2}$ | $4,99\cdot10^{-2}$ | 137,8 | 4868 | 0,672 |
| | $C_2$ | 0,245 | 0,223 | 0,266 | 22,86 | | |
| | $T_{max}$ | 37,09 | 36,81 | 37,37 | 266,3 | | |
| | $Y_{XN}$ | 7,045 | 6,898 | 7,193 | 95,56 | | |

estadística, mientras que cuando se emplea la ecuación [15], únicamente el parámetro $T_{min}$ no presenta sentido estadístico. Debido a que el citado parámetro es un sumando en dicha expresión, se puede tomar como cero, quedando, por tanto, tres parámetros en el modelo: $C_1$, $C_2$ y $T_{max}$. Los resultados obtenidos del ajuste a la ecuación [19], empleando la expresión [15] con los tres parámetros citados, se recogen en la Tabla 4, donde se observa que los valores de los parámetros estadísticos son muy elevados y la reproducción de los datos experimentales muy buena. Por último, debido a que el parámetro $Y_{XN}$ no depende de la temperatura, se decidió obtener su valor también por regresión, empleando la ecuación [19] junto con la [15] con tres parámetros, calculándose en total cuatro parámetros. En la Tabla 4, se observa que los valores de los parámetros obtenidos correspondientes a la variación de $k_X$ con la temperatura son muy similares a los que se obtienen con tres parámetros, siendo de destacar que se produce un ligero aumento en los valores de las t de Student correspondientes. El ajuste realizado con cuatro parámetros presenta valores con sentido tanto físico como estadístico, comprobándose que $Y_{XN}$ permanece constante con la temperatura.

En la Fig. 1 se recoge el análisis de residuos para los experimentos llevados a cabo variando la temperatura. Se observa la ausencia de tendencia en el error y el nivel aceptable de dicho error.

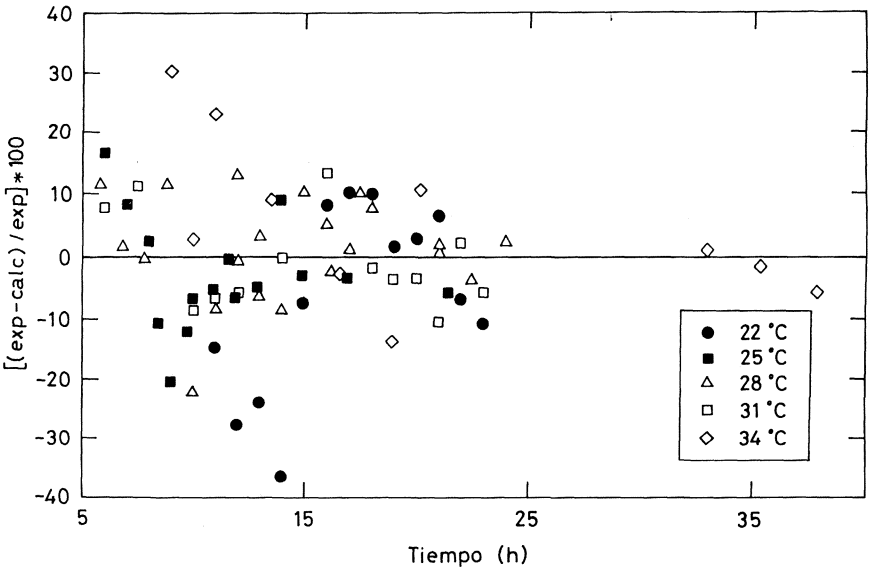



FIG. 1. Análisis de residuos de los experimentos realizados a diferente temperatura.

## Aplicación al cultivo continuo

La característica principal de un reactor tanque agitado continuo, denominado quimiostato, es la obtención a la salida del reactor de una corriente con concentraciones de los diferentes componentes del sistema idénticas a las que existen en el interior del reactor (debido a la buena mezcla); es decir, se acepta un flujo en mezcla perfecta ideal. El balance de materia para la biomasa responde a la ecuación:

$$C_{X_0} \cdot \frac{Q}{V} - C_{X_s} \cdot \frac{Q}{V} + \frac{dC_X}{dt} = 0 \qquad [20]$$

siendo V/Q lo que en ingeniería química se conoce como "tiempo espacial" o "tiempo de residencia", que corresponde a la inversa de la "velocidad de dilución", pudiéndose escribir el balance de la forma:

$$C_{X_0} \cdot D - C_{X_s} \cdot D + \frac{dC_X}{dt} = 0 \qquad [21]$$

En el caso del quimiostato la alimentación no suele llevar microorganismos, es decir, $C_{X0} = 0$, por tanto, se obtiene:

$$C_{X_s} \cdot D = \frac{dC_X}{dt} \qquad [22]$$

sustituyendo la expresión general de Monod (ecuación [8]) en la ecuación anterior, se llega a:

$$C_{X_s} \cdot D = \frac{\mu_m \cdot C_{S_s}}{K_S + C_{S_s}} \cdot C_{X_s} \therefore D = \frac{\mu_m \cdot C_{S_s}}{K_S + C_{S_s}} \quad [23]$$

Debido a que en estado estacionario $C_{S_s}$ es una constante, cuyo valor sólo depende de las condiciones de entrada al reactor y del uso que el microorganismo haga del citado substrato limitante (empleo para crecimiento, mantenimiento, producción, etc.), la ecuación [23] se puede simplificar a cualquiera de los casos correspondientes mostrados anteriormente, simplificaciones de la ecuación de Monod. Por tanto, se puede utilizar una expresión como la siguiente:

$$D = \mu \quad [24]$$

es decir, similar al caso, relativamente frecuente, en que la velocidad de crecimiento del microorganismo se da por una expresión de Malthus, que no es utilizable para describir procesos como los tratados en este trabajo, ni desde luego, para realizar un aumento de escala.

En el caso del substrato limitante, el balance de materia presenta la forma:

$$C_{S_0} \cdot \frac{Q}{V} - C_{S_s} \cdot \frac{Q}{V} + \frac{dC_S}{dt} = 0 \quad [25]$$

sustituyéndose en la velocidad de producción del citado substrato las expresiones [6], [11] ó [12], para los casos en los que el substrato limitante del crecimiento se emplee sólo para crecer (ecuación [6]); para crecer y en labores de mantenimiento (ecuación [11]); y para crecer, en mantenimiento y en la producción (ecuación [12]).

## Nomenclatura

- $B$ : Constante de Blackmann (1905). Ecuación [4] (gS·h/l).
- $C_1$ : Constante del modelo de Ratkowsky et al., (1983). Ecuación [15] ($h^{-2}$).
- $C_2$ : Constante del modelo de Ratkowsky et al., (1983). Ecuación [15] ($°C^{-1}$).
- $C_i$ : Concentración del componente i (g/l).
- $D$ : Velocidad de dilución ($h^{-1}$).
- $E_i$ : Energía de activación de la reacción i (cal/mol).
- $F$ : F de Fischer, parámetro estadístico.
- $H_i$ : Entalpía de la reacción i (cal/mol).
- $k_X$ : Velocidad específica máxima de crecimiento referida a la concentración del substrato limitante del mismo (l/gS·h).
- $k_{0i}$ : Factor preexponencial de la reacción i (l/gS·h).
- $K$ : Constante del modelo de Tiessier. Ecuación [7] (gS/l).
- $K_S$ : Constante de saturación del modelo de Monod (1949 y 1950). Ecuación [8] (gS/l).
- $m_s$ : Coeficiente de mantenimiento ($h^{-1}$).
- $N$ : Número de datos experimentales.

Q : Caudal volumétrico (l/h).
R : Constante de los gases (cal/mol·K).
SRC : Suma de residuos al cuadrado.
t : Tiempo (h).
$t_s$ : t de Student, parámetro estadístico.
T : Temperatura (°C, K).
$T_{min}$, $T_{max}$ : Parámetros del modelo de Ratkowsky et al. (11) (°C).
$T_0$ : Parámetro expresión de Shu y Yang (12). Ecuación [16] (°C).
V : Volumen de reacción (l).
$X_1$, $X_2$ : Parámetros de la ecuación de Shu y Yang (12). Ecuación [16] (gX/l).
$Y_{ij}$ : Rendimiento macroscópico de componente j en el componente i (gj/gi).

## Subíndices

N : Referido al substrato nitrogenado.
m : Valor máximo.
P : Referido al producto.
s : Referido a la salida.
S : Referido al substrato S, en general.
X : Referido a la biomasa.
0 : Referido al tiempo cero y a la entrada al reactor.
1 : Referido a la reacción 1.
2 : Referido a la reacción 2.

## Letras griegas

$\Delta$ : Incremento.
$\mu$ : Velocidad específica de crecimiento ($h^{-1}$).
$\mu_m$ : Velocidad específica máxima de crecimiento ($h^{-1}$).

## Bibliografía

1. Bailey, J. E., Ollis, D. F. (1983). Biochemical Engineering Fundamentals, pp. 457–500. Mc.Graw-Hill, New York, NY.
2. Esener, A. A., Röels, J. A., Kossen, N. W. F. (1983). Theory and application of unstructured growth models: kinetic and energetic aspects. Biotech. Bioeng. **25**, 2803–2841.
3. García-Ochoa, F., Romero, A., Santos, V. E., Rodríguez, C. O. (1989). Estudio del método diferencial en la determinación de modelos cinéticos de reacciones complejas. An. Quim. **85**, 542–556. [Traducido al Internat. Chem. Engng. **33**, 634–648 (1993)].

4. García-Ochoa, F., Romero, A., Santos, V. E. (1990). Comparación de métodos de determinación de parámetros cinéticos en reacciones complejas An. Quim. **86**, 625–637. [Traducido al Internat. Chem. Engng. **32**, 538–551 (1992)].
5. García-Ochoa, F., Santos, V. E., Frisch, A. P. (1992). Nutritional study of *Xanthomonas campestris* in xanthan gum production by factorial design of experiments. Enzyme Microbiol. Technol. **14**, 991–996.
6. Marquardt, A. W. (1963). An algoritm for least-squares estimation of nonlinear parameters. J. Soc. Indus. Appl. Math. **11**, 431–441.
7. Monod, J. (1949). The growth of bacterial cultures. Annu. Rev. Microbiol. **3**, 371–394.
8. Monod, J. (1950). La technique de culture continue theorie et applications. Ann. Inst. Pasteur **79**, 390–410.
9. Pinches, A., Pallent, L. J. (1986). Rate and yield relationships in the production of xanthan gum by batch fermentations using complex and chemically defined growth media. Biotech. Bioeng. **28**, 1484–1496.
10. Pirt, S. J. (1956). Maintenance energy of bacteria in growing cultures. Proc. Royal Soc. London B. **163**, 224–231.
11. Ratkowsky, D. A., Lowry, R. K., Stokes, T. A., Chandler, R. E. (1983). Model for bacterial culture growth rate throughout the entire biokinetic temperature range. J. Bacteriol. **154**, 1222–1226.
12. Shu, C.-H., Yang, S.-T. (1991). Kinetics and modeling of temperature effects on batch xanthan gum fermentations. Biotech. Bioeng. **37**, 567–574.
13. Sinclair, C. G. (1987). Microbial process kinetics. *In* Bu'lock, J., Kristiansen, B. (ed.), Basic Biotechnology, pp. 100–103. Academic Press, London.
14. Weiss, R. M., Ollis, D. F. (1980). Extracellular microbial polysaccharides. I. Substrate, biomass and product kinetic equations for batch xanthan gum fermentation. Biotech. Bioeng. **22**, 859–873.

MICROBIOLOGÍA SEM **11** (1995), 485–490

# Sensibilidad in vitro de cepas de *Mycobacterium chelonae* frente a diversos antimicrobianos

Amelia M. Hernández García,[1]* Ángeles Arias,[1] Ana Felipe,[1]
Roberto Álvarez,[1] Antonio Sierra[1, 2]

[1] *Cátedra de Medicina Preventiva y Salud Pública, Universidad de La Laguna, Tenerife*
[2] *Servicio de Microbiología del Hospital Universitario de Canarias, España*

Recibido 26 enero 1995/Aceptado 16 octubre 1995

**Summary**

The in vitro susceptibility of 32 *Mycobacterium chelonae* strains to 10 antimicrobial agents was determinated. The sources of the different strains were: clinical samples from patients treated at the Hospital Universitario de Canarias and Hospital del Tórax (General and Chest facilities) and from environmental sources (water supply, sewage, swimming pools and the sea). The susceptibility tests were performed by a broth microdilution method (Mueller-Hinton Broth). The results showed amikacine as the most effective antimicrobial agent against *M. chelonae* isolates, then ofloxacin and cefoxitin. However no statistical difference was detected among them. The least effective was imipenem, followed by ciprofloxacin and norfloxacin.

**Key words**: *Mycobacterium chelonae*, in vitro susceptibility, microdilution broth, amikacine, ofloxacin

**Resumen**

Se determinó la sensibilidad in vitro de 32 cepas de *Mycobacterium chelonae* frente a 10 agentes antimicrobianos. Las cepas pertenecen a muestras clínicas, procedentes de pacientes asistidos en el Hospital Universitario de Canarias y Hospital del Tórax, y de muestras ambientales (aguas de abastecimiento público, residuales, piscinas y de mar). El método utilizado fue el de microdilución en caldo, usando como medio base el Mueller-Hinton Broth. Los resultados mostraron que la amicacina es

* *Correspondencia*: Amelia M. Hernández García. Cátedra de Medicina Preventiva y Salud Pública. Facultad de Medicina. Universidad de La Laguna. 38071 Tenerife. España. Tel.: +34-22-603511. Fax: +34-22-603407.

el antimicrobiano más activo frente a las cepas de *M. chelonae*, seguido por ofloxacina y cefoxitina, aunque no se detectaron diferencias significativas de actividad entre los tres. El menos activo fue imipenem, seguido por ciprofloxacina y norfloxacina.

## Introducción

El papel de las micobacterias atípicas como agentes productores de infecciones humanas ha quedado claramente establecido para algunas especies; particularmente en pacientes inmunodeprimidos (1, 2). Las micobacteriosis raramente se transmiten por contacto directo entre los individuos; la infección en la mayoría de los casos es a partir del medio ambiente natural en el que se localizan las distintas especies.

Entre las micobacterias de crecimiento rápido, una de las que se aísla con más frecuencia en clínica es *Mycobacterium chelonae*. Este microorganismo ubicuo sobrevive fácilmente a la falta de nutrientes y a las temperaturas extremas. Puede ser recuperado del suelo, polvo y agua. Se ha aislado de agua de abastecimiento, fuentes de aguas municipales, áreas húmedas de los hospitales, materiales biológicos contaminados, acuarios, animales domésticos y organismos marinos (3).

La mayoría de las infecciones humanas se adquieren por traumatismo accidental, cirugía o inyección, y no se conocen casos de transmisión en forma interpersonal directa. La vía cutánea es la puerta de entrada habitual de *M. chelonae* (6). Además de este mecanismo puede haber otras formas más raras de transmisión, la ocular en la queratitis, infecciones de válvulas cardíacas o mamoplastias y otras técnicas hospitalarias o medidas agresivas (1, 3, 9). Igual que ocurre con la mayoría de las especies de micobacterias atípicas, *M. chelonae* es resistente a los tuberculostáticos clásicos. Por ello, en nuestro estudio ensayamos la actividad de distintos antimicrobianos frente a cepas de esta especie.

Basándonos en la revisión bibliográfica, se seleccionaron los antimicrobianos más activos frente a este grupo de micobacterias. También ensayamos antimicrobianos de distintos grupos terapéuticos. Finalmente, otros fueron incluidos por no existir estudios previos sobre su actividad frente a este tipo de microorganismos.

## Material y métodos

**Cepas.** En el presente estudio hemos empleado un total de 32 cepas de *M. chelonae*, de las subespecies *chelonae* y *abscessus*, mantenidas a temperatura de congelación (–70°C) en caldo de conservación (Caldo Dubos Base [Difco]) y clasificadas a nivel de subespecie. Estas cepas forman parte de un estudio que se realiza en nuestro departamento y fueron obtenidas a partir de muestras clínicas, procedentes de pacientes asistidos en el Hospital Universitario de Canarias y Hospital del Tórax, valoradas como colonizaciones (6), y de muestras ambientales (aguas de abastecimiento público, residuales, piscinas y de mar).

**Antimicrobianos.** Los antimicrobianos ensayados son los siguientes: ofloxacina, norfloxacina, ciprofloxacina, imipenem, amicacina, tobramicina, eritromicina, estreptomicina, cefoxitina y teicoplanina.

Estos antimicrobianos fueron diluidos en el disolvente apropiado. Norfloxacina lo fue en NaOH 0,05 M, imipenem en tampón fosfato a pH 7, eritromicina en tampón fosfato 0,1 M (pH 8) y el resto de los antimicrobianos en agua destilada estéril neutra hasta alcanzar una solución inicial de 1280 µg/ml.

Las concentraciones críticas para interpretar las micobacterias ensayadas como sensibles o resistentes fueron: ofloxacina (8 µg/ml), norfloxacina (1 µg/ml), ciprofloxacina (2 µg/ml), estreptomicina (32 µg/ml), amicacina (32 µg/ml), tobramicina (8 µg/ml), imipenem (8 µg/ml), cefoxitina (32 µg/ml), eritromicina (4 µg/ml) y teicoplanina (8 µg/ml).

**Estudio de la sensibilidad in vitro.** La sensibilidad a los antimicrobianos se determinó calculando la concentración mínima inhibidora (CMI) por el método de microdilución en caldo (19, 20), ya que la mayoría de las cepas estudiadas crecen mejor en caldo que en agar (13). Se utilizó como medio de cultivo el Mueller-Hinton Broth (Difco), recomendado por varios autores (13, 16, 18). El medio se dispensa mediante una pipeta multicanal Titertek en placas de microdilución de 96 pocillos Microtiter (M24A). Posteriormente, se añade el antimicrobiano y se realizan las diluciones dobles progresivas. De esta manera obtenemos una serie de diluciones de 128 µg/ml a 0,125 µg/ml. El volumen final en cada pocillo de medio más antimicrobiano es de 125 µl. Al último pocillo no se le añade antimicrobiano, quedando como control de crecimiento. Las microplacas así preparadas se mantienen a una temperatura de –70°C, durante un máximo de 7 días, y se descongelan en el momento de la utilización.

Para la preparación del inóculo, a partir del cultivo en Lowenstein-Jensen se transfiere con asa de platino una porción de cultivo a tubos que contienen 10 ml de Middlebrook 7H9 Broth (Difco) con perlas de vidrio estériles. Estos tubos son homogeneizados mediante agitador mecánico e incubados a 30°C durante un período de 3 a 5 días, hasta obtener una densidad óptica o turbidez equivalente al 0,5 de la escala de McFarland (aproximadamente $10^7$–$10^8$ unidades formadoras de colonia [UFC]/ml) (6).

La inoculación de las placas se realiza utilizando un pipeta multicanal, dispensando en cada pocillo 10 µl de inóculo, que tiene una concentración bacteriana de $10^4$–$10^5$ UFC/ml del aislado ensayado. Las microplacas ya inoculadas, son tapadas con parafilm, para evitar la evaporación, e incubadas a 30°C por un período de 3 a 5 días, hasta que haya crecimiento bien visible en los pocillos control.

La CMI se considera la menor concentración de antimicrobiano frente a la cual la micobacteria ensayada no exhibe desarrollo visible. El criterio de crecimiento es una turbidez definida. Al juzgar el punto final es importante considerar el crecimiento (o falta de crecimiento) en el pocillo en estudio, comparándolo con las características de crecimiento en el pocillo control (sin antimicrobiano).

## Resultados

En la Tabla 1 se exponen los valores de CMI en porcentajes acumulados de inhibición de las 32 cepas de *M. chelonae* que fueron ensayadas frente a los 10 antimicrobianos seleccionados.

El 100% de inhibición se alcanza a una CMI de 2 µg/ml de ofloxacina, 4 µg/ml de norfloxacina y 32 µg/ml de ciprofloxacina. Las cepas estudiados muestran una baja sensibilidad a imipenem, obteniéndose el 100% de inhibición a una CMI mayor de 128 µg/ml. Frente a cefoxitina la totalidad de las cepas son inhibidas a CMI inferiores a 32 µg/ml.

TABLA 1. Porcentajes acumulados de inhibición de las 32 cepas de *Mycobacterium chelonae* frente a 10 antimicrobianos

| Antimicrobiano | % de inhibición a las distintas concentraciones (µg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0,125 | 0,25 | 0,5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | >128 |
| Ofloxacina | 15,62 | 53,12 | 81,25 | 90,62 | 100 | | | | | | | |
| Norfloxacina | 6,25 | 18,75 | 53,12 | 78,12 | 87,5 | 96,87 | 100 | | | | | |
| Ciprofloxacina | | | | 3,12 | 9,37 | 43,75 | 78,12 | 90,62 | 100 | | | |
| Imipenem | | | | | | | | 15,62 | 43,75 | 53,12 | | 100 |
| Cefoxitina | | | | 15,62 | 46,87 | 87,5 | 96,87 | 100 | | | | |
| Eritromicina | 3,12 | 15,62 | 31,25 | 50 | 68,75 | 75 | 87,5 | 93,75 | 100 | | | |
| Tobramicina | | | 6,25 | 28,12 | 40,62 | 87,5 | 96,87 | | 100 | | | |
| Estreptomicina | | 3,12 | 9,37 | 15,62 | 25 | 46,87 | 62,5 | 84,37 | 96,87 | 100 | | |
| Amicacina | 9,37 | 37,5 | 59,37 | 93,75 | 96,87 | | | | 100 | | | |
| Teicoplanina | | | | 34,37 | 68,75 | 75 | 87,5 | 96,87 | | 100 | | |

## Discusión

En la actualidad, el estudio de la sensibilidad in vitro de antimicrobianos frente a micobacterias atípicas no está estandarizado, y por lo general se realiza en laboratorios especializados o de referencia. El método empleado por nosotros está ampliamente difundido. La microdilución en caldo (13, 19, 20) es un método que permite ensayar un mayor número de antimicrobianos por aislado en estudio (8 en cada placa), ahorrando tanto medio de cultivo como antimicrobiano, debido al pequeño volumen que requiere el llenado de las microplacas.

Para analizar de manera global la actividad de los diferentes antimicrobianos frente a las cepas de *M. chelonae*, se realizó un análisis de varianza no paramétrico en dos fases (8, 11).

(i) Aplicamos el método del estadístico Q de Cochran, para detectar diferencias significativas entre los antimicrobianos, utilizando el número de cepas que alcanzan el valor crítico para cada antimicrobiano por separado.

Los resultados de las comparaciones múltiples, en esta primera fase, se expresan en la Tabla 2. Los antimicrobianos se ordenan de más a menos activo.

(ii) Se aplica el método de comparaciones múltiples de Friedman, para establecer diferencias basadas en los valores que separan a cada aislado del valor crítico de cada antimicrobiano. Este método se aplica sobre aquellos antimicrobianos entre los que no se detectan diferencias significativas con el estadístico Q de Cochran.

El grupo formado anteriormente en la primera fase, queda dividido en cinco subgrupos. El primer subgrupo lo forman tres antimicrobianos, amicacina, ofloxacina y cefoxitina. El segundo subgrupo incluye desde ofloxacina a estreptomicina. Desde estreptomicina a eritromicina son los antimicrobianos que constituyen el tercer subgrupo. El cuarto subgrupo incluye desde tobramicina hasta norfloxacina. Por último, el quinto subgrupo lo forman norfloxacina, ciprofloxacina e imipenem.

TABLA 2. Resultados de las comparaciones múltiples de 10 antimicrobianos frente a 32 aislados de *Mycobacterium chelonae*

| Antimicrobianos | $C_j$* |
|---|---|
| Ofloxacina | 32 |
| Cefoxitina | 32 |
| Amicacina | 32 |
| Estreptomicina | 31 |
| Tobramicina | 31 |
| Teicoplanina | 28 |
| Norfloxacina | 25 |
| Eritromicina | 24 |
| Ciprofloxacina | 3 |
| Impenem | 0 |

* Número de aislados que no sobrepasan la concentración crítica (para cada antimicrobiano).

El orden de actividad de los antimicrobianos frente a las 32 cepas de *M. chelonae*, de más a menos activo, fue: amicacina, ofloxacina, cefoxitina, estreptomicina, tobramicina, teicoplanina, eritromicina, norfloxacina, ciprofloxacina e imipenem. Ninguna de las cepas de *M. chelonae* estudiadas fue resistente a amicacina, ofloxacina o cefoxitina. De ahí que no apreciemos diferencias significativas de actividad entre ellos, aunque amicacina se comportó como el más activo de estos agentes, teniendo en cuenta las bajas concentraciones necesarias para inhibir la totalidad de las cepas (muy por debajo de su concentración crítica de 32 µg/ml).

Para la mayoría de los autores el rango de concentraciones de amicacina frente a las cepas de *M. chelonae* oscila entre 1 y 16 µg/ml (9, 13, 17, 20).

De manera general, Wallace et al. (17) sugieren que el 91% de los cepas de *M. chelonae* tienen una CMI de 4 µg/ml, o superior. Según estos resultados, nuestras cepas se comportaron de manera más sensible frente a amicacina que las de estos autores. Ofloxacina inhibió a la totalidad de las cepas de *M. chelonae* a una concentración de 2 µg/ml. Este resultado difiere de los obtenidos por otros autores (2, 7, 14) que para alcanzar el 90% de inhibición necesitan concentraciones superiores a 16 µg/ml de ofloxacina frente a numerosas cepas de *M. chelonae*. Para inhibir dichas cepas, según Tomioka et al. (15), se necesitaban concentraciones mucho más altas de ofloxacina.

Todas las cepas de *M. chelonae* ensayadas fueron sensibles a concentraciones menores de 16 µg/ml de cefoxitina. Al comparar los resultados obtenidos frente a cefoxitina con los de otros autores, observamos discrepancias con algunos y coincidencias con otros. Así, Wallace et al. (20) obtienen una CMI de cefoxitina mayor de 64 µg/ml; en otro de sus trabajos (18), afirma que todos las cepas de *M. chelonae* son resistentes a cefoxitina. En cambio, para otros autores estas cepas se comportan de manera más sensible, inhibiéndose la mayoría a concentraciones inferiores a 32 µg/ml (19).

Ciprofloxacina, junto con norfloxacina e imipenem, fueron los antimicrobianos que menos actividad tuvieron frente a las cepas de *M. chelonae*. La marcada resistencia de estas cepas a ciprofloxacina es confirmada por la mayoría de los autores consultados (2, 4, 10, 13, 14). Algunos de ellos (5, 12) plantean que la diferente sensibilidad a ciprofloxacina puede utilizarse para diferenciar taxonómicamente *M. chelonae* de *M. fortuitum*.

## Bibliografía


1. Bartsch, P., Fraselle, R., Koemoth, P. (1991). Les mycobacterioses incidence croissante en pathologie humaine. Revue Médicale de Liège **10**, 542–553.
2. Casal, M., Rodríguez, F., Villalba, R., Benavente, M. C., González, A. I. (1987). In vitro susceptibility of *Mycobacterium fortuitum*, *Mycobacterium chelonae* and *Mycobacterium avium* against some quinolones. Chemother. **6**, 431–433.
3. Casal, M. (1992). Micobacterias. *In* Perea, E. J. (ed.), Enfermedades infecciosas y microbiología clínica, vol. 1, pp. 745–753. Doyma S.A., Barcelona.
4. Collins, C. H., Uttley, H. C. (1988). In vitro activity of seventeen antimicrobial compounds against seven species of mycobacteria. J. Antimicrob. Chemother. **22**, 857–861.
5. Collins, C. H., Yates, M. D., Uttley, A. H. C. (1985). Differentiation of *Mycobacterium chelonei* from *Mycobacterium fortuitum* by ciprofloxacin susceptibility. J. Hyg. Camb. **95**, 619–621.
6. Hawkins, J. E., Wallace Jr., R. J., Brown, B. A. (1991). Antibacterial susceptibility test: mycobacteria. *In* Balows, A., Hausler Jr., W. J., Isenberg, H. D., Shadomy, H. J. (ed.), Manual of Clinical Microbiology, 5th. ed., pp. 1138–1152. American Society for Microbiology, Washington, D.C.
7. Leysen, D. C., Haemers, A., Pattyn, S. (1989). Mycobacteria and the new quinolones. Antimicrob. Agents Chemother. **33**, 1–5.
8. Martin, A., Luna, J. D. (1989). Análisis de la varianza. *In* Bioestadística para las ciencias de la salud, 2a. ed., pp. 380–340. Ediciones Norma S.A., Madrid.
9. Sanders, W. E., Horowitz, E. A. (1991). Otras especies de micobacterias. *In* Mandell, G. L., Douglas, R. G., Bennett, J. E. (ed.), Enfermedades Infecciosas, tomo 1, 3a. ed., pp. 2026–2238. Editorial Médica Panamericana, Buenos Aires.
10. Sanz, J., Giron, A. J., Guerrero, A., et al. (1989). Infección por *Mycobacterium chelonei*. Rev. Esp. Microbiol. Clin. Junio, 372–376.
11. Siegel, S. (1990). Pruebas estadísticas paramétricas y no paramétricas. *In* Estadística no paramétrica. Aplicada a las ciencias de la salud, 3a. ed., pp. 52–55. Editorial Trillas, México.
12. Steele, L. C., Wallace Jr., R. J. (1987). Ability of ciprofloxacin but not pipemidic acid to differentiate all three biovariants of *Mycobacterium fortuitum* from *Mycobacterium chelonae*. J. Clin. Microbiol. **25**, 456–457.
13. Swenson, J. M., Wallace Jr., R. J., Silcox, V. A., Thornsberry, C. (1985). Antimicrobial susceptibility to five subgroups of *Mycobacterium fortuitum* and *Mycobacterium chelonae*. Antimicrob. Agents Chemother. **28**, 807–811.
14. Texier-Maugein, J., Mormede, M., Fourche, J., Bebear, C. (1987). In vitro activity of four fluoroquinolones against eighty-six isolates of mycobacteria. Eur. J. Clin. Microbiol. **6**, 584–586.
15. Tomioka, H., Sato, K., Saito, H. (1991). Comparative in vitro and in vivo activity of fleroxacin and ofloxacin against various mycobacteria. Tubercle **72**, 176–180.
16. Wai Yew, W., Yuk Lin Kwan, S., Keung Ma, W., Sang Lui, K. (1990). Minimal bactericidal and inhibitory concentrations of ofloxacin on *Mycobacterium fortuitum* at pH 7 and 5: therapeutic implications. Tubercle **71**, 205–208.
17. Wallace Jr., R. J., Nash, D. R., Steele, L. C., Steingrube, V. (1986). Susceptibility testing of slowly growing mycobacteria by a microdilution MIC method with 7H9 broth. J. Clin. Microbiol. **24**, 976–981.
18. Wallace Jr., R. J., Brown, B. A., Onyi, G. O. (1991). Susceptibility of *Mycobacterium fortuitum* biovar *fortuitum* and two subgroups of *Mycobacterium chelonae* to imipenem, cefmetazole, cefoxitin, and amoxicillin-clavulanic acid. Antimicrob. Agents Chemother. **35**, 773–775.
19. Wallace Jr., R. J., Brown, B. A., Silcox, V. A., et al. (1991). Clinical disease, drug susceptibility, and biochemical patterns of the unnamed third biovariant complex of *Mycobacterium fortuitum*. J. Infec. Dis. **163**, 598–603.
20. Wallace Jr., R. J., Swenson, J. M., Silcox, V. A., Bullen, M. G. (1985). Treatment of nonpulmonary infections due to *Mycobacterium fortuitum* and *Mycobacterium chelonei* on the basis of in vitro susceptibilities. J. Infect. Dis. **152**, 500–514.

MICROBIOLOGÍA SEM **11** (1995), 491–498

# Breve historia de la Sociedad Española de Microbiología, II. De 1971 a 1977

Concepción García Mendoza

*Centro de Investigaciones Biológicas, CSIC, Madrid, España*

*Este artículo es el segundo de la serie que dedica la autora a narrar sucintamente la historia de la Sociedad Española de Microbiología (SEM), con motivo de la celebración de su cincuenta aniversario. El primero fue publicado en el número anterior de la revista* (Microbiología SEM **11**: 359–368).

## Bienio 1971–1973: III Congreso Nacional de Microbiología y creación de diferentes Grupos de trabajo

El III Congreso Nacional, organizado por Agustín Pumarola Busquets, se celebró en los locales del Colegio Oficial de Médicos y de la Academia de Ciencias Médicas de Cataluña y de Baleares (Paseo de la Bonanova, Barcelona), los días 3 a 5 de junio de 1971. Pronunció la conferencia inaugural R. Sohier, profesor de la Universidad de Lyon y Socio de Honor de la SEM. El Congreso incluyó dos simposios sobre "Enseñanza de la Microbiología" y "Genética bacteriana", así como diez sesiones de comunicaciones. Se presentaron unas doscientas comunicaciones orales y participaron unos cuatrocientos microbiólogos.

La Junta General Ordinaria de la Sociedad tuvo lugar durante el Congreso. En la misma se acordó, por primera vez, organizar el pago de la cuota social, dada la dispersión de sistemas de cobro existentes hasta el momento. Seguidamente, se debatió la creación de los grupos de trabajo dentro de la Sociedad, tema ya propuesto anteriormente a la Asamblea, aprobándose la consolidación de la Delegación Regional de Cataluña como broche de oro a la finalización del Congreso. Respecto a la ubicación del siguiente Congreso Nacional, se decidió que tuviese lugar en Granada, en 1973, y que fuese organizado por Vicente Callao y José Olivares. Dada la necesidad cada vez más imperiosa de distribuir información sobre las diferentes actividades de la Sociedad a sus socios, se propuso editar un *Boletín Informativo*. Julio R. Villanueva se comprometió a realizarlo en su departamento de la Universidad de Salamanca.

*Correspondencia*: Concepción García Mendoza. Centro de Investigaciones Biológicas. CSIC. Velázquez, 144. 28006 Madrid. España. Tel.: +34-1-5611800. Fax: +34-1-5627518.

La renovación parcial de la Junta Directiva dio el siguiente resultado: Presidente, David Vázquez Martínez; Tesorero, Domingo Rodríguez Sánchez; Bibliotecario, Emilio Ronda Laín; y Vocales, Fernando Baquero Mochales, Jorge López Tello, Baldomero Íñigo Leal y Julio R. Villanueva.

Recién constituida la nueva Junta Directiva, su Secretario, Julio Pérez Silva, presentó su dimisión, por haber accedido a la Cátedra de Microbiología de la Facultad de Biología de Sevilla. Fue nombrado Secretario en funciones Antonio Portolés Alonso. La misma Junta Directiva nombró a Fernando Baquero vocal en el Consejo de Redacción de la revista *Microbiología Española*, en sustitución de Juan Santa María, en un intento más de conseguir la inclusión de dicha revista en los "Citation Indexes" de Biología.

Pendiente todavía de ser aprobada la enmienda de los Estatutos de la Sociedad por el Gobierno Civil, y habida cuenta de la consolidación de la Delegación de Cataluña y Baleares como Grupo de trabajo independiente dentro de la misma, se constituyó una comisión formada por Ramón Parés, Antonio Portolés, Fernando Baquero y Jorge López. Ésta debía realizar el anteproyecto relativo a las relaciones entre las distintas Delegaciones y Grupo de trabajo con la Sociedad, para su posterior inclusión en los nuevos Estatutos. La Delegación Regional de Cataluña y Baleares nombró su correspondiente Junta Directiva: Presidente, Ramón Parés Farràs; Vicepresidente, Amadeo Foz Tena; Secretario, Luis Arcalís Arce; Tesorero, Alberto Ramos Cormenzana; y Vocales, Agustín Pumarola Busquets, Francisco González Fuste y Antonio Rodríguez Torres.

Paralelamente, y con motivo de la celebración en Madrid de la I Reunión de Virólogos Españoles en 1971, Agustín Pumarola, presidente de la I Ponencia en la misma, comunicó a la Junta Directiva de la Sociedad la posibilidad de organizar un Grupo Especializado sobre Virus. Esta iniciativa se vio totalmente respaldada, por entrar dentro de las actividades emprendidas en ese momento por nuestra Sociedad. A estos efectos, y dado que la Sociedad estaba preparando un simposio sobre infecciones microbianas, el entonces presidente, David Vázquez, propuso incluir en el mismo otro simposio dedicado a los virus. Por ello, Agustín Pumarola que, durante el II Congreso Internacional de Virología (Budapest, 1971) se había comprometido a organizar en España el siguiente para 1975, propuso invitar a Peter Wildy (Birmingham, Gran Bretaña) en su calidad de miembro del Comité Internacional de Virología. Para la organización de este simposio se nombró una comisión formada por Florencio Pérez, Emilio Ronda, Miguel Rubio e Isidro Valladares.

Igualmente, Julio R. Villanueva, que había asistido como delegado de la SEM al V Congreso Latinoamericano de Microbiología (Punta del Este, Uruguay, 1971), informó sobre los acuerdos relativos a la celebración de los próximos Congresos Latinoamericanos. Así, en 1973 tendría lugar en Caracas (Venezuela), en 1975 en Argentina, y en 1977, tentativamente, en España. Próxima la celebración en Salamanca del III Simposio Internacional sobre "Protoplastos de levaduras", cuyo patrocinio había sido solicitado anteriormente a la Sociedad, su organizador, Julio R. Villanueva, presentó el programa, que incluía a numerosos científicos españoles y extranjeros de renombre internacional. La Junta Directiva acordó que su felicitación quedase reflejada en la correspondiente acta. Seguidamente, ante tal cúmulo de actividades, Julio R. Villanueva solicitó su relevo en el Comité de Redacción de la revista *Microbiología Española*. Fue sustituido por Agustín Pumarola, con lo que se mantenía la tendencia a incluir representantes de lugares distintos de Madrid en las diversas actividades de la SEM.

Continuando la política de creación de secciones especializadas dentro de la Sociedad, la Junta Directiva apoyó la propuesta de Carlos Ramírez de organizar un Grupo Especializado de Micología.

Siguiendo la trayectoria iniciada por las anteriores Juntas Directivas relativas a la celebración de simposios coordinados entre la Sociedad y la industria, Fernando Baquero, que estaba preparando un Simposio sobre "Infecciones bacterianas" en Madrid, propuso la organización de otro sobre "Rifamicinas", en Málaga. Por su parte, Antonio Portolés presentó el programa preliminar del III Simposio sobre "Aplicaciones de la espectroscopía molecular en biología microbiana", que estaba organizando en La Laguna. Finalmente, a raíz de las fructíferas relaciones con Hispanoamérica, la Asociación Latinoamericana de Microbiología propuso a Julio R. Villanueva como delegado español en el Comité Ejecutivo de la Asociación.

En las elecciones reglamentarias de finales del año 1972, el entonces Presidente, David Vázquez, propuso a la Junta Directiva una candidatura única, que fue aceptada, por lo que entraron a formar parte de la misma, como Vocales, Ramona Beltrá Martínez de Velasco, Jesús M. Repáraz Martínez de Azagra, José Olivares Pascual y Emiliano Esteban Velázquez.

El primer *Boletín Informativo*, redactado bajo la dirección de Julio R. Villanueva y Federico Uruburu en el Departamento de Microbiología de la Universidad de Salamanca, apareció también a finales de 1972. Quedó encargada de la revisión del mismo Ramona Beltrá, quien se ofreció, además, para organizar el Grupo Especializado de Fitopatología. Paralelamente, se propuso la constitución del Grupo Regional del Noroeste, de cuya organización se encargó Benito Regueiro Varela. Igualmente, encontrándose en gestación el Grupo Especializado de Microorganismos Patógenos e Inmunología, fueron nombrados como comisión gestora del mismo Fernando Baquero y Antonio Portolés, instándose a Jorge López Tello para que comenzase a estructurar el grupo de Microbiología Industrial. Los Presidentes de los Grupos Especializados y Regionales, ya consolidados o en formación, pasaron a formar parte de la Junta Directiva como Vocales natos de la misma.

Por otro lado, y a petición de la IAMS, se nombraron tres delegados de la Sociedad para las nuevas secciones de esta entidad internacional: Fernando Baquero, por Bacteriología, José Luis Fernández, por Micología y Emilio Ronda, por Virología, en caso de que el Grupo Especializado de Virus, todavía en formación, no tuviese presidente ya elegido. También, Jesús M. Repáraz, a la sazón Vocal de la Junta Directiva, propuso fundar un Centro Unificado de Microbiología en Pamplona, con la total aceptación por parte de la misma.

Tras la aprobación previa de la enmienda de Estatutos por parte de la Dirección General de Política Interior, y siguiendo las normas preceptuadas por la legislación vigente, se hizo necesaria la convocatoria de la correspondiente Junta General Extraordinaria para aprobar el texto definitivo de los citados Estatutos. En cuanto a la revista *Microbiología Española*, se produjo un recorte de financiación por parte del CSIC. Se decidió entonces la separación de tesorerías entre la revista y la SEM, pero ésta continuó subvencionándola, a la vez que participaba en sus distintos órganos de gestión.

El Grupo Regional del Noroeste, recién constituido, celebró su primera Reunión Científica, sobre "Microbiología hospitalaria", en Santiago de Compostela; fue dirigida por Benito Regueiro. Paralelamente, el Grupo Especializado de Virus presentó su Junta Directiva recién constituida: Presidente, Florencio Pérez Gallardo; Vicepresidente, Agustín Pumarola Busquets; Secretario, Rafael Nájera Morrondo; Tesorero, Ángel García Gancedo; y Vocales, Ángel M. Ordás Álvarez, Luis Valenciano Clavel, Guillermo Suárez Fernández, Eladio Viñuela Díaz y Antonio Peña Iglesias.

Con motivo de la próxima celebración del IV Congreso Nacional en Granada, David Vázquez y Julio R. Villanueva propusieron el nombramiento de Socios de Honor de nuestra Sociedad a Nikolas Van

Uden, del Centro de Estudios Avanzados de la Fundación Gulbenkian, en Oeiras (Portugal), y a Mark Richmond, de la Universidad de Bristol (Gran Bretaña), para que ambos nombramientos fuesen refrendados en la Asamblea a celebrar durante el citado Congreso Nacional.

## Bienio 1973–75: IV Congreso Nacional de Microbiología y actividades de los Grupos Especializados

Organizado por José Olivares, Secretario del Comité Organizador, por fallecimiento reciente de Vicente Callao Fabregat, Presidente del mismo, el IV Congreso Nacional tuvo lugar durante los días 3 a 5 de octubre de 1973 en la Facultad de Ciencias de la Universidad de Granada. El Congreso contó con un simposio de varias sesiones sobre "Genética microbiana" y ocho sesiones de comunicaciones. En estas sesiones se presentaron alrededor de doscientas comunicaciones orales y participaron más de cuatrocientos microbiólogos. Estos Congresos Nacionales comenzaron a ser el punto de encuentro obligado entre los distintos grupos de microbiólogos distribuidos a lo largo de la geografía nacional.

En la Asamblea celebrada en Granada, durante el IV Congreso Nacional, se aplazó la renovación parcial de la Junta Directiva debido a que la candidatura propuesta no consiguió difusión suficiente. Por primera vez los Grupos Regionales de Cataluña y Baleares y del Noroeste, así como el Especializado de Virus, presentaron un resumen de sus actividades. Como era habitual en estas reuniones plenarias, se refrendaron los nombramientos de Socios de Honor y se expusieron las diversas incidencias de la Sociedad ocurridas durante el bienio. Se acordó que el siguiente Congreso Nacional se celebrase en Salamanca, en 1975.

La Sociedad nombró como sus representantes en el Comité de Ecología Microbiana de la IAMS a Fernando Baquero y Domingo Rodríguez. Este último fue nombrado también delegado en la organización de una Federación de Sociedades Europeas de Microbiología (FEMS).

El Grupo de Microbiología de Alimentos, en formación, propuso organizar una reunión en León en 1974, y así poder consolidarse en el futuro. Dicho encuentro fue celebrado finalmente como II Reunión Científica del Noroeste sobre el tema "Intoxicaciones y toxiinfecciones alimentarias de origen bacteriano" y fue organizada por J. Burgos González, B. Moreno García, S. Ovejero del Agua y J. Sala Trepat.

La renovación parcial de la Junta Directiva, efectuada a principios de 1974, dio el siguiente resultado: Presidente, Fernando Baquero Mochales; Tesorero, Domingo Rodríguez Sánchez; Bibliotecario, Jorge López Tello; y Vocales, Federico Uruburu Fernández y Amadeo Foz Tena. En un intento más de impulsar la revista *Microbiología Española* y de que se cumpliesen los plazos de publicación, se nombró a Jorge López Tello, en su calidad de Bibliotecario de la SEM, Vocal de la Junta Gestora de la misma. Se decidió igualmente la publicación de un número especial de la revista sobre el I Simposio sobre "Infecciones bacterianas", celebrado en Madrid en 1972. Encontrándose todavía sin avanzar la constitución del Grupo Especializado de Micología, se decidió encargar de su reactivación a José Luis Arribas Llorente y Aurora Sánchez Sousa.

Federico Uruburu, director de la Colección Española de Cultivos Tipo (CECT) en Salamanca, con motivo de su traslado a la cátedra de Microbiología de la Universidad de Bilbao, informó a la Sociedad acerca de las posibilidades de reorganizar esta Colección en dicha localidad, a la que podría sumársele el Servicio de Identificación de Cepas Bacterianas. Esta iniciativa fue totalmente respaldada por la Junta

Directiva. En relación con el Grupo de Infecciones Bacterianas, aún en constitución, se decidió ampliar su Junta Gestora con la incorporación de Julio Casal y Evelio Perea, cambiando su anterior nombre por el de Grupo Especializado de Microbiología Clínica. Paralelamente, y para gestionar el Grupo Especializado de Microbiología de Alimentos, igualmente en formación, se nombró otra Junta Gestora formada por Bernabé Sanz, Cándida González, Antonio Rodríguez y Manuel Rodríguez. También, ante la ambigüedad de la denominación de nuestra Sociedad, se decidió empezar a utilizar las siglas SEMIC en lugar de SOEMI, de las que sólo se había hecho uso esporádico.

Durante este período se intentó que, tanto en Madrid como en distintos puntos de las Grupos Regionales, se celebrasen Reuniones o Mesas Redondas mensuales sobre temas de tipo general y, a poder ser, que las de Madrid coincidieran con las Juntas Directivas. El Grupo de Virología, recién creado, celebró su I Reunión Científica en Madrid en 1974, contando con la participación de Peter Wildy, de quien ya hicimos mención anteriormente. Con motivo de la celebración en Madrid de la Mesa Redonda sobre "Transmisión de la información genética en microorganismos" se publicó un libro, recogiendo todas las aportaciones presentadas, que constituyó el primer volumen de la serie de Monografías Básicas de la SEM. Tratando de impulsar la formación del Grupo de Micología, se propusieron como Socios de Honor de la Sociedad a J. Acar (Hospital Broussais, París, Francia) y a E. Drouhet (Instituto Pasteur, París, Francia), eminentes micólogos que formaron parte de la I Reunión Nacional de Micología, celebrada en Toledo en 1975. La participación de nuestra Sociedad en la FEMS como una de las Sociedades fundadoras, actuando Domingo Rodríguez como delegado español, siguió su curso hasta la consolidación de dicha Federación en 1974, con la elección de André Lwoff (Instituto Pasteur, París, Francia) como Presidente. Así mismo, la Sociedad se integró con las Sociedades Españolas de Fisiología, Bioquímica y Genética para formar la Asociación de Sociedades de Biología Experimental (ASBE), que posteriormente se constituyó como Federación y se denominó FESBE.

## Bienio 1975–77: V y VI Congresos Nacionales de Microbiología y vicisitudes de los Grupos Especializados y Regionales

El V Congreso Nacional, organizado por José A. García Rodríguez, se celebró durante los días 1 a 4 de octubre de 1975, en los edificios de la antigua Universidad de Salamanca (Fig. 1). El Congreso incluyó tres simposios internacionales sobre "Quimioterapia antibacteriana", "Estructura y función en microorganismos" y "Gérmenes anaerobios", así como nueve sesiones de comunicaciones. Contribuyeron más de doscientas comunicaciones orales y asistieron más de quinientos microbiólogos, con una considerable participación internacional.

La Junta General correspondiente tuvo lugar en Salamanca durante el Congreso Nacional y en ella se presentaron las múltiples actividades llevadas a cabo por la Sociedad durante el bienio anterior. Éstas se referían a los Grupos Regionales de Cataluña y Baleares y del Noroeste, así como a los Grupos Especializados (bien los ya consolidados, como el de Virología, bien los que tenían un funcionamiento casi normalizado, como los de Fitopatología y Microbiología Clínica, o bien los que estaban en fase de gestión, como Microbiología de Alimentos y Micología). El Grupo de Virología participó muy activamente en el III Congreso Internacional de Virología, realizado en septiembre de 1975 en Madrid, y organizado conjuntamente con la Sección de Virología de la IAMS. El Grupo de Microbiología Clínica

FIG. 1. Diversos miembros del Departamento de Microbiología de la Universidad de Salamanca, durante el V Congreso Nacional (Salamanca, 1975). De izquierda a derecha, Ángel Domínguez, M. Victoria Elorza, Tahía Benítez, César Nombela, Fernando Laborda, Sra. de Nicolás, Gregorio Nicolás, Julio R. Villanueva, Isabel García Acha, Rafael Sentandreu, Sra. de Hardisson, Carlos Hardisson, Paloma Liras, Juan Francisco Martín, Dolores García López, Carlos Lostau y Federico Uruburu, y abajo, Francisco del Rey, Tomás Santos, Eugenio Santos, Santiago Gascón y César de Haro.

realizó distintas Reuniones de carácter monográfico para su total consolidación, y el de Fitopatología estaba gestionando su I Reunión Científica.

En relación con las publicaciones de la Sociedad, se hizo especial mención a las dificultades de cofinanciación que estaba sufriendo *Microbiología Española* por parte del CSIC. Referente al *Boletín Informativo*, adscrito al Departamento de Microbiología de la Universidad de Salamanca, su coordinación fue encomendada a César Nombela.

A su vez, el Presidente, Fernando Baquero, presentó la propuesta de nombramiento de Socios de Honor de la SEM a Peter Wildy, J. Acar y E. Drouhet, para que fuese respaldada por los socios allí presentes. Se decidió que el próximo Congreso Nacional tuviese lugar en Santiago de Compostela, en 1977, y fuese organizado por Benito Regueiro.

Con motivo de haberse integrado nuestra Sociedad en la FESBE, y continuando con sus actividades habituales, la Junta Directiva propuso el tema "Relación huésped-parásito" como aportación al I Congreso organizado por la citada Federación, que se celebró en Madrid en 1976. Posteriormente, esas aportaciones fueron publicadas como el segundo número de las Monografías Básicas de la SEM. Además, en 1976 tuvo lugar el 3rd European Meeting on Bacterial Transformation and Transfection, patrocinado por nuestra Sociedad y organizado por Antonio Portolés en Granada. En relación con la constitución del Grupo de Microbiología Industrial, se aprobó la propuesta de Juan Francisco Martín

para hacerse cargo de la gestión del mismo. El Grupo de Microbiología Clínica propuso, para 1977, la celebración de una Reunión sobre "Brucelosis" en Valladolid, organizada por Antonio RodríguezTorres y, con la colaboración del Grupo del Noroeste, otra Reunión sobre "Salmonelosis" en Barcelona, a cargo de Amadeo Foz, y otra en Sevilla, gestionada por Evelio Perea, sobre "Control de calidad en microbiología clínica". Por otra parte, las relaciones entre la SEM y la revista *Microbiología Española* llegaron a su límite debido a que la Sociedad tenía que hacer frente a todos los gastos. Se acordó entonces que aquélla sería la última aportación si no se producían cambios radicales en las relaciones sobre este tema entre la SEM y el CSIC.

La propuesta de un nuevo Grupo Especializado de Microbiología del Suelo, con José Olivares como gestor, vino a sumarse a los Grupos ya en marcha. En este sentido, el Grupo Regional de Cataluña y Baleares mostraba escasa actividad científica, mientras que el del Noroeste ya había organizado su III Reunión Científica (Oviedo, 1976), sobre "Morfogénesis y diferenciación microbianas", bajo la dirección de Carlos Hardisson, y además colaboró con el Grupo de Microbiología Clínica en la Reunión sobre "Brucelosis" antes mencionada. Finalmente, nuestra Sociedad, de común acuerdo con los gestores de *Microbiología Española* del Instituto Jaime Ferrán, ante la falta de financiación por parte del CSIC, se desvincularon de los acuerdos preestablecidos, decidiendo que la SEM creara su propia revista.

Correspondiendo la renovación parcial de la Junta Directiva, ésta misma presentó una candidatura única en la Asamblea celebrada durante el I Congreso de FESBE (Madrid, 1976), que fue aceptada . Dio los siguientes resultados: Secretario, Emilio Muñoz Ruiz; y Vocales, Enrique Hernández Giménez, Carmen Rubio Calvo, José Miguel Barea Navarro y Eulalia Cabezas de Herrera Sánchez. En la misma Asamblea, los Grupos Especializados y Regionales presentaron las actividades realizadas durante el bienio. Como novedades citaremos a Eloy Mateo-Sagasta, Presidente del Grupo de Fitopatología, que anunció la celebración de la I Reunión Científica para 1977, y a Eladio Viñuela, en nombre del Grupo de Virología, que presentó el proyecto de un Catálogo de Virus de Interés Agropecuario.

La nueva Junta Directiva prosiguió sus actividades habituales incorporando el Grupo Especializado de Microbiología Industrial, en fase de gestión, a cargo de Juan Francisco Martín, con la colaboración de Federico Uruburu y Paloma Liras. Éstos propusieron, y así se aceptó, que el citado grupo se incorporara a la recién constituida Sociedad Europea de Biotecnología. El Grupo de Virología, durante este tiempo encabezado por Eladio Viñuela, contribuyó con la elaboración del Catálogo de Líneas Celulares, y el Grupo de Microbiología de Alimentos, al mando de Cándida González, presentó el programa de la I Reunión Científica del grupo, a celebrar en Madrid en 1977, organizada por Bernabé Sanz. El Grupo de Microbiología del Suelo, en fase de gestación, a cargo de José Miguel Barea, decidió discutir sus posibles relaciones con otros Grupos Especializados durante el próximo Congreso Nacional. Dada la ausencia de actividades por parte de la junta gestora del Grupo de Micología, se propuso la reorganización del mismo. Por su parte, el Grupo de Microbiología Clínica, dada sus relaciones con grupos internacionales de Quimioterapia, solicitó ampliar su denominación como Microbiología Clínica y Quimioterapia. Esta propuesta sería debatida durante el Congreso Nacional de Santiago de Compostela.

Con respecto a la edición de una nueva revista a cargo de la Sociedad, se acordó denominarla *Biología Microbiana*, nombrando editor de la misma a Jorge López Tello. Dicha revista podría aparecer por vez primera después de la celebración del siguiente Congreso Nacional, en 1977. En este Congreso, se introdujo la novedad de organizar una Mesa Redonda específica por parte de cada Grupo Especializado, así como la presentación de comunicaciones libres en forma de paneles. Se decidió, además, que en el

FIG. 2. Nombramiento de Doctores "Honoris Causa" a H. Umezawa y Sir E. Chain durante el VI Congreso Nacional de Santiago de Compostela, en 1977, con su organizador Benito Regueiro (a la izquierda de la foto) y Manuel Domínguez Carmona (a la derecha).

Congreso se hiciera entrega de la Primera Medalla de la Sociedad a Lorenzo Vilas, uno de los fundadores de la SEM (véase *Microbiología SEM* **11**(3), p. 360), como homenaje a su labor a lo largo de treinta años. Finalmente, y a requerimiento de la IAMS, que había propuesto su transformación en Unión Internacional de Sociedades de Microbiología (IUMS) en lugar de proseguir como Asociación, la Sociedad tuvo que participar en la enmienda de los correspondientes Estatutos.

El VI Congreso Nacional se celebró en Santiago de Compostela durante los días 6 a 9 de julio de 1977 y fue organizado por Benito Regueiro Varela. La conferencia inaugural fue pronunciada por Sir E. Chain (Gran Bretaña), quien fue investido Doctor "Honoris Causa" de la Universidad de Santiago, junto con H. Umezawa (Japón) (Fig. 2). El Congreso incluyó dos Simposios Internacionales, sobre "Microbiología Marina" y sobre "Resistencia a los antibióticos", a cargo de diferentes especialistas nacionales y extranjeros. Se organizaron seis Mesas Redondas, por los correspondientes Grupos Especializados, sobre: "Microbiología Clínica", "Micología", "Fitopatología", "Virología", "Microbiología de Alimentos" y "Microbiología Industrial". A su vez se estableció un Coloquio sobre Educación Microbiológica, cuatro sesiones demostrativas (sobre Identificación de Hongos Patógenos, Identificación de Protozoos, Diagnóstico de Infecciones por Anaerobios y Control de Infecciones Hospitalarias), y catorce sesiones de comunicaciones libres. Éstas se distribuyeron en comunicaciones orales y paneles. Hubo cerca de cuatrocientas aportaciones entre los diferentes tipos de sesiones y asistieron unos seiscientos microbiólogos.

---

Y hasta aquí, la segunda entrega de esta breve historia de la SEM. En el número siguiente de la revista aparecerá la continuación (años 1977 a 1983).

MICROBIOLOGÍA SEM **11** (1995), 499–502 **OPINION**

# Reflections on scientific evaluation. Some comments on the 8th Conference of the International Federation of Science Editors

Salvador Reguant

*Department of Dynamic Geology, Geophysics and Paleontology, School of Geology, University of Barcelona, Barcelona, Spain*

From July 9 to 13, 1995, the 8th Conference of the IFSE (International Federation of Science Editors) took place in Barcelona under the motto "Science, Culture and Communication for the 21st Century". Among the different issues on which IFSE focuses its activity, some major aspects of the current scientific evaluation systems were discussed. Eugene Garfield, from the Institute for Scientific Information (ISI), Philadelphia, presented a contribution under the title "The impact of cumulative impact factors". Besides, a session on the Peer Review System was held, and some other contributions dealt with the role of national journals. Most of those contributions will be published in the corresponding Proceedings.

I do not intend to sum up all things stated in that Conference. However, I would like to write my reflections on the current situation and efficiency of the scientific evaluation systems in the light of some approaches discussed at the meeting.

At present, the evaluation systems used in most developed countries are based on two formulae frequently interlinked: the "peer review" or analysis of the research to be published (or already published) or to be granted, and the indirect valuation of the results both through the relevance given to the journal of publication and through the quantitative analysis of the citations. The latter, which is a double indirect valuation, theoretically different, frequently turns out to be redundant.

## Analysis of the results obtained as a method for the evaluation of an article

This method is as old as science, and the current peer review, which is undertaken before the publication process, is just one of the formulae used. The selection procedures to incorporate new professors or researchers in a center are based on the same grounds. In fact, this process is a common procedure whenever excellence is sought. Such is the case, for example, when a soccer club hires those players that have obtained the best results in previous performances.

In the last few years, a certain novelty has been the establishment of a systematic "filtering" before publishing the results in any well known scientific journal, i.e. the peer review system. In fact, in a less formal way, this was already a

*Correspondence to*: Salvador Reguant. Departament de Geologia Dinàmica, Geofísica i Paleontologia. Facultat de Geologia. Universitat de Barcelona. Martí i Franqués, s/n. 08028 Barcelona. España. Tel.: +34-3-4021596. Fax: +34-3-4021340.

tradition; the editor of the journal might or might not accept a paper basing his decision either on his/her own judgement or on the judgement of some consultant. The system has brought about many mistakes, as many famous scientists like to mention while showing the letter received from a major journal rejecting his/her (later) famous paper. However, as someone at the IFSE-8 pointed out, like democracy, the peer review is a bad system, yet it is the least bad of all known systems of evaluation.

I for one agree to accept the peer review system. What I do not consider to be right is the lack of critical analysis of the mistakes which this system favours. Such a critical analysis would allow the appropriate modifications, leading to an improvement in the outcome of the method. As a matter of fact, science is also a "commercial product" which must be diffused. Without diffusion, there is no science. Thus, the "seller" of the product, i.e. the scientific editor, must assess its quality so that his/her "shop" has the prestige which is required to make the product acceptable to the "consumer", i.e. the scientist. Obviously, the location of the "shop" is of great importance. The sales are not the same in shops located downtown, on the outskirts of a town, or in a small village. Regardless of the actual quality, scientific products are more appreciated if they are released in countries with a high density of both scientific institutions and researchers of well known prestige. Therefore the papers published in journals from the richest countries, and in English, are considered good by definition. This is a complex topic, to which I do not want to pay much attention now, but which I have previously discussed (7).

Let us come back to the failures of the system, some of which are egregious. In the history of the introduction and acceptation of the theory of the plate tectonics, we can find an illustrative anecdote. The idea of the plate tectonics arose from the adequate interpretation of magnetic anomaly bands found on the bottom of some oceans, which were parallel to the ocean trenches. The interpretation was that the ocean bottom expanded from those trenches. Two researchers, Lawrence Morley, from the Canadian Geological Survey, and Fred Vine, from Cambridge University, independently understood that mechanism. Morley's article on the issue was rejected by *Nature* in June 1963. Nor was it accepted by the *Journal of Geophysical Research* (USA). Luckily for science, Vine was more fortunate, and he and Matthews had their article published in *Nature* later that year (3). Other cases have occurred, some of which come to lay people's notice through the press.

To sum up, I think it is absolutely necessary that either previously or after their publication, the scientific results obtained by a researcher be evaluated by other scientists. This will guarantee not only that the published results are scientifically sound, but also that money granted for research work is well invested. This must be complemented by a continuous evaluation to correct any failure in the review and valuation (6).

## Evaluating science through the evaluation of the journals. Impact factors

Recently criteria based on quantitative analyses have produced lists of journals which judge the "product" by the rank of its "container". The category given to a paper depends on both the journal where it has been published and how many citations it has deserved.

This system is actually more convenient because it does not imply an examination of the "product". It is commonly assumed that journals of well known prestige select the papers most carefully and on serious grounds, like prestigious companies, which usually sell only products of high quality. Besides, the use of the citation rate is based on a rational assumption. In fact, the better the results described in an article, the more cited the article tends to be.

Stating that articles published in a "good" journal are reliable, and that the number of citations of a given paper is a good index of its quality, usually coincides with reality. What is absolutely unsound is the opposite statement, to assume that articles published in "not so good" journals are not reliable, and that articles rarely cited are of no scientific value. In any case, these criteria are an unsound approach and are more likely to errors and deviations than both the analysis of the results themselves and the peer review are.

These errors and deviations may have a multiple origin. First of all, one must take into account the fact that the researchers more likely to have their articles published in "good" journals are those who can easily keep in contact with those journals. Those who have a good command of English also have an advantage. In the first case it is something obvious, which can be explained by the rules of human behavior. It is easier for an American to be in contact with an American journal of recognized prestige than it is for a Spaniard, for example, unless the latter has established previous special contacts with some person responsible for the journal (1). As far as language is concerned, all "good" journals are written in English. Thus, articles written in English are more likely to be cited than articles written in any other language. This fact is important, especially in the case of researchers having English as a first language, and it has been reported by different authors (4, 5). One wonders whether English journals are actually "good" because they have higher standards or because their articles are more cited due to the linguistic illiteracy of many researchers, unable to cite articles which have not been written in English.

Besides, deviations may also be due to the accepted view that all sciences work the same way as far as the publication of results is concerned. Such a statement is not at all true. As a matter of fact, there is not only one science, but multiple sciences, not only when we take into account the task of discovering and collecting data and results, but also when these results are communicated through lectures, contributions to meetings, and the publication of articles. In the case of sciences based on observation, the obtainment and the qualitative interpretation of data is more significant that any later mathematic processing of the data, which can even be useless. Stephen J. Gould (2) has informed against the stereotype of considering non quantitative descriptions as being non-scientific and somewhat valueless if compared with quantitative methods, which play a major role in some scientific fields. Gould sums up his thoughts wondering "if we will ever get past the worst legacy of IQ theory in its unilinear and hereditarian interpretation—the idea that intelligence can be captured by a single number, and that people can be arrayed in a simple sequence from idiot to Einstein". Besides, the best results in such non-quantitative disciplines are not usually published in articles in journals, but in long monographs, which are not taken into account when calculating most indexes of scientific excellence.

Another kind of mistakes is closely related to the above. It refers to the excessive simplicity with which data published by the authors, either regarding the impact indexes or the ranking of journals according to these indexes, are taken into account. In fact, some fields of science deal with issues of general interest, some of them closely related to human beings, such as topics dealing with health and with technological development. Thus, the scientific results most cited are usually related to these topics. There are thousands of researchers interested in the functioning of the human kidney, and in its pathological condition. However, rarely will you find many scientists who carry out their research work on the excretory apparatus of, let us say, such uninteresting animals as frogs. In a simplistic way, we might state that any paper on the human kidney would be considered to be better than any other dealing with the

kidneys of some amphibian. At the IFSE-8, Eugene Garfield himself illustrated this distorted vision with an example. He distributed a list of the articles most cited that had been produced by researchers from the scientific institutions in Barcelona. Eight out of ten articles cited dealt with medical issues, whereas the other two dealt with physics. When considering the authors, medical researchers also get the highest ranks. And the same happens when considering the institutions: sixteen out of nineteen institutions with articles having reached high standards are centers for medical research. I indeed acknowledge the relevance of the medical research carried out in Barcelona, but am reluctant to admit that 80% of the best researchers have developed their activities in the field of medicine. Eugene Garfield also distributed a list of journals considering their cumulative impact factor over a six-year period. The list rankeds 120 scientific journals. The only journal on Geology included was ranked 90th.

Finally, we must admit that a valuation system based on impact indexes, citation and other similar variables makes it difficult to assess the results of what is known as "technological transfer". A patent, for example, cannot be compared to a publication in any journal considered to be "good" or not so good. However, getting a patent means a lot of work to obtain results that cannot be published. Besides, patents are cited only in very specialized circles, and force the researcher to introduce him/herself into domains very different from his/her own speciality. Yet, the incidence of this kind of work is particularly excellent and efficient, and has a great interest for the country in which it is produced. Besides, it frequently means that researchers have reached a high standard.

## Conclusion

Obviously, research work can only be evaluated through its results, which are explicated in some kind of publication, usually in a journal or in a monograph. The analysis of the value and rightness of the several methods of evaluation makes us consider not only the positive aspects of the systems currently used, but also their negative aspects. In general, any system is exposed to mistakes either by action or omission, because of the unavoidable imperfection of any human activity. Nevertheless, the evaluation based on the examination of the contribution itself seems more serious and less error-prone than that based on the assumed excellence of the vehicle that conveys the contribution. Unfortunately, common usage is precisely the opposite. More value is given to the container than to the content. It is another kind of simple-mindedness.

## References and notes

1. Crusafont-Pairó, M., Reguant, S. (1970). The nomenclature of intermediate forms. Systematic Zoology **19**, 254–257. On p. 257 it is stated "This paper was translated from the Spanish by G. G. Simpson". As a matter of fact it was the friendship between Dr. Crusafont and the prestigious paleontologist G. G. Simpson, and not any other circumstance, which made this work be accepted, translated by Simpson himself and published in a journal which has been considered to be the best in its field for years. Simpson was an honorary member of the society publishing the journal.
2. Gould, S. J. (1989). Wonderful Life. W. W. Norton, New York, NY.
3. Hallam, A. (1985). Grandes controversias geológicas. Ed. Labor, Barcelona.
4. Kennedy, M. J, Reader, S. L., Swierczynski, L. M. (1994). Preservation records of microorganisms: Evidence of the tenacity of life. Microbiology **140**, 2513–2529.
5. Reguant, S. (1993). What lingua franca? Nature **361**, 107.
6. Reguant, S. (1994). Diez avisos para el científico español. Microbiología SEM **10**, 435–438.
7. Reguant, S., Casadellà, J. (1994). English as lingua franca in geological scientific publications. A bibliometric analysis. Scientometrics **29**, 335–351.

MICROBIOLOGÍA SEM **11** (1995), 503–508 **OPINION**

# Science, culture and communication for the 21st century.*
## Report on the Eighth Conference of the International Federation of Science Editors**

Karen Shashok

*Translator and Author's Editor, Granada, España*

In July 1995 the School of Biology of the University of Barcelona was the venue for the International Federation of Science Editors' eighth meeting. Professionals from virtually all branches of science publishing and information transfer enjoyed exchanging views on science communication between scientists themselves, and between scientists and the public. With the able assistance of a cadre of students from the School of Biology of the University of Barcelona, the Observatory of Scientific Communication of the Pompeu Fabra University, as well as the School for Scientific Communication in Santa Maria Imbaro, Italy, and a room full of computers with Internet and World Wide Web connections, IFSE brought together about 288 scientists, editors, journalists, and other communicators from 47 different countries. Ricard Guerrero, as Chairperson of the meeting, reminded participants that science, like literature, should be considered an important part of everyone's culture, and observed that current and future technologies for information transfer and electronic communication will be important in ensuring equal access to adequate scientific knowledge.

Sir John Kendrew, from Cambridge University, United Kingdom, stressed that we are about to see drastic changes in the editing and publishing of scientific literature, and warned that we had not yet faced the problems raised by the rapid transformation of peer review, page charges, copyright, and the durability of information. In his lecture titled "Good and bad manners in science" (1, 3, 4), Sir John Maddox explained that he had learned from his personal experience as editor of *Nature* how the conduct of science had changed since he had started his own academic career. Instead of openness, disregard for establishing priority, and generosity in sharing and acknowledging acquired knowledge, there was now a proliferation of slanted or selective citation of the authors' (and their competitors') previous work, failure to cite non-English literature, inappropriate claims of priority, and intentionally murky writing. Maddox believes that authors have been pressured to resort to these

* Based on a report published in European Science Editing 1995, **56**, 11–14.

** The IFSE can be contacted through World Wide Web at: http://www.cmns.mnegri.it/ifse

*Correspondence to*: Karen Shashok. Compositor Ruiz Aznar 12, 2–A. 18008 Granada. España. Tel.: +34-58-812305. Fax: +34-58-132354.

practices by the inherent conflict between the need to publish so that science can progress, and the fact that publication has become a measure of personal success.

When asked about the high rejection rates of manuscripts submitted by authors whose native language is not English, Maddox conceded that he was ashamed that *Nature* had done so little to respond to the unhappiness of non-English authors. In closing, Sir John Kendrew concluded that the problem of "poor manners" was more widespread than what was previously believed.

Mario Bellardinelli spoke on the dissemination of scientific and technical knowledge from European Commission (EC) Directorate-General XIII (for Telecommunications, Information Market and Exploitation of Research). He emphasized that the results of research supported by the EC must be disseminated to all those who can use them—a potential readership that includes not only scientists, but also politicians and the general public. Information.tools the EC has developed to disseminate information to a wider public include the Community Research and Development Information Service (CORDIS), a group of nine databases the provide information on all aspects of European Union research; Euroabstract, a monthly periodical that gives information on publications arising from European Union research programs; and VIPS, a new service that now provides about 500 scientific journalists all over Europe with information on the most interesting aspects of European research.

When the discussion turned to how the public uses scientific information, Miriam Balaban, president of IFSE, noted that the people should be actively involved in mediating between technology and its uses, and Sir John Kendrew reminded us that the communicators must communicate not only with scientists, but also with politicians. Bellardinelli lamented that although communication and dissemination receive ample moral support, very little money is actually spent on ensuring dissemination to the public.

Jonathan Piel (former editor of *Scientific American*, New York) introduced two eminent researchers who have successfully introduced new theories into their disciplines. Alan H. Guth, of the Massachusetts Institute of Technology, spoke on the theory of eternal cosmic inflation, and explained how the processes it entails imply the perpetual creation of infinite universes. He noted that this model is infinite into the future, although we do not know whether it is infinite into the past, or whether indeed it had a beginning. However, inflation must have started sometime, otherwise the universe would be unobservable. His conclusion was that exponential expansion is more important that a hypothetical beginning. The imaginative concepts Prof. Guth described as part of the infinite universe theory were thus shown to play a productive role in the creative process of science.

William H. Calvin, of the University of Washington (Seattle), then gave us an introduction to his theory of the brain as a Darwinian machine. Drawing parallels between the processes that shape new species over millennia and those that give rise to new antibodies over the days or weeks of an immune response, he suggested that neurological connections in the brain developed, copied, and produced variations of patterns that competed against each other for occupation of an anatomically limited space. Their success in this competition is influenced by a multifaceted environment, and their survival to reproductive maturity is understandably skewed.

The relationships between science and the general public were examined in sessions titled "Philosophy, Sociology and Policy in Science", and "Communicating Science to the Public". Because of the public's curiosity about science, and their fear and ignorance of quickly evolving areas such as genetic engineering and biotechnology, there is a growing need for dissemination

of science and technology to the public through all available media. Ramon Folch, of *Enciclopèdia Catalana*, Barcelona, warned that as science advances, ignorance increases. To prevent a new form of enlightened despotism from emerging, scientists must help to close the fast-growing gap between their accumulating new knowledge and the public's increasingly outdated knowledge. Therefore, scientists need to understand the sociological significance of their activities, and play a more active role in disseminating new knowledge to the public.

Jonathan Piel described how networked computers and the CD-ROM give journalists excellent opportunities to reach the public in ways that are advantageous to both the information provider and the user. Alfred Giner-Sorolla, of the University of South Florida in Tampa, traced the effects of crucial scientific discoveries on society. He noted that the discoveries of Copernicus and Galileo had no immediate effect on society, whereas Diderot's Encyclopedia marked the establishment of the concept of scientific revolution. Since then, Newton's *Principia*, the works of Darwin, and the development of nuclear weapons, have had profound effects on humankind.

Eugene Garfield, of the Institute for Scientific Information (Philadelphia), spoke on absolute and cumulative citation counts, and identified specific variables that influence these figures. In a lecture packed with data on citation counts, he compared the ranking of the top 34 research journals based on their 1993 impact factor with the journals' cumulative impact (all citations between 1981 and 1993) for articles published in 1981, and their very different ranking based on cumulative impact. Garfield cautioned that it is better to use a 5-year or 6-year window instead of a 1-year window to study a journal's performance, because data for a brief period of citation was likely to be skewed by short-term fluctuations in citation trends and possibly by the effect of a single super-cited paper. He also noted that different types of articles (i.e., research articles, reviews, and letters) should be identified and analyzed separately. He reminded us (yes, many still need to be reminded!) that comparisons between fields were not valid; for the purposes of evaluation, it is necessary to separate not only disciplines, but even fields within disciplines. Recalling that the initial paper on cold fusion received hundreds of citations, Garfield also insisted that research productivity should not be based on impact factors, because these figures lump together favorable and unfavorable citations.

In the session titled "Reviewing the Peer Review System", three editors of biomedical journals offered some bold views on how current dissatisfactions might be addressed. Drummond Rennie, West Coast Editor of the *Journal of the American Medical Association*, announced that it was time for a change in the long-standing principle of reviewer anonymity. Pointing out that anonymity is a perfect recipe for power dissociated from responsibility, he called for open peer reviewing, in which all referees would sign their critiques and be identified to the authors. Disputing claims that neither referees nor authors are ready for the referee's identity to be revealed (2), Rennie pointed to evidence that reviewers are more constructive and polite, and authors more satisfied, when review is open. Referees who refuse to sign are probably too used to expressing strong opinions without providing evidence in support of their views; the opinions they express about the manuscript are not likely to be very useful to the editor. Rennie also recommended that editors be more conscientious in choosing well-qualified referees and taking final responsibility for the decision to accept, reject, or request revisions, rather than trying to blame an unfavorable review for a decision to reject—a type of behavior typical of the passive "potted palm" or "signpost" editor.

Richard Smith, editor of the *British Medical Journal*, pointed out that most of our assumptions

about peer review were based more on myth than on evidence. Peer review does not separate good from bad, it is not free from bias, it does not protect against fraud, it is probably not the best system we have for deciding which paper to publish and which grant proposals to fund, it does not necessarily make a journal superior to one that does not use peer review, one cannot trust papers in peer-reviewed journals, and in fact, there is no evidence that peer review even works! In the spirit of encouraging research into peer review, Smith also denied that it is impossible to research, that we need to define it first, that all systems are alike, that senior colleagues provide the best opinions, that signed reviewing could not work, that peer review will avoid problems of conflict of interest and will ensure the quality of drug-company-sponsored supplements, that research on it is a waste of resources, that there is no alternative to peer review, and that we know all that we need to know about it. He illustrated some of his points with examples from the files of the *British Medical Journal*, and declared, as further proof of the inefficacy of peer review, that the medical literature was filled with poorly designed and interpreted studies (a.k.a. "rubbish")!

In his challenging and elegantly illustrated talk titled "The Rhetoric of Peer Review", Richard Horton, editor of *The Lancet*, contrasted surface interpretations and deeper readings of paintings and scientific articles. Because the intertextual web is constantly changing, and because the analysis of a text on different levels will always be a subjective process, a scientific paper cannot be assumed to be reliable or objective from the start—the only proof is replication or refutation. The traditional purpose of peer review has been to ensure the internal validity (i.e., the correctness and authenticity) of each study that is published, but now it needs to set itself a much more urgent goal: defining the external validity, i.e., the generalizability—or rather, the limits to generalizability—of the data. In other words, the research must be applied to a real-life setting, and the degree to which it conserves or loses its validity must be specified. Horton warned us that there are no objective criteria to judge generalizability, and that editors should require that the reasons for choosing one interpretation over another must be made clear when that data of, say, a clinical trial are being analyzed. Drawing us neatly into the theme of communicating science to the public, Horton then gave us an example of how a science article in the "lay press" had made an inappropriate generalization from insect (fruit fly) to mammalian (human) behavior, even though the journalists, in preparing their article, had had the cooperation of the authors of the original study.

These three talks from eminent authorities on editing and peer review raised hopes that at least some editors were ready to admit that the criteria used to accept and reject manuscripts needed to be examined closely and revised. It was therefore disappointing in the extreme to hear all three speakers admit that they do not preach what they practice, and that editors were two-faced (or even three-faced). Part of the problem might be tackled if publishers would give editors more money to investigate the other, unspoken factors that affect the fates of manuscripts.

An imaginative lecture by Lynn Margulis, University of Massachusetts, Amherst, stood as a good example of the importance of creativity in scientific thinking. She introduced us to Darwinian time, Vernadskian space, the biosphere concept and the protoctists, and with the help of her video "From Gaia to Microcosm" gave us a sweeping view of how life on earth can be understood in terms of the biosphere concept. The speaker reminded us that science requires honesty, and that scientists must describe the world as they have experienced it directly. A scientist might consider his or her system of belief to be "truth" or "science", but regardless of what it is called, the scientific

enterprise must be open to discussion, exchange and criticism, and is necessarily international. Margulis ended with a pointed observation that editors who act as censors are counterproductive to the scientific enterprise, and warned us not to let the marketplace determine the quality of science.

Perhaps the sessions that best represented the themes of this year's IFSE conference were those devoted to "Education in Science and Science Communication", "Training of Science Editors and Journalists", and "Scientific Activity and Communication in Emerging Regions" (including Wales)! Participants from many different countries and cultural backgrounds reported their experiences in running journals and training editors, teaching science writing, communicating science to the public, and disseminating research from developing regions—and this is only a small sample of the topics we heard presentations on. Among the highlights of these sessions were Jorge Avendaño-Inestrillas's report of his very successful courses on the teaching of medical writing at the Universidad Nacional Autónoma in Mexico City, and a summary by Bernhard C. Adelmann-Grill, of the European Initiative for Communicators in Science (EICOS) program offered at the Max-Planck-Institut für Psychiatrie in Martinsried, Germany. Peter T. Haskell, of the University of Wales in Cardiff, United Kingdom, and Joe Menyonga, of Safgrad, Burkina Faso, reported on a study being undertaken by the Technical Centre for Agricultural and Rural Cooperation to determine the causes of, and find solutions for, the suspected high rejection rate for papers submitted to international journals by agricultural research scientists from developing countries.

John E. Anderson, of BIOSIS, Philadelphia, described the forces that will mold the scientific information industry in the next five years. In his lecture titled "Technology and the evolution of scientific publishing", he predicted that publishing will be transformed into electronic scientific information distribution in response to the evolving needs of scientists themselves. Information distributed electronically can benefit from editorial input, and indeed the function of editors must be provided for in the "brave new world" of electronic publishing, but editors will need to take the initiative and make sure that their ability to help guarantee the quality and accuracy of science communication in the new media is recognized. He warned that if scientists and editors do not act quickly to uphold editorial standards in electronic publishing, someone else will move in and provide what may be less than optimal solutions. The next four to five years will be a period of turmoil as the strong (and potentially damaging) economic forces that are driving science publishing try to steer scientific information, and thus scientific activity. Anderson warned that governments may take over science publishing to guide information processing, just as governments now control funding for research. Two things urgently needed to facilitate the electronic movement of information are a standardized bibliographic format, and a new definition of data ownership, i.e., copyright.

Robert A. Day, University of Delaware, Newark, defined international English as English that is understandable to as many readers as possible. Noting that most currently active scientists have a native language other than English, he suggested that science editors and journals should ensure that the language is as simple and concise as possible without compromising accuracy. He emphasized that it is not necessary to have a huge vocabulary in English to be able to use the language clearly and elegantly—if the writer has a sound understanding of the parts of speech, together with good sense, these will help him or her use words as tools for communication with simple language.

Science writer and consultant Bernard Dixon, Middlesex, United Kingdom, spoke on the journalist as proselytiser. Under some circumstances, he felt that was inadequate and irresponsible for a

science journalist to merely transmit information. There are times when readers might want or need to be persuaded; this would require the journalist to use a more questioning, crusading or pioneering approach when researching and writing up the story. However, Dixon recognized that investigative journalism can sometimes be unreasonable and dangerous: if the journalist is too close to his or her source, the public can be mislead. In the end, what most readers want is simple, reliable, factual information, and honest reports covering all sides of the story when controversy exists.

Despite the emphasis on effective communication, this meeting, like any other professional gathering, was not without its share of unintelligible presentations. A few talks were outstanding, and many were well organized and clearly delivered, but some were dreadful. Why? Because some speakers, who presumably use their communication skills as an indispensable tool of their trade, failed to follow some very basic rules. We were at times subjected to awful overheads with too much text and lettering, which were sometimes too small to be read even from the first rows. Some of the graphics used, although probably successful on the printed page, did not project well when used as an overhead. And handwriting needs to be banned once and for all from overheads! Finally, would all speakers in future please enunciate clearly and not rush! Given the variety of accents heard at international meetings, we should try to remember that the ear needs some time to adjust to different voices. If many in the audience are unable to understand, they will miss important information, and may feel that they are not getting a fair return on their very considerable investment in attending.

On the other hand, one of the natural-born communicators we had the pleasure of listening to was Jorge Wagensberg, Director of the Science Museum in Barcelona. Swinging himself on lianas of enthusiasm and excellent examples from his own experience, he sailed self-assuredly over the churning rapids and yawning crevasses of English, and although he sometimes got a bit splattered and scratched, his lecture, titled "Science Museums in a Democratic Society", was one of the best of the conference. He envisioned science museums as places that provide an interactive environment that stimulates the public to think and form opinions about scientific issues, and encourages scientists and the public to seek each other out.

On the day after the conference, several workshops were run to broaden the participants' perspectives on training—for the authors of research papers, journal editors, and the trainers themselves of both collectives, as well as for science journalists and science educators.

## References

1. Dewey, W. L. (1995). Bad manners, not good. Nature **376**, 545.
2. Fabiato, A. (1994). Anonymity of reviewers. Cardiovascular Research **28**,1134–1139.
3. Koehn, R. K. (1995). Good manners. Nature **378**, 10.
4. Maddox, J. (1995). Restoring good manners in research. Nature **376**, 113.

MICROBIOLOGÍA SEM **11** (1995), 509–511 **OPINION**

# Getting the message across. Main topics of the Eighth Conference of the International Federation of Science Editors

Michael Dolan

*Departament of Biology, University of Massachusetts, Amherst, Massachusetts, USA*

The increasing use of electronic communication and the need to include research published in non-English journals in the scientific literature written in international English were two of the major themes of the 8th Conference of the International Federation of Scientific Editors (IFSE) held at the School of Biology of the University of Barcelona, on July 9–13, 1995, under the chairmanship of Ricard Guerrero, of the University of Barcelona. The IFSE meetings, which are more international than most society meetings, with 288 participants this time from 47 countries, are forums for discussing standards in scientific publishing such as peer review, the latest technologies for the international dissemination of scientific information, and the culture of publishing scientific research results. One conclusion of the meeting was that developments in digital technology can reduce the isolation of scientists in the underdeveloped world, but that it will not necessarily reverse the predominant flow of information from the center to the periphery.

John Maddox, retiring this year as editor of *Nature*, the journal which epitomizes the scientific establishment, suggested in his opening talk that the central concern of scientific publishing is the need to promote "good manners" and to discourage "bad manners". The former he eloquently characterized as centered around the principle that, "the results of research are valid only when they have been published, which means that researchers are expected to give away their discoveries without charge, and that they are rewarded only by the esteem in which they are held professionally." He included as bad manners various practices of withholding data, publishing incomplete results and committing fraud, which he illustrated with examples he encountered during his long career at *Nature*. He also suggested that the use of non-English references should be encouraged by editors, but was opposed to any quota system guaranteeing non-native English speakers space in *Nature*'s pages. Maddox suggested that *Nature* might create an international advisory committee to review the journal's practices and advocated, apparently in a losing cause, the retaining of standard British English as the international language of science.

*Correspondence to*: Michael Dolan. Department of Biolo y. University of Massachusetts. Amherst, MA 01003. USA. Tel.: +1-413-5453244. Fax: +1-413-5453243. E-mail: pbi@bio.umass.edu

This question of an international English for use in scientific publications was taken up later in the conference by microbiologist and English professor Robert Day who argued that English is essentially a simple language and that, "science editors and journalists should make every effort to insure that the English used in scientific publications is understandable to as many readers as possible." He also recommended that the "vocabulary should be limited to clear words of certain meaning. Specialized words and jargon must be eliminated or explained. Idiomatic language should be avoided or translated into words or phrases that can be universally understood." Many others participants agreed with Day's proposal.

One of the most interesting aspects of the conference because of its inherently futuristic context was the discussion on electronic communication. Topics discussed in this area were: the integrity of electronic publishing, the dissemination and storage of such electronically-produced products, changes in indexing and referencing procedures and the total disintegration of paper-based communication in favor of multimedia computer screens. John Maddox expressed concern, as did many others, that electronic publishing would lead to a flood of unreviewed papers of little quality. David Blackburn, of the *Bureau International de Poids et Mesures* in Sèvres, France, described the changing authority relationships under electronic publishing, with the roles of referee, editor, printer, publisher and librarian replaced by those of telecommunications company and database owner. He portrayed an essentially anarchistic world of scientific publishing, but suggested that smaller journals would have to go electronic to survive. John Anderson of BIOSIS, the publisher of *Biological Abstracts*, said that scientific publishing will be mainly electronic within five years, and that problems with bibliographic information, necessary for tracking down references, will be multiplied. He also suggested that electronic journals could serve as the disseminators of abstracts from other journals as printed abstract compilations become obsolete.

The most visionary of the speakers on electronics was Prof. Michael Hawley, of the Massachusetts Institute of Technology, to whom the word "enthusiast" would be a gross understatement. To Hawley the ability to cram huge quantities of information into small places is inherently good. One of his heroes is a character from an Arthur Clark novel who made his fortune "selling the shoebox-sized, bootleg edition of the Library of Congress," a place Hawley characterized today as "filled with dead trees." He stressed the rapid innovation in computer technology through which a machine just a few years old is already obsolete. He concluded his talk with his new computer program for embroidering cloth and a comment that such a program could be used to insert computer wires into our clothing, our furniture, even our doorknobs. We face an accelerating polarization between rich and poor, but at least we will have intelligent doorknobs.

Christopher T. Zielinski, of the World Health Organization in Egypt, saw the electronic communication world differently. He pointed out that electronic information systems have exploited and marginalized the South and that the growing dominance of such technology is likely to bring more of the same. "Users of electronic media are able to pinpoint information they need with deadly accuracy and extract all the value without having to pay anything for them," he said citing a case of a database created by the United Nations which was sold very cheaply to a company making CDs which were then sold back to countries at a high price. Zielinski suggested that a category of "essential information" should be recognized and that this information should be provided to developing countries at cost. He also spoke against information tagging systems and ones that include an encryption lock as being contrary to the principle

of the free flow of information. Zielinski suggested that discussion of electronic information systems should take place within the context of the displacement of Third World science: only three percent of the Science Citation Index comes from the Third World while the North focuses on only 3,000 of the 100,000 journals printed in the world.

On the topic of the Science Citation Index, Eugene Garfield, founder of the Index as well as of *Current Contents* and the newspaper, *The Scientist*, discussed impact factors, calculations of the relative influence of journals. He noted that journals with the highest current impact factor, for the most recent few years, are often not the same journals with the highest cumulative impact factors, which go back fifteen years. The impact of Spanish journals, while relatively low, has doubled in recent years, and now accounts for 2 percent of the world references per field on average. In a related matter Garfield noted that the longevity of citations is steadily decreasing each year with papers published recently not being cited as continuously as papers published thirty years ago.

The IFSE conference was also notable for the discussion of science journalism and science writing in general. Luís Reales of the newspaper *La Vanguardia* in Barcelona told of the benefits to journalists of using the Internet to develop contacts with expert scientists so that accurate information can be obtained for news stories. Monica Starendahl, president of the Swedish science journalists association, said that journalists should strive to be able to evaluate scientific research, adding that journalists are very interested to learn about the world of scientists, but that the opposite was not true and that science communication to the public suffers as a result. Ramon Folch, of the *Enciclopèdia Catalana*, spoke of the need for scientists to explain things comprehensibly to the public. He said that research and communication should be inseparable and that, "what is needed is not more isolated information but a general culture of science."

In "A Message of Peace through the Sharing of Scientific Knowledge and Communication," his review of the history of the universe and our place in it, Joan Oró suggested several principles of human conduct as informed by science: (i) Humility—we are all made of stardust; (ii) Solidarity—we are all sisters and brothers of the mitochondrial Eve; (iii) Conscience of Knowledge—conscience of our knowledge should free us from dogmatism; (iv) The Golden Rule of Human Conduct—love each person as yourself.


# Centro de Documentación en Acuicultura

## CSIC

## Centro de Información y Documentación Científica (CINDOC)

Este Directorio constituye la cuarta versión impresa del banco de datos elaborado por el Centro de Documentación en Acuicultura. Recoge información sobre empresas y centros de investigación que desarrollan su actividad en el sector de la Acuicultura, o en apoyo de este sector, en España.

**Contenido**

Centros de Investigación (Universidades, Ministerios, Comunidades Autónomas y Administraciones Locales), Empresas (Relación alfabética, Empresas agrupadas por sector de actividad), Especies censadas (Índice nombre científico/nombre común, Índice nombre común/nombre científico), Índice alfabético de Centros de Investigación, Índice de Centros por Comunidades Autónomas, Índice de Empresas por Comunidades Autónomas.

**Enviar el cupón de petición (o fotocopia) acompañado de su pago a:**

CENTRO DE DOCUMENTACIÓN EN ACUICULTURA
CINDOC
C/ Joaquín Costa, 22. 28002 Madrid
Tel.: 91-5635482. Fax: 91-5642644.

Precio: 4.680 PTA
ISBN: 84-00-07475-0
Núm. de páginas: 295

## CUPÓN DE PETICIÓN

Pago mediante: ❑ Cheque a nombre del CINDOC
❑ Giro postal (en este caso, adjunte resguardo del mismo)

Nombre: .....
Dirección: .....
.....
.....
Código postal: ..... Población: ..... País: .....
Teléfono: ..... Fax: ..... E-mail: .....

MICROBIOLOGÍA SEM **11** (1995), 513–518

# Revisión de libros

*La sección de* Revisión de libros *de este número de la revista está dedicada a diversas publicaciones relacionadas con la biología del medio acuático, comprendiendo tanto obras de carácter básico o fundamental como aplicado o tecnológico. Animamos a los lectores a enviarnos sugerencias sobre libros que podrían ser comentados en números sucesivos, especialmente aquéllos de los que sean autores socios de la SEM.*

## Limnology in Brazil

José G. Tundisi, Carlos E. M. Bicudo, Takako Matsumura-Tundisi (ed.)

*Brazilian Academy of Sciences, Brazilian Limnological Society, Rio de Janeiro, 1995. 376 pp. ISBN 85-85761-05-9.*

A lo largo de los últimos quince años se ha producido un espectacular avance en las posibilidades técnicas para determinar y medir variables fisicoquímicas en un amplio margen de escalas. Sin embargo, existe la creencia de que tales avances (debidos a la mejora de la instrumentación científica y técnica) tienen su aplicación, con muy pocas excepciones, en oceanografía, pero no en limnología. Esta autolimitación en la aplicación de medios técnicos, hace que se estén desaprovechando las posibilidades de experimentación controlada que ofrecen los lagos, en su condición de sistemas cerrados.

Entre los objetos de estudio de la limnología se incluyen la física de los movimientos del agua, el conocimiento de las plantas acuáticas y del plancton, la ecología de los invertebrados, la contaminación de las aguas interiores, la química del agua, la biomasa y la producción primaria de diversos sistemas. Todos esos aspectos, referidos al conjunto de ese inmenso país que es Brasil (tiene más de 8,5 millones de quilómetros cuadrados, con 146 millones de habitantes), se desarrollan a lo largo de los 17 capítulos del libro. Éste constituye una excelente síntesis de los estudios de los ecosistemas acuáticos brasileños, incluyendo la estructura y funcionamiento de las comunidades de agua dulce y las aplicaciones técnicas y metodológicas. Los aspectos aplicados se ocupan de los métodos de protección y recuperación de los ecosistemas, de los procesos de eutrofización, del modelado y del empleo de sensores remotos como instrumentos para la gestión y conservación de lagos y embalses.

El libro aporta considerable información sobre ecosistemas naturales y artificiales y los problemas relacionados con los mecanismos de funcionamiento, conservación–recuperación, biodiversidad y organización de las cadenas alimentarias. El conocimiento de la geomorfología de los ríos de montaña, y su consideración como laboratorios naturales, y el funcionamiento de pequeños ríos tropicales, incluidos en grandes cuencas hidrográficas, son elementos clave de la limnología actual. Existe una gran presión social para intervenir en estos sistemas, pero el escaso conocimiento que se tiene impide prever las consecuencias de esa acción humana. Se ha aducido que, en muchos casos, el desconocimiento se debe a la multiplicidad de ambientes, a la extensión geográfica y a las carencias de infraestructura.

El XXVI Congreso de la Societas Internationalis Limnologiae (SIL) se celebró durante los días 23 al 29 de julio de 1995 en São Paulo, Brasil, y contó con una asistencia superior a los 900 participantes, de los cuales 370 constituían la representación brasileña. La SIL, fundada en 1922, conserva su nombre en latín (además de su versión inglesa, International Association of Theoretical & Applied Limnology), pero su publicación congresual, por decisión tomada en el congreso de São Paulo, finalmente ha dejado de denominarse *Verhandlungen*, en alemán, para pasar a su equivalente en inglés, *Proceedings*. (Por ello, la publicación dejará de citarse como *Verh. Internat. Verein. Limnol.* para ser *Proc. Soc. Internat. Limnol.*) Tradicionalmente, los congresos de la SIL son motivo para que el país organizador prepare una publicación que recoja el estado de la investigación en él. Los editores de *Limnology in Brazil*, dirigidos por José Galizia Tundisi, han mantenido el compromiso, ofreciendo en este volumen el trabajo intenso y extenso de un nutrido grupo de investigadores.

La inmensidad geográfica de Sudamérica propicia una gran diversidad climática, geomorfológica y de ambientes acuáticos, que da lugar a una extraordinaria biodiversidad. No son muchas las zonas, consideradas en sus límites nacionales o continentales, que como el caso de Brasil, pueden ofrecer tan amplia gama de variaciones ambientales y climáticas (tropical, desértico, húmedo, llano, montañoso, costero, interior) en extensiones considerables de terreno. Por esta razón, todas y cada una de las áreas de estudio propias de la limnología pueden ser desarrolladas en el país. Por esa razón, también el libro ofrece una perspectiva completa de esta disciplina científica que tiene en buena parte de Sudamérica, y especialmente en Argentina y Brasil, una extensa tradición. En el caso concreto de Brasil, sus inicios, implantación y desarrollo sirven de base al capítulo introductorio del libro.

John R. Vallentyne, también conocido como "Johnny Biosphere", afirma que en sus inicios, la limnología dio sus primeros pasos centrada en su propio objeto sin considerar el conjunto de relaciones interactuantes. Pero, añadimos nosotros, esto ha sido así en muchas otras disciplinas o al menos en la concepción experimental de ellas cuando no han tenido en cuenta los factores que podían influir en sus características. En el caso concreto de la limnología, pudo ser la constante presión de los desplazamientos humanos, su consecuente contaminación e impacto sobre la biota de los ecosistemas acuáticos lo que alertó sobre la necesidad de evaluar el alcance de esas alteraciones. La concepción global, que ha afectado igualmente a otras disciplinas, se iría imponiendo paulatinamente en limnología.

En España la tradición y el interés en la limnología se pone de manifiesto con los ya clásicos libros y artículos de Ramon Margalef, publicados ya desde los años cuarenta, los numerosos trabajos de otros investigadores, a partir de los sesenta, y la existencia de la Asociación Española de Limnología. Barcelona fue en 1992 la sede del XXV congreso SIL y, en el mismo año, Sevilla acogió el I Encuentro de Limnólogos Iberoamericanos. Sin olvidar los prestigiosos cursos que durante los años de 1977 a 1993 organizó el Instituto Agronómico Mediterráneo de Zaragoza. Por ello, no cabe duda de que el libro de Tundisi (actualmente presidente del Consejo Nacional de Investigaciones, CNPq, de Brasil) y sus colaboradores será muy útil en nuestro país, para el nutrido grupo de personas, investigadores, técnicos, docentes, estudiantes y profesionales de diversas disciplinas relacionadas con la limnología, porque el libro constituye una aportación excelente a la actualización de diversos aspectos de esta ciencia multidisciplinar.

*Ricard Guerrero*
*Universidad de Barcelona*

# Molecular Ecology of Aquatic Microbes

Ian Joint (ed.)

*NATO ASI Series, vol. G 38, Springer-Verlag, Berlin, 1995. 416 pp. ISBN 3-540-60134-1.*

Dentro de las reuniones de especialistas que regularmente organiza el NATO Advanced Study Institute (NATO ASI), en septiembre de 1994 tuvo lugar en Il Ciocco, Italia, una dedicada a la ecología molecular de los microorganismos acuáticos. Tanto el encuentro como este volumen, que recoge lo allí tratado, estuvieron a cargo de Ian Joint (del Plymouth Marine Laboratory, Gran Bretaña). Diversos asistentes a la reunión escribieron las restantes contribuciones.

El encuentro se dedicó a estudiar los cambios experimentados recientemente por la ecología microbiana acuática, tras la incorporación de las técnicas recientes de biología molecular. Los autores evalúan el progreso y resultados de esos cambios en la concepción experimental de esta materia. Durante años, por falta de metodologías apropiadas, temas como la diversidad filogenética, bases moleculares de las aclimataciones fisiológicas, transducción de señales ambientales y respuesta de los organismos, carecían de un enfoque experimental capaz de producir resultados comprensibles. Por eso, los avances de la biología molecular y el desarrollo de técnicas como la reacción en cadena de la polimerasa (PCR), al permitir una evaluación cuantitativa de la microbiota, han supuesto un avance en la comprensión de los mecanismos de funcionamiento de las comunidades microbianas.

El volumen, que consta de veintiún capítulos, trata importantes aspectos de las comunidades de microorganismos. El primer artículo, The potential of molecular ecology, a cargo de I. Joint, expresa la opinión del editor sobre las limitaciones de la ecología molecular. El resto de artículos se dedican a los siguientes temas (los autores van entre paréntesis): Understanding the molecular ecology (P. G. Falkowski); Bacteria in oceanic carbon cycling (F. Azam et al.); Regulation of microbes in sediment nitrogen cycle (H. Blackburn); Taxonomic probes for bacteria in the nitrogen cycle (B. B. Ward); Mixotrophy in pelagic environments (B. Riemann et al.); Successional changes in planktonic vegetation (C. S. Reynolds); Molecular techniques and the species concept (L. K. Medlin); Adjust of cyanobacteria to their environment (J. Houmard); Cell expression of nutrient limitation (N. H. Mann); The problem of excess and/or limitation of the habitat conditions (R. Guerrero y J. Mas-Castellà); Signal transduction in microorganisms (M. I. Simon); Microbial diversity in oceanic systems (S. J. Giovannoni et al.); Ecological role of the viruses (G. Bratback y M. Heldal); Molecular analysis of plastid evolution (W. Löffelhardt); Estimating bacterial growth from ribosomal RNA content (P. F. Kemp); Cell cycle of phytoplankton (D. Vaulot); Response of photosynthetic microorganisms to changing of $CO_2$ (M. Ronen-Tarazi et al.); Nitrogen fixation in the sea (J. P. Zehr); Molecular ecology of marine metanotrophs (J. Colin Murrell y A. J. Holmes); y Microbial culture and natural populations (N. G. Carr).

Finalmente, diremos que la exposición, estilo y profundidad de los capítulos son excelentes y mantienen una gran homogeneidad, independientemente de la diversidad temática. El libro está muy bien presentado, las ilustraciones son muy claras y la bibliografía es suficiente y está actualizada. Por todo ello, resulta un texto muy conveniente para investigadores dedicados a temas relacionados e imprescindible para especialistas del campo de la microbiología acuática y de la ecología molecular.

*Mercè Piqueras*
*Societat Catalana de Biologia*

# Los paisajes del agua: terminología popular de los humedales

Fernando González Bernáldez

*J. M. Reyero Editor, Madrid, 1992. 258 pp.*
*ISBN 84-604-3757-6.*

El reconocimiento de los humedales como componentes únicos del paisaje y de su importancia como zonas de recreo, protección de la vida salvaje y áreas de gestión y conservación del agua ha ido en aumento en los últimos años. No se ha llegado a tiempo, sin embargo, de evitar la destrucción de una gran cantidad de ellos, por desconocimiento, incompetencia o descuido, no sólo en nuestro país, sino en muchos otros. Y con graves consecuencias. Un humedal persiste gracias a un delicado equilibrio dinámico que abarca no solamente la masa de agua sino todo su entorno. Por tanto, cualquier acción que se emprenda ha de basarse en criterios globales y en substituir beneficios a corto plazo por objetivos de equilibrio a largo plazo.

*Los paisajes del agua* es el último libro de Fernando González Bernáldez. Una hermosa contribución a esos criterios globales de conservación del medio que el autor, naturalista hasta el fin (nació en 1933 y murió en 1992), defendió combativamente en el ejercicio de su actividad docente e investigadora. Es un libro pequeño, del llamado formato de bolsillo, donde se recoge la terminología popular de los humedales. El autor consideraba esencial conocer la expresión con que designan los componentes del entorno quienes tradicionalmente han estado ligados al mismo por una relación de subsistencia. Junto al patrimonio natural que son los humedales, sitúa el autor el patrimonio cultural, esos términos con los que, en el medio rural, se expresa el saber ligado a las formas de utilización del suelo y sus componentes. Era esencial recoger esta terminología popular antes de su completa desaparición, habida cuenta de que el paulatino abandono de las zonas rurales conduce al olvido de las denominaciones geográficas.

En todos los casos se pone de manifiesto la gran precisión y coherencia del autor. Al estilo de los diccionarios, junto a cada acepción el lector encontrará la procedencia del término, la explicación del significado y las variaciones locales. Aunque la mayoría de voces son castellanas, el libro recoge una buena cantidad en otras lenguas (catalán, portugués,euskera, inglés, francés, alemán, árabe, bereber, ruso, y dos términos en lengua G/wi, de los bosquimanos del Kalahari central). Finaliza con un índice de los términos agrupados por tipos de ecosistemas y por los aspectos estructurales y funcionales de los humedales.

Abre el libro un prólogo de Ramon Margalef, con una semblanza del naturalista y del amigo para tantos de nosotros. Particularmente entrañable resulta la mención que hace Margalef de "las deliciosas viñetas, principalmente de mariposas, con que a menudo me favoreció en sus cartas...", porque en el caso del firmante de esta revisión (que conoció a Fernando cuando éste redactaba su tesis doctoral) las viñetas eran de flores y plantas, auténticas delicias que se mantienen vivas sobre el papel en el que quedaron plasmadas.

Adecuado para especialistas, este particular diccionario es igualmente recomendable para cualquiera que sienta respeto e interés por esa parte de la naturaleza que son los humedales. Sería necesario que el editor, J. M. Reyero, de quien nos consta su afición a los temas ecológicos, se planteara la reedición de este libro, ya difícil de conseguir. Hay que evitar la paradoja que supondría la pérdida o el olvido de una obra cuya intención fue precisamente conservar un patrimonio cultural.

*Ricard Guerrero*
*Universidad de Barcelona*

# Estudio epidemiológico de zonas de baño de la provincia de Málaga

Juan J. Borrego, Fernando J. Mariño

*Junta de Andalucía, Consejería de Salud, 1995. 217 pp. ISBN 84-87247-81-4.*

Para un país como España, con una importante parte de su economía basada en el turismo, el control de la sanidad de sus playas es esencial, tanto desde el punto de vista de la competitividad con otras áreas turísticas, como desde el más elemental sentido de la higiene de los lugares de utilización pública. De ahí la necesidad y utilidad de un estudio epidemiológico de tales zonas.

Además de los aspectos de sanidad e higiene de las zonas de recreo, los autores incluyen los criterios que rigen la sanidad y calidad de las aguas de baño en relación a la normativa oficial. Por la amplitud, profusión y rigor de los datos que se ofrecen, el libro tiene un gran interés y resulta de utilidad para un amplio sector de público, estudiantes, técnicos y profesionales en medio ambiente y en control y análisis de aguas, vertidos, etc. Hay que destacar su interés para investigadores y responsables de las administraciones con competencias en sanidad y control ambiental.

Se analizan los niveles de contaminación microbiológica del agua de mar y arenas de playas de la provincia de Málaga, junto con un estudio sanitario y epidemiológico de la población expuesta. A la vez, se propone una revisión de los criterios de calidad para las aguas de baño y recreativas. La metodología describe detalladamente, en todos sus aspectos, el tratamiento microbiológico de las muestras, sondeo epidemiológico, elección de las zonas de estudio y de la población, y tratamiento estadístico de los datos. El trabajo constituye un modelo a seguir en otros estudios en el área mediterránea.

El contenido se distribuye en los apartados clásicos de todo trabajo científico: introducción, objetivos, materiales y métodos, resultados, discusión y conclusiones. En ellos, entre otros aspectos, se describen los principales microorganismos contaminantes de las aguas marinas, procedencia, vías de entrada, patogenicidad y condiciones que permiten su supervivencia. Las infecciones más comunes asociadas a la contaminación de las zonas de baño se tratan con un excelente apoyo bibliográfico y de tablas y figuras. La información obtenida en este estudio exhaustivo permite la elaboración de criterios y definición de objetivos para la obtención y mantenimiento de la calidad microbiológica de las aguas de zonas recreativas en el área mediterránea. Se exponen los resultados de estudios epidemiológicos de otros países.

Resultados y datos aportan importantes conclusiones sobre las características fisicoquímicas y microbiológicas de las aguas, la calificación sanitaria de las principales playas de la provincia de Málaga, y la distribución e importancia de las enfermedades debidas a hongos, bacterias y virus. Destacan las características demográficas de la población, análisis de la morbilidad y su relación con la calidad del agua.

Sin lugar a dudas, las conclusiones de este completo estudio serán de gran utilidad para la futura actuación de los responsables de la sanidad pública. El mantenimiento de la calidad de las aguas recreativas exige un conocimiento previo de la situación y estado de las mismas. La obra, además de proporcionar una idea clara de la situación de las aguas y playas de la provincia de Málaga, puede ser un eficaz instrumento para mejorar la sanidad de éstas, una ayuda para los profesionales de medio ambiente, y una guía para estudiantes y público.

*David Isamat*
*Universidad de Barcelona*

# Tratamiento de aguas residuales

Rubens S. Ramalho

*Editorial Reverté, S.A., Barcelona, 1993. 705 pp. ISBN 84-291-7975-5.*

Abordar un tema esencialmente práctico, como es el tratamiento de las aguas residuales, exige una cuidadosa selección de los métodos, técnicas e información disponibles. Al hacerlo, el autor no ha intentado presentar una síntesis de los múltiples procedimientos existentes, sino tratar de una manera global todo lo realmente significativo. El libro se dirige a un público bastante amplio, desde estudiantes con deseos de conocer las múltiples aplicaciones biológicas en la industria, hasta profesionales que aplican sus conocimientos en los distintos sectores de la misma.

El contenido del libro se divide según los diferentes pasos o procesos que se realizan en los tratamientos de aguas. El primer capítulo explica la importancia de estos procesos para reducir al mínimo el impacto de las aguas residuales en el ambiente, desde sus efectos, hasta la clasificación de los contaminantes que puedan contener y el interés de la reutilización de estas aguas para los balances económicos. El segundo capítulo describe los diferentes métodos para la caracterización o evaluación de las aguas residuales domésticas o industriales, con el objeto de decidir la utilización de un proceso u otro.

El libro tiene ocho capítulos. A partir del tercero se describen las diferentes fases de los tres tratamientos continuos que sufren dichas aguas, para que éstas vuelvan a ser introducidas al medio, sin que ello implique un aumento de la eutrofización del hábitat acuático, o un peligro potencial en la reutilización para el consumo humano. Hay un concienzudo detalle en la explicación de los tratamientos a los que se someten los lodos obtenidos durante los primeros procesos, con aplicación de las más modernas técnicas, tales como la ósmosis inversa o el intercambio iónico, para proporcionar a dichas aguas unas últimas propiedades determinadas, que dependerán de la finalidad que se le vaya a dar a esa agua en su reutilización.

Además de las divisiones temáticas de los capítulos, el autor agrupa el contenido en tres apartados generales: resumen de la teoría necesaria para el estudio del proceso en cuestión, definición de los parámetros de diseño para el proceso y determinación experimental de éstos, y desarrollo de un procedimiento sistemático para el diseño definitivo de la unidad de tratamiento. No cabe duda, con todo ello, de la idoneidad del procedimiento a seguir para seleccionar y diseñar un tratamiento de aguas residuales.

En resumen, a diferencia del resto de libros dedicados al mismo tema, que describen los estudios de los procesos realizados en conjunto, éste abarca un punto de vista totalmente diferente: se basa principalmente en el estudio de las operaciones y procesos unitarios, de manera que, en cada caso, se puede seleccionar o diseñar un determinado proceso para un tipo específico de agua residual.

El objetivo del libro es que el lector se forme una idea clara de las características necesarias para diseñar un proceso o tratamiento determinado. Por ello, el libro ofrece una completa información actualizada sobre estos tratamientos, lo que constituye un campo importante dentro de la investigación, tanto básica como aplicada. Se describe detalladamente la utilización de los microorganismos para resolver el problema de la contaminación de las aguas en los medios acuáticos. Ello hace que este libro sea de obligada consulta para los microbiólogos dedicados, o simplemente interesados, al tratamiento de las aguas residuales.

*Antoni Navarrete*
*Universidad de Barcelona*

LIBROS REVISADOS
VOLUMEN 11

## Atlas of Clinical Fungi

G. S. Hoog, J. Guarro (ed.)
*Centraalbureau voor Schimmelcultures/ Universitat Rovira i Virgili, 1995. 720 pp. ISBN 90-70351-26-9.*
Revisado en 11(1), 125–126.

## Biostabilization of Sediments

W. E. Krumbein, D. M. Paterson, L. J. Stal (ed.)
*Universität Oldenburg-Verlag, Berlin, 1994. 526 pp. ISBN 3-8142-0483-2.*
Revisado en 11(1), 129–130.

## Cowan and Steel's Manual for the Identification of Medical Bacteria

G. I. Barrow, R. K. A. Feltham (ed.)
*Cambridge University Press, Cambridge, 1993, 3rd. ed. 331 pp. ISBN 0-521-32611-7.*
Revisado en 11(1), 127.

## Cuadernos de microbiología

Publicación periódica patrocinada por Biocheck, S.A.
Revisado en 11(1), 131.

## Early Life on Earth

**Nobel Symposium No. 84**
Stefan Bengtson (ed.)
*Columbia University Press, New York, 1994. 630 pp. ISBN 0-231-08088-3.*
Revisado en 11(2), 278–279.

## Estudio epidemiológico de zonas de baño de la provincia de Málaga

Juan J. Borrego, Fernando J. Mariño
*Junta de Andalucía, Consejería de Salud, 1995. 217 pp. ISBN 84-87247-81-4.*
Revisado en 11(4), 517.

## Guía de los lagos y humedales de España

Santos Casado, Carlos Montes
*J. M. Reyero Editor, Madrid, 1995. 256 pp. ISBN 84-605-3109-0.*
Revisado en 11(3), 411–412.

## Les origines de la vie

Marie-Christine Maurel
*Syros (Pub), Paris, 1994. 208 pp. ISBN 2-84146023-1.*
Revisado en 11(2), 281–282.

## Limnology in Brazil

José G. Tundisi, Carlos E. M. Bicudo, Takako Matsumura-Tundisi (ed.)
*Brazilian Academy of Sciences, Brazilian Limnological Society, Rio de Janeiro, 1995. 376 pp. ISBN 85-85761-05-9.*
Revisado en 11(4), 513–514.

## Limnology Now

**A Paradigm of Planetary Problems**
R. Margalef (ed.)
*Elsevier Science B. V., Amsterdam, Netherlands, 1994. 553 pp. ISBN 0-444-89826-3.*
Revisado en 11(1), 128–129.

LIBROS REVISADOS
VOLUMEN 11

## Los paisajes del agua:
**terminología popular de los humedales**
Fernando González Bernáldez
*J. M. Reyero Editor, Madrid, 1992. 257 pp.*
*ISBN 84-604-3756-6.*
Revisado en 11(4), 516.

## Microbial Biotechnology
**Fundamentals of Applied Microbiology**
Alexander N. Glazer, Hiroshi Nikaido
*W. H. Freeman and Co., New York, 1995. 662 pp.*
*ISBN 0-7167-2608-4.*
Revisado en 11(3), 413.

## Molecular Ecology of Aquatic Microbes
Ian Joint (ed.)
*Nato ASI Series, vol. G 38, Springer-Verlag, Berlin, 1995. 416 pp. ISBN 3-540-60134-1.*
Revisado en 11(4), 515.

## Plastics from Microbes
David P. Mobley (ed.)
*Hanser Publishers, Munich, 1994. 270 pp.*
*ISBN 3-446-17367-6.*
Revisado en 11(3), 414.

## Tratamiento de aguas residuales
Rubens S. Ramalho
*Editorial Reverté, S.A., Barcelona, 1993. 705 pp.*
*ISBN 84-291-7975-5.*
Revisado en 11(4), 518.

## Vital Dust
**Life as a Cosmic Imperative**
Christian de Duve
*Basic Books, a division of Harper Collins Pub. Inc., 1995. 362 pp. ISBN 0-465-09044-3.*
Revisado en 11(2), 277–278.

## What is Life?
Lynn Margulis, Dorion Sagan
*Simon & Schuster, New York, 1995. 208 pp.*
*ISBN 0-684-81326-2.*
Revisado en 11(2), 280.

# Normas para los autores

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

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

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

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, NY.

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

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

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 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.

ARTÍCULOS 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, 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. Posteriormente, caso de ser aceptado, se pedirá también una versión en disco de ordenador.

# Instructions to authors

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Editorial Board addresses/Direcciones de los miembros del Consejo Editorial

*Viene de p. 424*

Juan Iriberri
Departamento de Microbiología
Universidad del País Vasco
Apartado 644
48080 Bilbao

Germán Larriba
Departamento de Microbiología
Universidad de Extremadura
06071 Badajoz

Paloma Liras
Área de Microbiología
Facultad de Biología
Universidad de León
24071 León

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

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

M. Benjamín Manzanal
Dpto. Interfacultativo de Microbiología
Facultad de Medicina
Universidad de Oviedo
33071 Oviedo

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

José Olivares
Estación experimental del Zaidín, CSIC
Profesor Albareda, 1
18008 Granada

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

J. 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

Harold W. Rossmoore
Department of Biological Sciences
Wayne State University
Detroit, MI 48202, USA

Editorial Board addresses/Direcciones de los miembros del Consejo Editorial

# AUTHOR INDEX
# VOLUME 11

# KEY WORD INDEX
## VOLUME 11

*UIB - GBF - CSIC - TUB SYMPOSIUM*

# Biodegradation of Organic Pollutants

***Mallorca, June 29 – July 3, 1996***

**Goals and Scope**

The aims of the Symposium are to present latest advances in the biodegradation of organic pollutans and bioremediation, to provide a lively forum for the conception of new ideas and approaches, and to stimulate the development of new collaborations.

**Conference Centre**

The Symposium will be held at the Hotel Palas Atenea, which is located only 12 km from the international airport and offers easy access to the city centre and local beaches.

**Abstracts**

Abstracts should be typed single-spaced in 12 point, preferably Times, within a 15 cm × 24 cm frame with title first in bold upper case letters, followed by the names and addresses of the authors in normal type. Crucial references may be given at the end. Up to two pages may be used.

**Sessions**

I: Aerobic Pathways. II: Anaerobic Pathways. III: Genetics and Pathway Engineering. IV: Protein Structure: Function and Engineering. V: Physiology, Bioavailability and Bioaccessibility. VI: Ecology of Pollution and Bioremediation. VII: Process Development and Bioremediation. VII: Politics, Economics and Business of Bioremediation. Closing Address: Wither bioremediation?

**Accomodation Costs**

Rooms have been reserved for most participants in the Hotel Palas Atenea. At current rates of exchange, full board will amount approximately DM 150 per person per day in a shared room and DM 190 in a single room. Some rooms have also been reserved for students in another hotel nearby, where the cost of half board will amount to approximately DM 60 per person per day in a shared room, and approximately DM 80 in a single room.

**Organized by K. N. Timmis, GBF, and J. Lalucat, UIB**

I would like receive more information about the Symposium

Name: .....

Affiliation: .....

Address: .....

.....

.....

Zip code: ..... City: ..... Country: .....

Tel: ..... Fax: ..... E-mail: .....

**Symposium Official Address:**

Professor K. N. Timmis. Division of Microbiology, GBF. Mascheroder Weg 1.
D-38124 Braunschweig. Germany

**Fax: +49-531-6181411. E-mail: kti@gbf-braunschweig.de**

# ERRATA

## Detection of *Bacillus larvae* spores in Argentinian honeys by using a semi selective medium

Adriana M. Alippi

*Laboratorio de Fitopatología, Departamento de Sanidad Vegetal, Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, La Plata, Argentina*

Volumen 11 (3), página 345, en el apartado Materials and methods, Development of a semi-selective medium which allows..., en todos los casos donde aparece mg/ml debería leerse µg/ml.

## Breve historia de la Sociedad Española de Microbiología, I. De 1946 a 1971

Concepción García Mendoza

*Centro de Investigaciones Biológicas, CSIC, Madrid*

Volumen 11(3), página 360, en la nota de pie de página, en lugar de: "celebrado en Berlin", debería leerse: "durante el II Congreso Internacional de Microbiología celebrado en Londres (organizado primeramente en Berlín en 1933, pero cancelado debido a los acontecimientos políticos de Alemania) cambió...".

# Microbiología SEM

PUBLICACIÓN DE LA SOCIEDAD
ESPAÑOLA DE MICROBIOLOGÍA

## BOLETÍN DE SUBSCRIPCIÓN, AÑO 1996

---

**Importe de la subscripción, 1996**
4 números, aprox. 600 pp. (Vol. 12)
España (I.V.A. incluido) ..... 12.000 PTA
Internacional ..... 111 US $

Enviar boletín de subscripción a:

Editorial Garsi, S.A.
Juan Bravo, 46
28006 Madrid (España)

**Dirección de envío**
Nombre y apellidos .....
Dirección .....
Código Postal ..... Población .....
Provincia ..... País .....
FORMA DE PAGO: ❑ Cheque nominativo que adjunto
❑ Domiciliación bancaria

---

**ORDEN DE PAGO. DOMICILIACIÓN BANCARIA**
Nombre del titular de la cuenta .....
Banco/Caja de Ahorros ..... C/C Nº .....
Sucursal ..... Calle .....
Código Postal ..... Población .....
Provincia ..... País .....

Ruego a Vds. tomen nota de que hasta nuevo aviso deberán adeudar en mi cuenta el recibo presentado anualmente por EDITORIAL GARSI, S.A.