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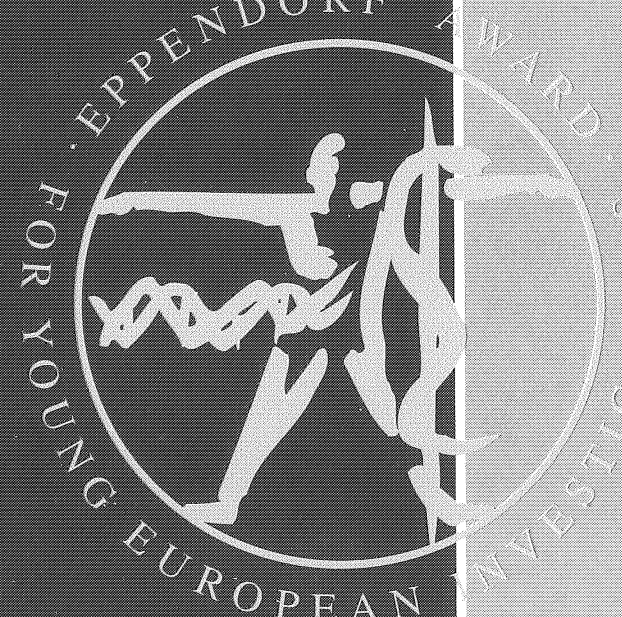

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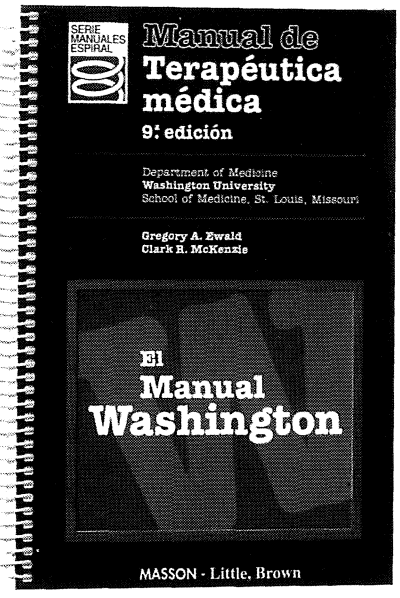

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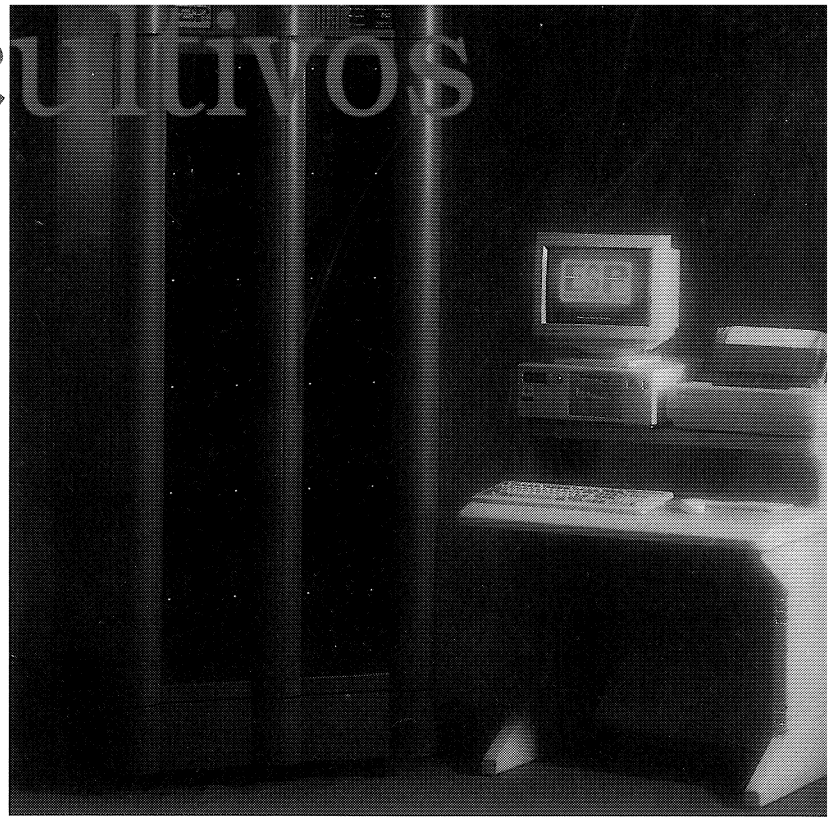

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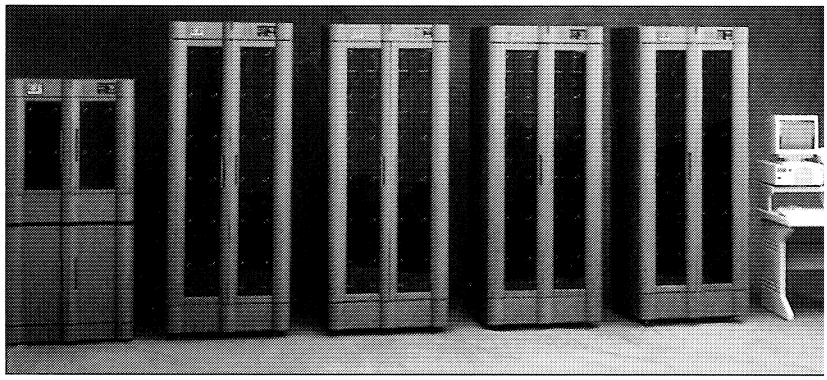

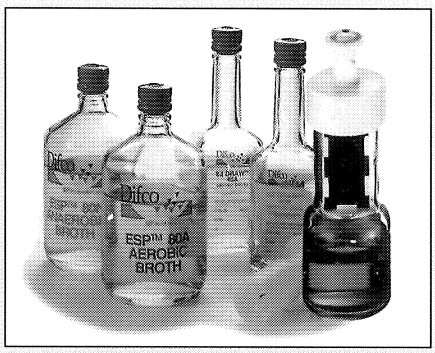

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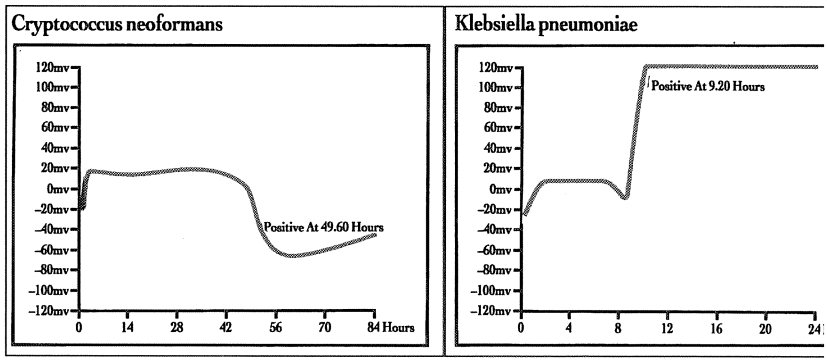

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(continued on next page)

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Editorial office address:

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

SEM Secretariat address:

Isabel Perdiguero
Sociedad Española de Microbiología
Vitruvio, 8
E-28006 Madrid, Spain
Tel.: +34-1-5613381. Fax: +34-1-5613299
E-mail: orgra46@fresno.csic.es

Preparation and layout of originals by:

Isabel Gallego
Arantzazu Gorostiza
Eulàlia Massana
Marta Montolio

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MICROBIOLOGÍA SEM **13** (1997), 127–130

Editorial*

La ciencia en América Latina desde la perspectiva chilena

La historia de la ciencia en Chile y en gran parte de América Latina, con excepción de Argentina, es breve y arranca en la segunda mitad de este siglo. Hubo sí, notables precursores, científicos hoy reconocidos, que la falta de continuidad dejó en casos aislados. Para este editorial me he servido del estudio *Análisis y Proyecciones de la Ciencia Chilena*, realizado por el Consejo Internacional de Uniones Científicas (ICSU) y por el Comité Nacional de ICSU de Chile. También me basé en ese informe para la conferencia inaugural que impartí, como presidente de la Academia Chilena de Ciencias, en la Asamblea General de ICSU (Santiago de Chile, octubre de 1993). Desde entonces nada ha alterado en esencia lo que allí expresé. He añadido datos sobre otros países de América Latina y sobre la relación de nuestra región con España y Portugal.

En Chile se desarrolla investigación de calidad y se publica en revistas científicas de difusión internacional. En los últimos doce años ha aumentado en 2,6 el número de trabajos en revistas citadas por el Institute of Scientific Information. En 1992 se publicaron cerca de 1300 trabajos, lo que sitúa nuestro país en cuarto lugar de América Latina, detrás de Brasil, Argentina y México. El cálculo de la productividad científica por habitante nos coloca en el primer lugar de la región. Por áreas, hay un crecimiento notable de publicaciones en matemáticas, física y química, mientras que en biología y ciencias de la Tierra se han estancado. La calidad de las publicaciones, medida por las veces que son citadas en la literatura científica, sitúa al país en el primer lugar de América Latina. Dentro de las disciplinas, hay que destacar el elevado nivel de la física y la astronomía. Pero en el contexto mundial la situación es muy diferente. América Latina, que se aproxima al 10% de la población mundial, produce sólo el 1,5% de la ciencia. La comunidad de científicos en Chile tiene calidad, pero es muy reducida.

En el Directorio de la Academia de Ciencias, cuyo requisito de inclusión fue la productividad continuada durante los últimos cinco años, aparecen sólo 1342 investigadores. Cerca del 50% de los investigadores y un alto porcentaje de las publicaciones son de ciencias biológicas, incluyendo biomedicina. Entre las materias más desarrolladas están fisiología, ecología y bioquímica, y hay

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* *Microbiología SEM* continúa en este número la serie de editoriales dedicados a comentar la situación de la ciencia en América Latina, en esta ocasión a cargo del prestigioso bioquímico chileno Jorge E. Allende.

también grupos de calidad en biofísica, biología celular, botánica, inmunología y biología de la reproducción, aunque, en conjunto, ha habido un estancamiento, y en los últimos años se han incorporado pocos científicos jóvenes. Sigue en importancia la química, con 107 grupos y un número de publicaciones en aumento, aunque ha habido también un estancamiento. Las áreas más desarrolladas son fisicoquímica y después química orgánica, química de productos naturales, química inorgánica y química ambiental.

Chile, balcón entre los Andes y el Pacífico, que debiera tener un gran desarrollo en ciencias del mar, sólo cuenta con 185 investigadores, la mitad en biología marina. A pesar de haberse duplicado su número, es preocupante que sólo haya 5 para oceanografía física, 8 en oceanografía química y ¡3! en contaminación marina. Considérese la enorme contaminación del Océano Pacífico y los más de 5000 kilómetros de costa chilena. En un país cuya principal fuente de ingresos es la minería, y con una larga y trágica historia de terremotos, hay 167 investigadores en ciencias de la Tierra. En ciencias ambientales, con un centenar de investigadores, 28 se ocupan del problema de la contaminación atmosférica, 6 de la contaminación de suelos y 11 del cambio global, tema destacado por ICSU, con miles de científicos trabajando en todo el mundo. Chile podría ser el país más afectado por las consecuencias del agujero de ozono.

Además del número preocupa la edad de los investigadores, pues sólo el 25% de los que aparecen en el Directorio tienen menos de 43 años. Las nuevas y más dinámicas áreas de las ciencias están pobremente representadas y preocupa pensar de dónde va a salir la generación de relevo para los próximos años. Los programas de doctorado, instrumentos para aumentar el número de científicos y paliar la fuga de cerebros, son de calidad, como atestiguan los evaluadores externos convocados por la Fundación Andes, en colaboración con la Universidad de Londres, la National Academy of Sciences de Estados Unidos y la Academia Chilena de Ciencias. Cada tesis doctoral da lugar a varias publicaciones en revistas de prestigio. También laboratorios extranjeros que han recibido becarios postdoctorales han confirmado su excelente capacidad y preparación.

Los aspectos más preocupantes son cuantitativos. Los programas de doctorado producen un reducido número de graduados, unos 35 por año en todas las áreas de las ciencias. Aumentar la productividad de esos programas requiere actuaciones políticas que asignen los recursos necesarios e instaure medidas sociales, políticas y educativas que: (i) animen a los jóvenes a dedicarse a la investigación científica, (ii) mejoren la valoración económica y social de los científicos, (iii) abran posibilidades de empleo para los jóvenes, y (iv) hagan participar a las industrias e institutos tecnológicos, a fin de ofrecer puestos de trabajo y diversificar y ampliar la oferta. Otro mecanismo que puede ayudar a más corto plazo es el retorno de los científicos que abandonaron el país; si en la década de los 70 se debió a motivos políticos, en años más recientes el motivo fue el desempleo, las bajas remuneraciones y el escaso financiamiento para la investigación. La gran mayoría de científicos ausentes están en Estados Unidos y Europa. El Directorio señala casi 400 sólo de ciencias naturales y exactas. Países como México, Colombia, Uruguay y otros han instaurado programas para facilitar el retorno de sus científicos que, en el caso de Chile, sería una posibilidad viable, ya que un porcentaje elevado ha expresado su deseo de regresar si pueden gozar de condiciones de trabajo y remuneración razonables.

Sigue haciendo falta el apoyo de la comunidad científica internacional para aumentar el número de investigadores. En muchas áreas no se dispone de la masa crítica necesaria para formarlos en el

nivel de doctorado y, en todas las áreas, es preciso que los jóvenes realicen estancias postdoctorales en buenos centros extranjeros. También se necesitan recursos para traer expertos que impartan cursos especializados, evalúen proyectos y tesis, y lleven a cabo investigaciones en colaboración. En los últimos diez años, el Fondo Nacional de Ciencia y Tecnología (FONDECYT), aportado por el Estado y administrado por CONICYT, ha aumentado notoriamente su presupuesto; actualmente es de 30 millones de dólares al año, que se reparten entre unos 1300 proyectos en ciencia y tecnología. El Fondo es el instrumento esencial de apoyo a la investigación de los diversos grupos. Con él se financia entre el 30 y 35% de los proyectos presentados, que son sometidos a evaluación paritaria y por grupos de expertos. Los criterios básicos son la excelencia del proyecto y el currículum de los candidatos.

La inversión en investigación y desarrollo es sólo del 0,7% del PIB, y en las universidades, donde se efectúa el 80% de la investigación, se han reducido los fondos. La crisis financiera y los cambios estructurales que sufren las universidades son, en gran medida, responsables de la falta de renovación y envejecimiento de los cuadros de investigadores que tiene el país. También es muy negativo el escaso financiamiento, alrededor del 10%, del sector privado, que, en los países industrializados es del 50 al 60%. Otra muestra es que de los 250 doctores en ciencia formados entre 1974 y 1992, sólo 14 fueron contratados por el sector privado. Del resto, 32 están en el extranjero y los demás en universidades chilenas. Una meta para el año 2000 sería elevar al 1% la parte del PIB destinada a investigación, con la participación del sector público y privado.

En la sociedad chilena hay un alto grado de analfabetismo científico, aun entre gente que se considera culta, lo cual influye en que los jóvenes capacitados no se interesen por la ciencia, no se valore económica y socialmente a los científicos y no se establezca una política científica. El nivel de enseñanza de las ciencias en la educación primaria y secundaria es bajo, y gran parte de la docencia universitaria de ciencia no está a cargo de investigadores. Esto ocurre también en otros países en desarrollo y, sorprendentemente, está sucediendo en los países industrializados. Es necesario combatir un oscuro pero real movimiento anticientífico que ha penetrado en muchas mentes y organizaciones. Se culpa a la ciencia de muchos de los males de la sociedad, sin pensar que los problemas surgen de la ignorancia y no del conocimiento. El estudio de la situación nos ha llevado a varias recomendaciones, las principales de las cuales serían: (i) Creación de un Consejo Asesor del Presidente de la República para Ciencia y Tecnología, formado por científicos y técnicos de alto nivel, y por representantes del gobierno y del sector productivo, para establecer una política científica nacional de desarrollo y coordinar la asignación de actividades y recursos. (ii) Creación de un sistema nacional que vele por la investigación de calidad y conceda estímulos financieros para mejorar las condiciones de sus investigadores y evitar la fuga de cerebros. (iii) Creación de un programa para aumentar los recursos humanos en ciencia y tecnología. La comunidad científica está trabajando en este proyecto, pero es necesario el apoyo político y social.

La comunidad científica internacional puede jugar un papel muy importante a la hora de elaborar y evaluar este proyecto. La situación chilena no es idéntica a la de otros países latinoamericanos, pues hay una amplia heterogeneidad en el desarrollo científico de los diferentes países. Por ejemplo, Brasil cuenta con recursos humanos (50.000 becarios de pre- y postgrado), mientras que Paraguay y Honduras carecen de programas de postgrado. Un número de doctores que es insuficiente para Chile resultaría una meta ambiciosa para Perú, Bolivia o Ecuador. La ciencia en Cuba, con respaldo

oficial y un gran número de jóvenes interesados en las carreras científicas, es deficiente en ciencias básicas debido al excesivo énfasis gubernamental en la aplicación de la ciencia. La ciencia latinoamericana, con 0,3 científicos por 1000 habitantes, constituye la décima parte de lo que tiene Estados Unidos. En productividad científica, el número de publicaciones de toda América Latina es poco mayor que la de Israel y muy parecida a la de Suiza. A pesar de la situación, los científicos latinoamericanos somos optimistas. El predominio de los regímenes democráticos, la mejora global de la economía de la región y los procesos de integración económica son buenos signos.

En 1994, con apoyo de UNESCO y el interés personal de su Director general, Prof. Federico Mayor, de ICSU y de la Academia de Ciencias de América Latina, iniciamos la constitución de Redes Científicas Latinoamericanas en las áreas de física, matemáticas, química, astronomía y ciencias de la Tierra, usando como modelo la Red Latinoamericana de Ciencias Biológicas (RELAB). El Comité Coordinador de Redes Científicas de Latinoamérica (CCRCLA) constituye una instancia representativa de la comunidad científica latinoamericana. Se ha creado un programa de intercambio de jóvenes científicos y se ha pedido a los gobiernos de los países participantes a que presten su apoyo político y financiero.

Una idea que ha arraigado con fuerza entre los científicos latinoamericanos es la de reforzar los vínculos con nuestros colegas de España y Portugal. En los años 60, el nivel científico que había en España no era muy diferente al de Argentina, Brasil, Chile o México en esa época. Personalmente, recuerdo que en el Congreso Nacional de Bioquímica (New York, 1964), se presentaron 4 trabajos de España y 4 de Chile. La situación ha cambiado y el panorama español es hoy superior al de América Latina y cercano al de los países europeos de mayor trayectoria y tradición. La colaboración entre nuestros países y las actividades de intercambio en la formación de los jóvenes científicos es algo deseable y mutuamente beneficioso. En un momento en que desaparecen fronteras, necesitamos integrarnos en una gran comunidad iberoamericana.

En la VI Cumbre Iberoamericana (Santiago de Chile, noviembre de 1996), el presidente chileno propuso la creación de un Fondo Iberoamericano de Integración Científica y Tecnológica. Los 29 mandatarios de los países latinoamericanos solicitaron al gobierno de Chile una proposición operativa para la VII Cumbre, que tendrá lugar este año en Venezuela. Esperemos que la propuesta incluya la opinión de los científicos de nuestros países y que constituya una eficaz herramienta de integración y desarrollo.

Jorge E. Allende
Universidad de Chile

Jorge E. Allende es profesor de bioquímica en la Universidad de Chile, en Santiago. Durante los últimos treinta años, paralelamente a su carrera científica, ha trabajado en favor de la integración científica y tecnológica de América Latina, y ha coordinado desde su inicio, en 1975, la Red Latinoamericana de Ciencias Biológicas. Ha sido Presidente de la Academia de Ciencias de Chile. Actualmente preside el Comité Coordinador de Redes Científicas Latinoamericanas.

Correspondencia: Jorge E. Allende. Departamento de Bioquímica. Facultad de Medicina. Universidad de Chile. Casilla 70086. Santiago 7. Chile. Tel. y Fax: +56-2-7376320. E-mail: jallende@abello.dic.uchile.cl

MICROBIOLOGÍA SEM **13** (1997), 131–148 **MINIREVIEW**

Two generations of spore research: from father to son

Harlyn O. Halvorson

Policy Center for Marine Biosciences and Technology, University of Massachusetts, Dartmouth, USA

Received 3 February 1997/Accepted 2 April 1997

Summary

Harlyn O. Halvorson has played an active and defining role in the field of microbiology for more than 40 years. In this article, he reminisces on the major influence that his father, H. Orin Halvorson, had in the direction of his professional life. The two H. O. Halvorson's, often confused in the literature, became one of the only two father and son pairs to head the American Society for Microbiology in 1954 and 1977, respectively. Major scientists of the era, their research and their collaborations, are remembered in relation to their main works. Harlyn O. Halvorson's interest in bacterial and yeast spores, and especially spore germination in bacilli, is the highlight of this memoir. His scientific research thrusts, leadership at Marine Biological Laboratories, Woods Hole, NACSEX (North American-Cuban Scientific Exchange) and elsewhere, his dedication to advanced students and numerous other endeavors, both scientific and political, are here told.

Key words: Harlyn O. Halvorson, H. Orin Halvorson (1897–1976), *Bacillus*, *Saccharomyces*, bacterial and yeast spores

Resumen

Harlyn O. Halvorson ha desempeñado un importante papel en la microbiología durante más de 40 años. En el presente artículo, rememora la enorme influencia que su padre, H. Orin Halvorson, tuvo en la dirección que su vida profesional tomó. Ambos H. O. Halvorson, a menudo confundidos en la literatura, fueron una de las dos únicas parejas de padre e hijo que dirigieron la American Society for Microbiology en 1954 y 1977 respectivamente. Importantes científicos de la era, sus investigaciones y colaboraciones son recordados en sus más importantes trabajos. Su interés en las

Correspondence to: Harlyn O. Halvorson. Policy Center for Marine Biosciences and Technology. University of Massachusetts, Dartmouth. North Dartmouth. MA 02747. USA. Tel.: +1-508-5401030. Fax: +1-508-5405441. E-mail: hhalvors@mbl.edu

esporas bacterianas y de levaduras, y especialmente la germinación de esporas en bacilos, son lo más destacado de este artículo. El dinamismo de sus investigaciones, su capacidad de dirección en el Marine Biological Laboratories, Woods Hole, NACSEX (North American-Cuban Scientific Exchange) y en otras instituciones, su dedicación a los estudiantes más avanzados, y otros numerosos esfuerzos, tanto en el campo científico como en el político, se explican en este artículo.

Introduction

Since microbiology involves so many aspects of our living experience, I feel fortunate to have had a career in the field. Microbiology relates to our health, infectious diseases, purity of water, food processing, and many other issues. Biotechnology started with fermentation and now includes studies of the fundamentals of living systems; molecular biology is built on experiments with microorganisms. Intermediate metabolism was revealed to a large extent by studying bacteria. The studies of evolution and of molecular systematics have also been built around the use of microbes. Such studies allow one to be in touch with a wide variety of biological processes fundamental to mankind.

Microbiologist, son of a microbiologist

One certainly is influenced, even if subconsciously, by one's parents. I was born to my parents, Selma K. and Halvor Orin Halvorson, in Minneapolis, Minnesota, USA, on May 17, 1925. Ours was an unusual family in that every Sunday afternoon we visited sewage treatment plants. When I was about 10 years old, I realized that none of my friends shared these experiences. This background led eventually to about 10 years of my own research on polyphosphate accumulation in microorganisms that predominate in sewage. Two big problems dominate sewage treatment. Phosphate removal to prevent algal blooms and nitrogen removal. In many older treatment plants the phosphate and nitrogen are removed by a chemical process. In a new method, the "anoxic/oxic (A/O) process", both phosphate and nitrogen are removed by microbial action (32). Phosphate is removed by creation of a unique compound, polyphosphate, with chain lengths of up to a thousand. Nitrogen is removed by denitrification. Both occur in a couple of cycles of the A/O processes. A number of microorganisms accumulate polyphosphate, including *Acinetobacter*. Although *Acinetobacter* does not metabolize glucose, it is a strong aerobe. In starting a wastewater treatment plant, if the oxygen is turned off every so often, after a few months a population of microbes develops of which *Acinetobacter lwoffii* is prominent. Playing the major role of phosphate removal, this microorganism accumulates 25% of its weight as polyphosphate. These polyphosphate granules have been known since 1805 as "volutin granules". When the biomass is removed so is the phosphate. This produces a good fertilizer (11). The A/O system enriches for microorganisms that clump; they settle out readily and are easily filtered off. The *Acinetobacter* produce surface proteins that adhere to each other. Since you have to disperse the clumps, it is a bit of a problem to study them.

In 1948, my father moved from the University of Minnesota to the chairmanship of the Department of Microbiology at the University of Illinois in order to revitalize the Department. He recruited a team of young scientists to use microorganisms to study biochemistry, genetics and bioregulation: I. C. Gunsalus, Salvador Luria

and Sol Spiegelman. In short order this was considered one of the best, if not the best, microbiology departments in the country. The next decade was a "golden era" in which many students were launched into the newly emerging fields of microbiology. In moving to Illinois, my father also decided to concentrate his personal research on a subject that emerged from his industrial interests with the Hormel Meat Packing Company, Austin, Minnesota: bacterial spores.

The Department at of Microbiology of the University of Illinois had been largely a food bacteriology department. He found in the icebox many strains of spore formers, including one with a very large spore. *Bacillus cereus* T is a strain that produces a large terminal spore, which is why "T" is in the name. These very large cells were selected because they are particularly easy to centrifuge and isolate in order to prepare a population of only spores. *B. cereus* T became for many years the favorite object for the study of spores and for some time becoming the *Escherichia coli* of the spore field.

I had initially started out working on induced enzymes in yeast, a career that went in a different direction from my father's. When I started to work on spores, I found I was confused with him in the literature since my father had given me the same initials as his own! His name was Halvor Orin, mine Harlyn Odell. He went by H. Orin Halvorson and I go by Harlyn O. Halvorson. Our names were randomly distributed in journal abstracts. Finally we published a paper together to show there were two Halvorsons, but that did not entirely resolve the conflict. Once in Paris, when I was at the Institut Pasteur with Jacques Monod in 1955. Working alone on a Saturday morning, I saw Monod talking to an exceedingly old gentleman. He was shaking his head. They finally came inside and the elder scientist asked me about formic hydrogen lyase, an enzyme my father had worked on in 1929. He had purified it from hospital samples in Minneapolis and, with his co-workers, determined for the first time an iron dependency of the enzyme. I said yes, I remember that very well. We talked a bit about my father's published experiments and I said to the elderly gentleman maybe he would like to know about some of the experiments that did not get into the paper. The last I saw of this gentleman he was walking out with his head still shaking. He said he could not believe that I was so young; I was born in 1925!

My father had a very active career. Like most scientists, he spent most of his time in the laboratory and at scientific meetings with his many microbiology students. There was very little money available to support students in the 1930's, so my father's graduate students usually lived with us. As a young boy I grew up living with a series of graduate students, people with whom I have kept in contact since. I would actually say that I am one of those fortunate people who went into science because of the influence of my father.

Born in Wisconsin in 1897, my father was a self-made man who came to the University of Minnesota from a small farming community (River Falls, Wisconsin) just prior to World War I. He trained initially as a chemical engineer. Through interests in engineering he eventually studied wastewater treatment and became drawn into microbiology. He was the first Ph.D. in the Department of Bacteriology at the University of Minnesota Medical School in 1925. He was always interested in applied science and from there became involved in a number of projects, all of which gave rise to research programs that looked for basic understanding of the problems encountered. He worked with George Hormel at the Hormel company where he helped to determine the number of bacteria on the surface of ham in the late 1920's, which helped lead to the development of Spam a decade later. His earlier

quantitative discoveries led to his discovery, as the sixth person to do so, of the Poisson distribution. George Hormel was sufficiently impressed with the utility of basic research in his company that he created the Hormel Research Institute (affiliated with the Universtiy of Minnesota) out of his barn in Austin after World War II. While at Hormel, my father also developed probability tables for dilution to estimate the most probable number of bacteria. These tables were widely used, and even Salvador Luria and Max Delbruck used them in determining the mutation rate of bacteria in the late 1930's.

He also worked with another company: the Minnesota Valley Canning Company (Green Giant peas and corn). This work brought him to problems of sterilization. Stemming from the Hormel company experience and his work with the Minnesota Valley Canning Company, he developed his own interest in bacterial spores. When he eventually took over the chairmanship of the Department of Microbiology at the University of Illinois in 1948, he decided to devote his research lab entirely to the study of bacterial spores.

I, of course, was exposed to his work as a young boy and visited his laboratory a number of times. He built a lab in our basement which gave me my early experience in science. My father was also deeply interested in education and public service. During his presidential public lecture at the American Society for Microbiology, in 1954, he reviewed the educational background of the leaders in microbiology, none of whom at that time had been trained as microbiologists. In fact, I would say he was basically more interested in training students than in publishing papers, although he had a good publication record.

Many years later, in 1977, I too became President of the American Society for Microbiology. We were the second father/son presidential combination. The Murrays (Robert Murray, University of Western Ontario, Canada, and before him his father, E.D.G. Murray, McGill University, Canada) previously had been presidents as well, and I have had occasion to compare notes with the younger Murray about this.

Yeast

After I graduated from college I took a Master's degree in nutrition. Through this part of my education I became interested in problems of protein synthesis and of regulation of cells in general. The study of cell regulation, it became obvious to me, was far too complicated a problem to be studied in intact animals. I sought a simpler system in which developmental changes could be studied. I encountered the work of Sol Spiegelman, who then, as a faculty member at the University of Minnesota, worked on induced enzyme biosynthesis in yeast. When he relocated to the University of Illiniois, Department of Microbiology, Urbana, with my father, I decided to join Spiegelman's laboratory and carry out studies with *Saccharomyces cerevisiae*.

This microorganism is a simple eukaryotic cell which undergoes a life cycle in response to starvation. The cells go through the reductive cell division process called meiosis which leads to the formation of four haploid spores. These spores then germinate and grow as haploid cells. A haploid cell may bud and grow perfectly adequately, but before sporulation is possible, a diploid organism must be formed by the fusion of haploid opposite mating types. A haploid culture of yeast does not sporulate. These yeasts have two mating types, *a* and α which conjugate to form the diploid cell. When the diploid cell undergoes meiosis, the life cycle is complete. In the life cycle of this eukaryotic single-celled organism the diploid state is followed by the reductive meiotic division and a morphogenetic

change to form spores called ascospores. When ascospores germinate, the cells return to the growing stage. This life history has certain similarities to bacterial spores, also a major part of my research career.

Much of my research career has been devoted to studying regulation of synthesis of specific proteins in *S. cerevisiae*. Not long after my interest in bacterial spores emerged I became intrigued by similarities that may exist between sporulation in yeast and in bacilli. An opportunity to make these comparisons arose when a Canadian graduate student at the University of Wisconsin, Paul Rousseau, developed an interest in yeast spores and learned how to produce them in quantity (26). Together we isolated sufficient amounts of yeast spores to enable a study of their physical properties and the conditions that would support their germination (29).

Many underlying biochemical events which happen in yeast cells in response to starvation, and in the formation of a semi-dormant state, are parallel to those in bacteria. However, the spore cortex and the unique dipicolinic acid of bacteria are missing from yeast cells. The dormant yeast spore has some metabolic activity; yeast spores are not as resistant as those of *Bacillus*. The yeast spore coat is quite similar to the normal cell walls of growing yeast, unlike the properties of bacilli. Sporulation is essentially a response to starvation—it is an aerobic process involved in acetate metabolism that requires depletion of nitrogen or some other component. Yet, like bacteria in response to simple chemical stimulants, yeast ascospores germinate to become more metabolically active (28). Less is known about these chemical stimulants in yeast. A sequence of events occur in ordered fashion comparable to those described for *Bacillus* (27). We have a basic behavior in a fungus (yeast) and a bacillus which may well have been derived through evolution from simpler bacterial systems.

Alanine and *Bacillus* spore activation

I have had a lifetime of experience working with bacterial and yeast spores. I had an advantage from the beginning. My father, while working at the Hormel company, became interested in bacterial spores on ham. Many experiments were done to both count the bacteria and to see if contamination of ham by bacteria could be controlled. Eventually my father began a research program on bacterial spores with one of his students, Babette Taylor. They demonstrated that spores, even though metabolically inert, had a surface enzyme (alanine racemase) that converted L-alanine to a mixture of L- and D-alanine. D-Alanine inhibited germination stimulated by L-alanine. The discovery of a specific inhibitor that blocked the germination process was to me fascinating. I watched their work while I studied nutrition as a graduate student at the University of Minnesota.

I had an early interest in using microorganisms to study development. I was influenced by Cornelis B. van Niel (1897–1985), of Stanford University, having taken his summer course (in 1950) at the Hopkins Marine Station, Pacific Grove, California, where we were introduced to a variety of interesting organisms. I also had the opportunity to attend a lecture series in embryology at the Hopkins Marine Station where I was intrigued with developmental trigger mechanisms which had been shown to operate in sea urchins and other animals. A microbial model, I thought, could provide a simplified system for study of developmental triggers.

Progress in spore research was promoted by the creation of a series of American Society for Microbiology (ASM)-hosted spore conferences. The origin of the ASM conferences can be found at the Leeds spore conferences, organized by the late Joseph Wolfe, that my father, one of my students, Brooks Church, and I attended in 1954

in Leeds, England. The success of this meeting led to an informal discussion my father organized at the SAB meeting in Houston, Texas in 1956. With the help of Roger Reid (ONR), the first Spore Conference was organized October 11–12, 1956, at Allerton Park, Illinois, sponsored by the University of Illinois and ONR. These conferences, held every 3–4 years, defined the subject for more than a decade. Starting with the 3rd Spore Conference in 1964, these were sponsored by ASM. Following my father's retirement in 1966, the conferences were moved to the University of Wisconsin (chaired by myself), Michigan State University (chaired by Phil Gerhardt), and back to the University of Wisconsin (chaired by Richard Hanson). I was involved in these conferences through the 1970's. The last Conference was held in 1992 at the Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts (chaired by Simon Silver).

In the late 1940's and early 1950's, Joan Powell, Medical Research Council, Porton, England, made the exciting discovery that one could germinate spores of *Bacillus* by simply exposing them to L-alanine. This was a simple trigger response. Bill Murrell, CSIRO, Homebush, Australia, who was doing his Ph. D. thesis at Oxford University, went on to describe the physiology of the germinating spores. By the early 1950's, spores of aerobic spore-formers were ready to be used as a model to study development. Three terms describe the transformation of a spore to a growing cell: activation, germination and outgrowth. Spores are "activated" by heat in a process that does not involve any viable structural change. "Germination," a degradative process, does not involve any macromolecule synthesis (Fig. 1). Materials are lost from the germinating spore, it becomes less refractile, and water is taken up. Germination is followed by the process of "outgrowth", which involves biosynthesis as RNA and proteins are synthesized and DNA eventually is replicated. The cell, during outgrowth, reverts to the vegetative, growing state. Spores were interesting at this time not only as a model for differentiation. They also excited interest because of the belief that they could survive prolonged periods of dormancy and could germinate immediately, within minutes, after years of dormancy.

As my first interest began with germination, I will discuss this first. In the 1950's I joined the University of Michigan faculty at Ann Arbor. One of my first students, Brooks Church, came from Fort Dietrich, Maryland. He had previously worked with *Bacillus globegii*, which was used as a control for spore dispersal. Colonies of *B. globegii* formed a red color, making it easy to identify them. Church was interested in studying the mode of action of ethylene oxide—a very toxic gas used to sterilize spores. We decided to solve this problem by studying the mechanism of germination (3). We returned to L-alanine as a trigger for germination and tried to estimate how few molecules might be involved in the process. In these studies we had a unique opportunity to study the spore collections amassed at Fort Dietrich and to assess the ability of these spores to survive for many years.

The dormancy analysis of the Fort Dietrich spores showed that the ability to survive declined slowly with time. Knox Harrell, another graduate student at the University of Michigan, also became interested in the L-alanine trigger (13). He presented spores with ^{14}C-labeled alanine and collected all the gas released from them. We washed the spores carefully and showed that they could germinate after just a few seconds exposure to alanine. The radioactive carbon on the surface of the spores was still present as alanine. The spores did not metabolize the alanine. More importantly, only a few molecules per spore sufficed to trigger the germination response. We did not have the tools then to

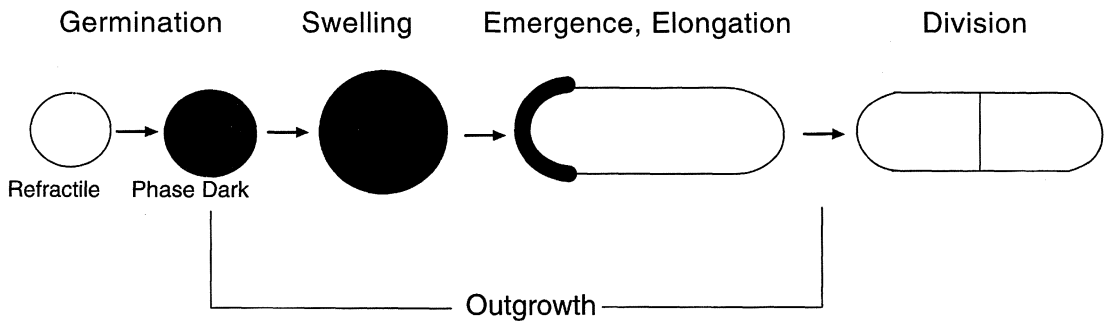

FIG. 1. Germination and growth of bacterial spores.

proceed further but we did define the problem in terms of the need for only a few L-alanine molecules to initiate germination.

In 1955, I took a sabbatical and went to the laboratory of Jacques Monod at the Institut Pasteur in Paris. At this time there was a great deal of interest on the mechanism of induced enzyme biosynthesis. An understanding of genetic control of induced enzyme formation was developing. From my own Ph.D. thesis experiments (8) and experiments of others, by 1955 it was established that enzyme adaptation both in yeast and in *E. coli* involved de novo protein synthesis. These responses were triggered in cells by the presence of a specific compound in the medium. This, too, was developmental biology.

On returning to the United States from the Institut Pasteur, I transferred to the University of Wisconsin at Madison. The Bacteriology Department was looking to bring in some new blood and Joshua Lederberg, then in the Genetics Department, expressed an interest in my being recruited. On relocating to the University of Wisconsin, I decided to return to the issue of alanine-induced germination and problems related to spore survival in dormancy. Without the presence of a series of students and postdoctoral fellows who were interested in bacterial spores it would have been impossible to study these problems. One of the first of these was Richard O'Conner, a very innovative student who, after a productive doctoral thesis and a promising career at Monsanto chemical company, tragically committed suicide. Richard undertook a new approach to the study of the alanine-induced response to germination (21). A variety of compounds were tested for their ability to support germination. The question was: Is there a specific site? Is the site responding to germination (as with bacterial adaptation) stereospecific? Is there a relationship to alanine dehydrogenase? (23). So we followed up with many experiments by Neil McCormick (20). Richard O'Connor purified alanine dehydrogenase (22). We studied its physical properties, its specificity and its pH optimum, to see whether the physiological process of germination, which is obviously very complicated, matched closely to the properties of alanine dehydrogenase. In fact it did. We also found that we could vary the level of alanine dehydrogenase in spores by different methods of producing the spores. Spores with different levels of alanine dehydrogenase varied their response to alanine-induced germination (19). Our hypothesis, one we held for many years, was that alanine dehydrogenase (or something very similar to it) is the initial receptor site for the first step in spore germination (24). Ultimately this proved not to be the case. Ernst Freeze, at the National Institutes of Health, Bethesda, Mary-

land, was able to obtain mutants deficient in alanine dehydrogenase that still germinated. The actual receptor site, whatever its identity, must share many properties in common with alanine dehydrogenase.

Kinetics of *Bacillus* spore activation

We also undertook a kinetic analysis of spore germination, together with Carl Woese, University of Illinois. He had for many years been working at General Electric on models for the origin of the genetic code and later went on to study the molecular basis of bacterial phylogeny. He became interested in some of the kinetic responses to germination. Together we derived a model, consistent with what we had seen earlier, that explained germination kinetics on the assumption that there were only one or two sites reacting per spore (37). Different species of spore-forming bacteria responded to other compounds besides alanine. One of these, interestingly enough, is a compound that is contained uniquely within bacterial spores and produced at some 20% of the dry weight of the spore: calcium dipicolinic acid. One can vary the level of dipicolinic acid and show that it affects heat resistance, dormancy and germination properties (17). We found with others that when dipicolinic acid was present in equal molar amounts to calcium in the medium it would germinate spores (16). One could bypass or at least provide an alternate way to the alanine-induced metabolically active system, a germination based on calcium dipicolonic acid. We speculated that there must be several routes that could initiate the degradative events in germination that would later have a common pathway.

That germination was complex became very clear to us when Ian Dawes, a postdoctoral fellow in my laboratory at Madison and later with me at Brandeis University, started to look at temperature-sensitive mutants that at the non-permissive temperature would be blocked at different stages in germination but at lower temperatures function normally. A series of temperature-sensitive mutants were produced that showed more than one pathway (5). That led to a collaboration over many summers that went on at MBL, with Alex Keynan, and later with an expert in trypsin enzymes, Walter Troll (2). Troll had a long interest in cancer research and the role trypsin inhibitors might play in the process of rapidly dividing cells. We found that a variety of compounds which inhibit trypsin-like enzymes would also inhibit germination (1). The spores would begin to germinate and then would stop—that is they would not go completely "phase gray", a term which refers to bacterial cells as they appear under phase contrast light microscopy. Dormant bacterial spores are highly refractile because they contain very little water (Fig. 2). They have the refractility of hair. As germination occurs the spores lose this refractility, take in water, become phase gray, and display a refractive index typical of a normal vegetative cell. If you look at the germination process, as one can by taking pictures of a given spore at brief intervals (34), or if you look at mutants or at cells which are inhibited, they go through part of the germination, lose part of their refractility, but then stop. We know germination involves a series of phases. At that time of course we did not have much of a clue as to what was happening biochemically other than we knew that the cells were releasing materials like cell wall components, dipicolinic acid and peptides. The compounds identified as bacterial cell wall components were in fact originally discovered when they were isolated in the early 1950's as products released from germinating spores. Both the mutants and the inhibitor experiments indicated to us that some process related to degrada-

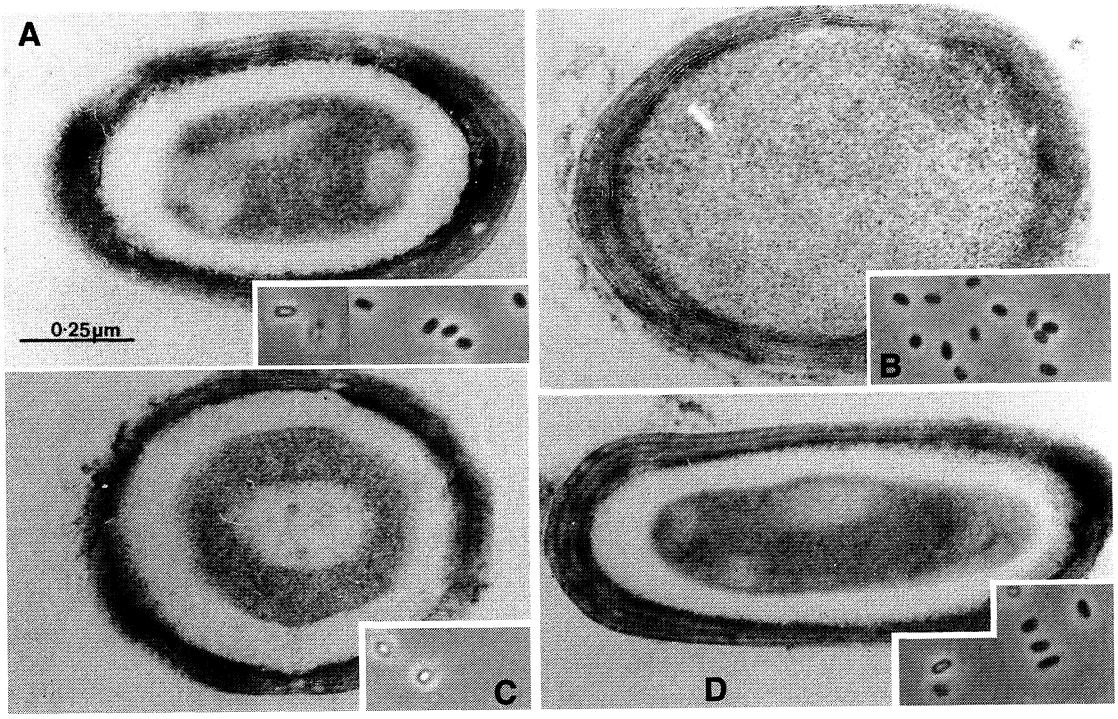

FIG. 2. Germination of bacterial spores. Electron micrographs of the following stages: (A) refractile spore; (B) swelling; (C) outgrowth; (D) elongation. (Insets are the corresponding light micrographs.) (Courtesy of Richard Warburg.)

tion was involved in germination (1, 2). This led us to look at enzymes that we could extract by grinding up spores. We searched for types of enzymes that were sensitive to the same inhibitors that blocked germination. The thought began to emerge that germination initiated a process that activated degradative enzymes. Through its activity, this barrier of a tight cortex—that holds the spore like a squeeze sponge—is broken open allowing water to enter and then releasing metabolic activities. That there are many steps in this process was also indicated by the ability to isolate numerous germination genes that were called *ger* genes. We spent some time on a more prominent one of these *ger* genes. Richard Warburg, who had previously studied germination mutants in D. Anne Moir's laboratory, Sheffield University, U.K., cloned the *ger* gene in 1986 and mapped the gene involved (35). There was a fairly extensive interest from a number of labs to characterize the genes involved in the germination process.

Germination of bacterial spores first requires activation. The late Brooks Church started spore research in my laboratory at the University of Michigan by correlating the metabolic activity of the spore with the extent of heat activation applied (4). The temperature and amount of heat required can be used as an index of dormancy. Spores stored for longer periods of time, or with greater quantities of dipicolinic acid, required more heat to be activated (Fig. 3). Once activated, spores could be returned to dormancy following refrigeration. The process of reactivation followed by dormancy could be repeated several times but eventually the ability of spores to respond was lost. A relation somehow existed between the need for heat activation and the

poising of the spore for germination. Another graduate student at Wisconsin, Roy Doi, extensively analyzed enzymes extractable from activated, dormant and growing cells (6). The enzymes were present both in dormant and activated cells; the mere act of rupturing the spore coats released most of the enzyme activity. In those days my lab also formed part of a group studying ribosomes and the mechanism of protein synthesis. Applying our knowledge to bacterial spores, we discovered that ribosomes derived from bacterial spores could not support protein synthesis in extracts from growing cells (18). Yashu Kobiashi, University of Tokyo, Japan, together with John Idriss, University of Wisconsin, demonstrated that spore ribosomes were defective. The restoration of active ribosomes following protein synthesis occurred as active germination proceeded (15).

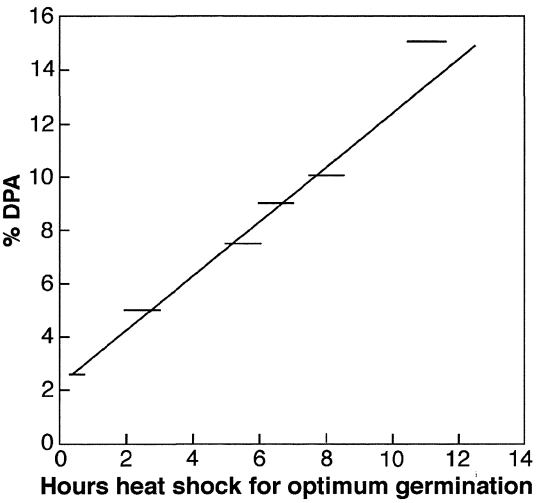

FIG. 3. Heat activation of bacterial spores: percent dipicolinic acid and hours of heating needed before germination. Redrawn from (19).

Bacillus spore DNA

The conversion of a germinated spore to a vegetative cell requires outgrowth. When I came to MBL in 1962 to participate in the microbial physiology course, I chose to use outgrowth as a subject of study. The MBL courses offer very intensive, hands-on experience where students also take on research projects. Two of my students, Bill Steinberg and Jim Vary, accompanied me to help in studies of the outgrowth of bacterial spores. Inhibitors of transcription did not inhibit germination but did prevent return of the spore to a growing cell (outgrowth) (9). Germination, we concluded, involved a degradative process followed by a biosynthetic process. An intriguing question was the structure of DNA in the dormant state within spores. Spores in some strains survive autoclaving: high pressure and well over 100°C, i.e., temperatures that denature DNA. We began researching ways to break spores to analyze the structure of their DNA. One postdoc at the University of Wisconsin who went on to the University of Michigan, Harry Douthit, extracted DNA from *B. cereus* spores and found an intriguing satellite DNA band (7). This DNA was present in dormant spores, but as they germinated and went through outgrowth, the band disappeared. In 1965 I spent a sabbatical in the CNRS Laboratories of Kiesel Szulmajster in Gif-sur-Yvette, France, south of Paris, where the year was spent isolating and characterizing dense satellite DNA from *B. cereus* (12). Like vegetative DNA, satellite DNA had the standard base residues. We did not find any unique bases, proteins or amino acids in the purified DNA. We did not understand the nature of the heavier density. This spore satellite DNA could not be used for transformation. When the satellite DNA reverted to a normal DNA density, it could then be used readily for transformation. Only much later did it become clear that we were studying a change in the DNA structure of spores.

Alex Keynan and I then studied the effects on spore outgrowth of DNA synthesis inhibitors.

We found that DNA only became transcribable after a transition that was subject to inhibition. Rajendra Rana, a postdoctoral fellow from Pantagar, India, showed that DNA repair occurs first before DNA synthesis. Our experiments with radioactive isotopes showed this period involved in the repair process to be about an hour before synthesis of DNA occurred (25).

Experiments from the Institut Pasteur and elsewhere made it clear that the *E. coli* genome in growing cells was always accessible to transcription. Growing *Bacillus* cells also behave this way. Ordered synthesis occurs in the process of outgrowth as shown by Bill Steinberg, together with Tore Hoyem, Veterinary College of Norway, Oslo, Sid Rodenberg, University of Pennsylvania, and Harry Douthit (14, 31). Using specific inhibitors, they found that 2 or 3 min before protein synthesis begins, transcription (RNA synthesis) proceeds. This paralleled the transcription/translation/protein synthesis sequence of growing cells. The proteins made by germinating spores did not represent the entire spectrum of the growing cell proteins, but rather they appeared in an ordered sequence. DNA taken from spores at different intervals demonstrated an order of transformability of these same DNA markers (31). DNA apparently was made accessible sequentially for translation. Once growth occurred the entire genome was accessible for transcription. Bill Steinberg went on to analyze this ordering in a series of interesting studies at the University of Virginia. His sudden, premature death from cancer at age 38 in 1976 brought these promising studies to an end, and reminds us of the limited time we each have to achieve our goals.

The outgrowth period has a clock mechanism built into it, as in other differentiation processes such as those found during the cell cycle of yeast. Some genes, such as those of the ribosomes or the histone proteins are transcribed all the time. Some other enzymes were sequential (Fig. 4). George Spiegelman, the son of my mentor Sol Spiegelman, came to my lab to work on the Spiegelman/Gillespie method of DNA hybridi-

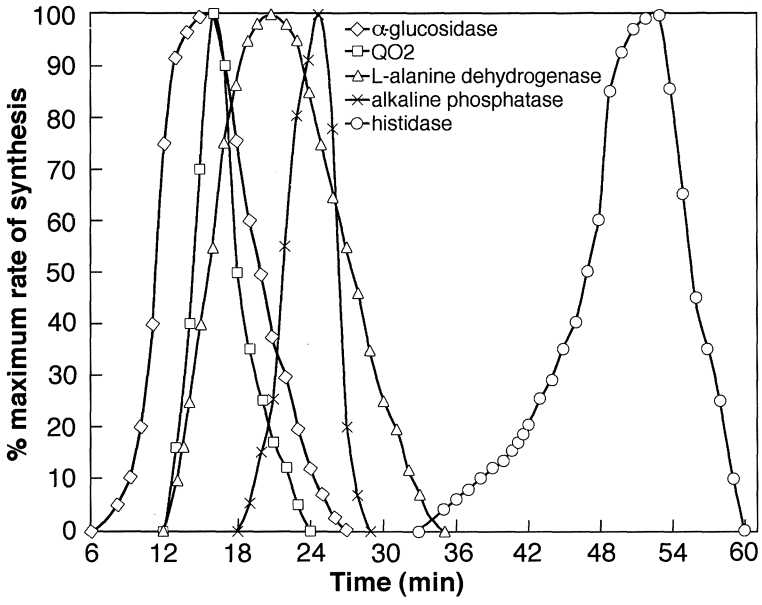

FIG. 4. Appearance of enzymes through time after spore germination. Redrawn from (31).

zation. Different messenger RNA populations were present at different times during outgrowth (30). They developed hybridization-competition experiments indicating that RNA messages were made in an ordered sequence. In growing cells, by contrast, the full population of messages was present. The ordering of transcription, as we have seen from enzyme activity data, is paralleled by an ordered translation. This transfer from dormancy to activity proceeds through a set of fundamental processes common to differentiation in other systems. Successful survival of an organism depends on the built-in ability to remain dormant or to germinate in the presence of appropriate germination stimulants.

Bacillus spore dormancy

One of my primary interests had to do with dormancy. What general features are expected in dormancy? Metabolic processes must be turned off during the formation of spores (sporulation). Bacilli ultimately generate a dry object in a liquid environment! Events must occur in sporulation that restrict and direct synthesis to unique spore components. Many studies on sporulation and its genetic control point not only to unique component synthesis but to changes in DNA structure. A set of proteins with dipicolinic acid make a new cortex, squeezing and tightly restricting the spore surface. A repair system must assure spores survive for long periods of time. During storage, much DNA damage is accumulated that needs repair. Furthermore, to respond to unique opportunities that may occur only briefly, once in many years, a mechanism for rapid germination must be in place. This germination system must not be too efficient. The survivors are those cells which germinate slowly. They must be able to persist for a second round of germination in case, after the first clue to a supportive environment, conditions are in fact unfavorable. In 1929, Elizabeth McCoy, at the Department of Microbiology, University of Wisconsin, micromanipulated individual spores into rich nutrient growth medium. She studied what happened to their ability to germinate. Most germinated immediately. Some germinated in a few days, some in a few weeks and very few in a year or more. She described this as "delayed dormancy." In spores isolated from nature, the germinated ones can be separated from the ungerminated. Upon reisolation of the ungerminated ones, one can select for delayed dormancy.

The quantity of dipicolinic acid and response to L-alanine varies. The spore composition reflects to some extent the conditions under which it is produced. Spore-forming bacilli have built-in delayed dormancy, a very interesting survival mechanism. Many claims were made in the literature that spores survive for long periods of time. Al Sussman, a scientist in the Botany Department at Ann Arbor, Michigan, and I shared an interest in dormancy when I was at the University of Michigan. We surveyed the literature and wrote a book, published in the '60's, on "cryptobiosis" (33) in which we evaluated the claims in the literature of dormancy of spores isolated from salt mines or other ancient systems. We could never have confidence that these ancient samples had not been contaminated. So while the claims were interesting they were not conclusive.

In the early 1960's Peter Sneath, Leeds University, England, developed a very clever approach to studying spore dormancy by going to the British Museum herbarium and collecting soil samples from plants that had been stored for periods up to 300 years. He had multiple collections from similar sites. He analyzed the bacterial content of these samples. As controls he used the paper in which the plants were wrapped. Most plants were poisoned with metals and kept

dry. The number of viable spores decreased with time. The kinetics suggested a rapid die-off in the first period, 50–100 years with a half-life of about 50 years. Older samples contained spores with a half-life of about 100 years. A relatively rich spore population could survive for several hundred years. He postulated that the killing rate was roughly that expected from the radioactive decay of the trace metals found in spores. This work was probably the best measurement of bacterial dormancy one could find. It paralleled studies from the Sahara Desert, where the viable number of spores decreased exponentially with depth. Survival of spores in soil/sand parallels those which were found in the British Museum herbarium samples.

In my own personal experience we have germinated spores which have been stored in the laboratory for 40 years or more. Clearly spores can survive for a long period of time, but how long we do not know. No one has studied a given spore suspension one hundred years old or more. Museum and other isolates from nature show protracted survival. For example, Alex Keynan and I, along with students of the Microbial Ecology Course at MBL, isolated many spores from samples supplied by Holger Jannasch at Woods Hole Oceanographic Institute, Woods Hole, Massachusetts, which came from the Puerto Rico trench, one hundred miles north of Puerto Rico, and the deepest part of the Atlantic Ocean. We have also taken samples from deep marine soils and only a few spores germinate. Estimation of the percentage of germinating spores is not possible since we only see the viable ones. Not only do these germinating marine spore-formers show resistance to heavy metals but they are frequently resistant to a variety of antibiotics, including synthetic antibiotics only recently developed! The Mercks and Duponts of the world cannot be accused of producing these antibiotic resistant strains that are older than these pharmaceutical companies. Why antibiotics and antibiotic resistance evolved is unknown. These ancient spores with their unique properties are potentially interesting and useful.

Long term survival of spores was one of the reasons that Svante Arrhenius hypothesized in 1908 that life arose on Earth by transfer of spores through space. Joshua Lederberg became intrigued with space research and the Arrhenius hypothesis in the late 1950's. Meanwhile I had just moved to Wisconsin with an interest in spores so we jointly responded to a request from the U.S. Army to design an experiment to capture spores in space. As the space capsules were not recoverable at that time we had to measure spore germination in space. We prepared a grant proposal and designed an instrument that could remotely collect spores on a tape rotated outside the space capsule, expose them to the environment, rotate them back in, and spray them with germinants. We would measure the refractility changes electronically to monitor germination. We were all excited to do these experiments, but began to wonder why the Army was carrying out essentially civilian research. We spent a fair amount of time contacting members of the U.S. House of Representatives and Senate from the states of Wisconsin, Minnesota and Illinois. Eventually we received a letter from the Army saying that they liked our research but the authority to do this kind of research had been taken away from them. That was in 1957–1958, when the National Aeronautics and Space Administration (NASA) was formed. We never had an opportunity to do those experiments, although we later became interested in the search for bacteria with the Moon and the Mars probes. But that brief and interesting experience led to further interest on survival in dormancy.

A bacterial spore is an object produced in a liquid environment and yet it is dry! The trick is to find ways in which water can be excluded

during sporulation. Experiments with deuterium labeled water by others showed that most of the volume of the spore can be penetrated by water. If the macromolecules in the spore are all bound up in larger structures, then one can imagine how heat resistance and loss of metabolic activity occurs in the dormant state. Scientists have attempted for many years to provide explanations for the dormant spore. Dipicolinic acid chelates proteins through calcium and could provide a clue to this question.

Both sporulation and spore germination involve a series of genes, many of which have now been isolated, that are unique to the two processes. Bacterial spores are the most resistant propagules of life that we know of, probably due to the absence of water. They have the greatest prospect for long term survival.

While we lack complete proof, I believe the following offers the most likely interpretation of what is known about the regulation of bacterial spores. Sporulation involves a selective turning off of transcription in the genome. In outgrowth the transcription turns back on. Genome inactivity is probably due to reversible conversion of an inactive form of DNA which then binds with newly synthesized proteins. The structure of the DNA is probably responsible for sequentially turning off DNA transcription and translation. This differentiation cycle is very exciting to study.

Replication normally involves a gyrase enzyme that unfolds DNA; it opens up the double helix for DNA replication and repair. The gyrase which is also involved in the repair process is a normal component of DNA synthesis.

The correlation of DNA structure with sporulation and activation has also been studied by Peter Setlow. His hypothesis is that DNA is in the Z-form in the dormant spore. Sporulation leads to the production of the inactive form. During germination of the spore DNA converts from the Z- back to the normal form.

Marine spore germination

Since the studies of Russell in 1891, spore-forming *Bacillus* have long been recognized in marine environments. Numerous laboratories (Newton, ZoBell, Kriss, Dias, Leadbetter, Fammy, and Rheinheimer) have isolated these, following germination and growth, on complex sea water. Based upon the finding from molecular studies that functions are conserved in evolution, one might expect dormancy, or cryptobiosis, to share common mechanisms. Alex Keynen and I, along with students in the Microbial Ecology course at MBL, Woods Hole (36), found interesting parallels when we compared dormancy in marine spore-forming bacteria with soil microbes. Both spores from marine environments and from soil contain calcium dipicolinate and require activation to germinate. Since one does not expect sharp changes of nutrient concentrations to occur in sea water, the germination requirements may well differ. The brief periods of elevated concentrations of L-alanine or glucose from the breakdown of natural products that occur in soil are not expected in ocean water. To explore this we investigated the germination of one particular spore former, 3A10, isolated from a marine marsh sediment. Two unusual features of germination not reported in terrestrial spores were observed in 3A10: NH_4^+ serves as a germinating agent, and germination is stimulated by Na^+. Many amino acids, including L- and D-alanine, will also replace the NH_4^+ requirement making very unlikely a germination mechanism like the L-alanine triggering of *B. cereus* spores. We isolated hundreds of pigmented spore from ocean waters and sediments. While only a few were chosen for germination studies, there does not seem to be a common germination mechanism, nor an insight into their ecological role in marine ecosystems. This remains an interesting area for study.

Biotechnology and international science

Both yeast and *Bacillus* studies have led to applications of tools that can be used in service of mankind. "Biotechnology," with its long history, is derived from microbiology. It is not surprising that I developed an interest in biotechnology because my father's career was originally built from the practical applications of his research, and from there he went to basic science. My career was somewhat the reverse: I became interested in applied aspects of science after I had many years of basic science involvement.

The interest in biotechnology came in part from my work with the professional societies. Public policy issues, where science can be used to help solve social problems, and the ability to communicate with scientists in other parts of the world, are aspects of professional microbiologist life that interest me. My sabbaticals in France and Scotland brought me in contact with many scientists gathered there at a unique time in the history of molecular biology. I have always been interested in international science exchange. ASM had an exchange program with the USSR in the 1970's with which I was involved. I have also participated in exchange programs in Japan and had long joint programs with scientists in Israel.

The most interesting international interaction occurred as a result of an introduction by Lynn Margulis, then at Boston University, to Ernesto Bravo, a biochemist from Cuba who spent a year in Boston (10). Ernesto wanted to develop interactions between Cuban and United States scientists. Margulis and I both visited Cuba in the summer of 1983. Shortly thereafter we were involved in creating an organization called NACSEX (North American/Cuban Scientific Exchange). NACSEX organized visits of scientists and science writers to visit Cuba and to exchange ideas and information. About 80 individuals were part of this program which continued in the 1980's and 1990's. This led to a series of training programs primarily where new molecular biology in the United States was brought to the attention of active young Cuban scientists. Courses were given in Havana. Advice was provided to a growing program where the Cuban medical community tried to build a basic science infrastructure in a very short period of time. The Institute for Genetic Engineering was one of the focal points of this Cuban activity. In the mid 1980's this led to the construction of a large research center built under the guidance of the Cuban Academy of Science. Since then other scientific institutes have appeared in Cuba. Cuba has begun to depend heavily on technology to provide the economic motor for the country. Cuba has also begun to provide leadership to the third world through science. Scientific interchange has proven valuable even in the face of political and language differences between the countries. The language of science is international and is not easily limited by borders. Microbiology and molecular biology have played a key role in scientific interchange and technology transfer.

After the Israeli War in 1948, Prime Minister Ben-Gurion, who knew Alex Keynan, Israeli Institute for Biological Science, very well, noted that every country has its own basic medical research center. "I want you to create such a basic medical research center" in Israel, Ben-Gurion told Alex. He assigned Alex to go out and find an empty house or villa, even though Alex at that point was still a graduate student. Alex's wife, Malka, had been in charge of security for Jerusalem and Alex had been in charge of assuring the quality of the water supply in Jerusalem during the war. When the war ended, Alex found an appropriate place in Ness Ziona, near the Chaim Weizmann Institute, where they created

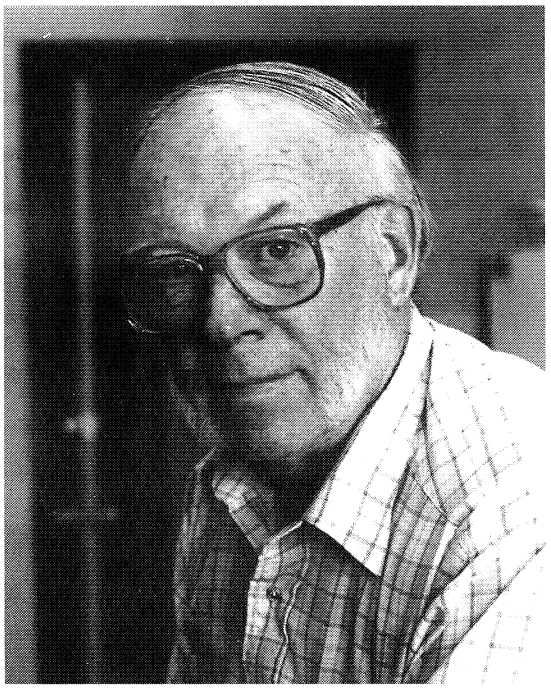

FIG. 5. Harlyn O. Halvorson. (Courtesy of MBL Information Office.)

the Israeli Institute for Biological Science. Alex became the director. Only many years later did he in fact receive his Ph.D. degree. I visited Ness Ziona a number of times while Alex was the director and I met many people. One of the most interesting was Alexander "Leshek" Cohen, a virologist and creator of the *Journal of Basic Irreproducible Results*. Alex hired many, many people including Ludwik Fleck, the Polish medical microbiologist who had survived life in the Nazi concentration camps. [See *Microbiología SEM* **12**, 131–136 and 659–660.] Fleck, in 1936, had written in German the masterpiece of the sociology of science "Genesis and Development of a Scientific Fact" (University of Chicago Press, 1979). I have continued to collaborate with Alex Keynan for nearly half a century (1, 2, 11, 36), through the 1988 MBL centenary, at which time I was still MBL director (Fig. 5).

The study of bacterial spores has provided many, my father and I included, an opportunity to explore the world around us. I feel fortunate to have had a career in a field such as microbiology which not only touches so many aspects of our lives, but also places a personal responsibility upon us.

William Golden, a New York banker, who designed the first science advisory organization for Henry Truman, said in his 1988 book, *Science and Technology Advice*: "More and more, every level of our government must take science and technology factors into account in policy formation in the long range and day-to-day decision making." But public officials are drawn, by-and-large, from outside the scientific community. They find themselves in the difficult position of having to explain their decisions to a public that is too often scientifically illiterate. Until we do something about the general level of science literacy among voters, it is hard to imagine very many public officials being guided in any serious way by good scientific and technological advice. Former U.S. Congressman George E. Brown, of California, said: "We should place scientific literacy very close to the top of our priority list in improving the conditions of science in this country. A scientifically literate public and scientifically literate Congress would do more for the health of science than anything else we could do."

Our society needs imaginative technicians —for which there is an increasing demand—and community and business leaders who are technology literate. Microbiologists have a unique opportunity to produce the leaders who will guide this country in the 21st century. History will judge us by what we *do* in this critical period in our history, and whether we *address* real society issues. We need to identify and articulate our national priorities—and that process should start here.

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MICROBIOLOGÍA SEM **13** (1997), 149–160 **MINIREVIEW**

Major viral diseases affecting fish aquaculture in Spain

Sara I. Pérez,* Sylvia Rodríguez

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

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Summary

The number of viruses isolated from fish has grown in the last few years as a reflection of the increasing interest in fish diseases, particularly those occurring in aquaculture facilities. Of all the described viruses, only a few are considered to be of serious concern and economic importance; they are described in this review, drawing special attention to the four families of viruses (*Birnaviridae*, *Rhabdoviridae*, *Iridoviridae* and *Reoviridae*) that have been reported in Spanish aquaculture. Infectious pancreatic necrosis virus, a member of the first family, is the most spread virus with a prevalence of 39%. Viral diseases are untreatable and because effective and safe vaccines for fish are not yet commercially available, a great care needs to be exercised when moving fish or eggs from one site or country to another. Some fish health control regulations have been legislated in Europe and USA.

Key words: fish viruses, fish diseases, rainbow trout (*Onchorhynchus mykiss*), turbot (*Scophtalmus maximus*), seabass (*Dicentrarchus labrax*)

Resumen

El número de virus aislados en peces ha aumentado en los últimos años, como consecuencia del creciente interés en estas enfermedades, especialmente en aquéllas que se producen en las instalaciones de acuicultura. De todos los virus descritos, sólo unos cuantos pueden considerarse graves y de impacto económico; ellos son los que se describen en esta revisión, con especial interés hacia las cuatro familias de virus (*Birnaviridae*, *Rhabdoviridae*, *Iridoviridae* y *Reoviridae*) cuya existencia ha

* *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

sido descrita en la acuicultura española. El virus de la necrosis pancreática infecciosa, un miembro de la primera de estas familias, es el virus más extendido, con una prevalencia del 39%. Las enfermedades víricas carecen de tratamiento y puesto que tampoco existen aún vacunas eficaces comerciales, son precisas grandes precauciones en los movimientos de peces o huevos embrionados entre zonas o países distintos. En Europa y EE. UU. se han legislado normas de control sanitario respecto a ello.

Introduction

Aquaculture has dramatically expanded worldwide during the past decade. Global aquaculture production is estimated at nearly 10 million metric tons annually, and it contributes more 12% of the total consumed fish and shellfish. Fish represents more than two thirds of the aquaculture output, and the industry is now projected to grow at an annual rate of 8% through the year 2000 by the United Nations Food and Agriculture Organization (22). Like other animals, however, fish are susceptible to microbial disease, and although the range of the pathogens appears to be rather restricted, and they can cause relative problems among natural fish stocks, they are devasting when fish are subjected to intensive culture practices, since it is under these conditions that extremely large numbers of fish can rapidily become infected and die. It has been estimated that 10% of all cultured aquatic animals are lost as a result of infectious disease (22). Bacterial diseases have been controled either by the use of antibiotics or by the successful application of commercially available vaccines, but neither effective therapeutics nor immunological control measures have been developed yet against viral infections.

The achieving of fish cell and tissue culture in the 1970s (42) brought about more and more descriptions of viruses of potential pathogenic significance in freshwater, warmwater and marine environments. In 1981 Wolf wrote a review on the viral diseases of fish. By then, 16 agents had been isolated in cell culture, and other 11 had been seen by electron microscopy. In his book published in 1988 (43), the numbers had grown to 34 isolated viruses and another 25 that had been observed but not yet isolated. As stated by Hetrick and Hedrick (16) during the period 1988–1993 another 35 new agents were described in the literature, including 9 aquareoviruses, 8 picornaviruses, 6 iridoviruses, 5 herpesviruses, 3 rhabdoviruses, 2 retroviruses, 1 paramyxovirus and 1 coronavirus. And they anticipated that these numbers would increase steadily as the numbers of both trained personnel and species under cultivation went on increasing. Not unexpectedly, research interests have focused mostly on viruses infecting fish of economic importance. As a result, much more is known about salmonid viruses than about all other viruses together. In fact, from all the described or isolated fish viruses, only a few are considered to be of serious concern. The list contains a birnavirus, infectious pancreatic necrosis virus (IPNV), three rhabdoviruses, viral hemorragic septicemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV), the spring viremia of carp virus (SVCV) and a herpesvirus, channel catfish virus (CCV). Some other viruses of special interest to Asian aquaculture belong to the families *Reoviridae* and *Coronaviridae* (1). The classification of viruses is based mostly on the type of nucleic acid, the viral morphology and the infected hosts (Fig. 1).

Five characteristics of viruses made them major infectious agents in aquaculture: (i) They

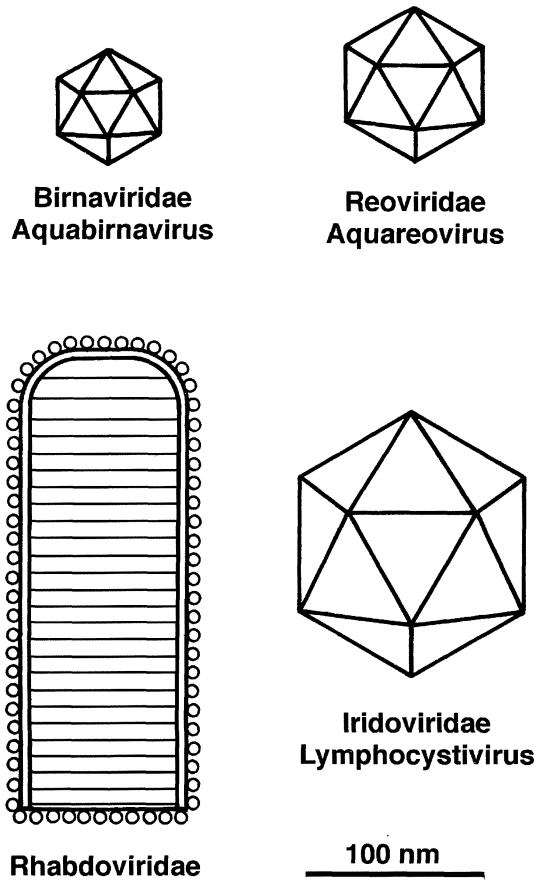

FIG. 1. Major families of viruses infecting fish.

are usually virulent, with clinical manifestation of the disease and high mortalities. The subsequent economic impact is not restricted to the died fish; many other problems (scoliosis, weight lost) have an influence on the market prize of the survivors. (ii) Viruses can usually induce persistent infections, the survivors of epizootics being life-long virus carriers. (iii) They are potential pathogens in the marine environment which must not be underestimated. (iv) Viruses may be involved in dual infections that are poorly known, and which have immediate implications in diagnostics and in the control of the disease. (v) Viral diseases have no treatment. Antivirals do not produce a marked decrease in the mortality rates, and their cost and efficacy make them impractical for applications in hatcheries.

This paper focuses on the description of the viruses and viral diseases that have been of concern in the Spanish aquaculture.

Viral diseases in Spanish aquaculture

Freshwater fish farming within the European Community is dominated by trout production that has more than doubled in just over 10 years, moving from 75,000 Tm to 150,000 Tm. Quantitatively, Spain is considered the fifth productive country, with an amount of about 18,500 Tm in 1994. Farms (141 private installations) are generally small or medium-sized enterprises and are heavily dependant on imported embryonated eggs, which means a serious risk of introduction of viruses or even new diseases.

During the last ten years, several diseases of viral etiology have been described in different species of reared fish in Spain. The infectious agents are from known virus families, and include members of the *Birnaviridae*, *Rhabdoviridae*, *Iridoviridae* and *Reoviridae* (Table 1).

Birnaviridae: Infectious pancreatic necrosis virus

The viral etiology of infectious pancreatic necrosis (IPN), an acute disease of young reared brook trout (*Salvelinus fontinalis* L.) and rainbow trout (*Onchorrynchus mykiss* Walbaum), was first confirmed in 1960, by Wolf (43), who isolated the IPN virus (IPNV) strain VR-299. IPNV has been recently defined as the type species of the genus *Aquabirnavirus* (29), and it might be the most widespread virus in aquaculture ponds. Salmonids under six months are the most commonly infected fish, but the virus has been

TABLE 1. Viruses in Spanish aquaculture

Family	Virus-Species	References
Birnaviridae	IPN Sp and AB strains	18, 21, 36, 38
	VR-strain	30
Rhabdoviridae	VHS	18
	IHN-like	39, 40
	SVCV	25
Iridoviridae	Lymphocystis	3
	VEN, VEI	34, 35
Reoviridae	Turbot reovirus	23
	(TRV)	10

isolated also from many other species such as pike, carp (43), molluscs and rotifers (7). In salmonids, the virus causes necrotic lesions in the pancreas. It is also found, without having caused lesions, in other organs and regions such as kidney, intestine or brain. Older fish can be naturally or experimentally infected, but show no clinical signs of disease. The survivors of an epizootic become lifelong carriers, maintaining the virus in the population by continuously shedding and transmitting it to their progeny or to other susceptible species. There are no known vectors.

IPN viruses have been constant subject of research since 1960, and many of their biochemical, molecular characteristics are well known. There are classic articles on the topic, as well as an excellent general revision on the pathogen itself and the IPN disease (9, 43). IPNV has the most persistent infectivity found among fish viruses. Under laboratory storage and tests conditions, it is unusually stable.

The virion is about 60 nm in diameter, single-shelled, non-enveloped icosahedron. About 132 morphological subunits make up the viral capside. The molecular biology of IPNV has been studied in detail for serotype 1 (VR-299, Jasper) (9). The IPNV genome contains two dsRNA segments (A and B) of 3092 basepair (bp) (A) and 2784 bp (B). Segment A encodes the structural proteins pre VP2 and VP3, and a non-structural protein (NS). Segment B encodes the RNA-polymerase (VP1) of the virus. Virions contain five polypeptides and no lipids.

Several authors have described different virulence for different strains of IPNV, even in the same species of fish. IPNV shows a high degree of antigenic diversity. Classifications of IPNV strains isolated from different aquatic organisms have not been yet well established. The first European isolates, Ab and Sp, were shown to be antigenically different from the original reference strain ATCC LWVRT60-1, the American strain VR-299. Until 1983 only these three major serotypes had been identified (27, 31). However, Hill and Way (17), on the basis of cross-neutralization assays, have identified six additional serotypes and two serogroups. A tenth serotype, represented by the N1 strains, was proposed by Christie et al. (5). All IPNV serotypes of serogroup I, tentatively designated A1 to A10 (6), showed some degree of cross-reaction. (Serogroup II comprises unrelated groups isolated from a variety of marine shellfish and teleost fishes [32, 36].) In 1995 Hill and Way (17) proposed only nine serotypes in serogroup A (A1–A9) and a single serotype in serogroup B, after having studied almost 200 IPNV isolates and revised serological procedures used for the serotype classification. The classification is be-

eing now established at the genomic level (15).

Birnavirus isolated in Spain. IPNV, considered the most worldwide prevalent virus of salmonid fish, has also a high (39%) prevalence in Spain, which is not surprising. Aquabirnavirus from Spanish aquaculture have been studied since 1988. As shown in Table 1, several groups of researchers have described and isolated the virus. In our laboratory, several surveys have been carried out for four years in the most representative geographic areas of salmonid culture (37, 38). We have determined the antigenic relationships between the wild isolates and the reference IPNV strains. The serotype most commonly isolated in Spain is IPNV Sp (Table 2).

In Spain three research groups (J. L. Barja and A.Toranzo, Santiago de Compostela; A. Figueras, CSIC, Vigo; and ourselves) are involved in the characterization of birnavirus (Fig. 2, 3). In our laboratory, new methodologies, such as RT-PCR assay and flow cytometry, have been applied to correlate genomic heterogenicity between the different IPNV reference strains and those isolated in Spain. Beside rainbow trout production, fishfarming in Spain is mainly devoted to the growing of marine fish such as turbot (*Scophthalmus maximus*), seabream (*Sparus aurata*) and seabass (*Dicentrarchus labrax*). Culturing of turbot in Spain is a relatively recent activity. The first attempt was carried out in Galicia in the 1970s, but the first production output on a commercial scale reached the market only in 1984. The excellent natural condition (12–18°C range of seawater temperature) have improved the development of this industry, and Spain has become the major turbot producer among European countries. The production has grown steadily from 40 Tm in 1986 to 1500 Tm in 1993, and the existing facilities have a potential production of 2375 Tm/year, which is envisaged to increase to 5000 Tm by the year 2000 (19).

In Galicia, Barja and Toranzo, have undertaken viral surveys in turbot and salmonid fish farms since 1986. As a result, they have isolated viruses not only from fish showing signs of disease, but also from apparently healthy animals. These viruses have been identified as members of the *Birnaviridae* and *Reoviridae* families (21, 36). The aquabirnaviruses isolated were of the serotypes Sp and Ab, which were the European IPNV strains. However, two birnaviruses of VR-299 serotype, a typical North American serotype of IPNV, were posteriorly described for the first time in Europe in turbot (30) by the same group in coordination with the lead by Figueras in the CSIC (Vigo). In wild marine fish species, the rate of mortality associated with aquabirnavirus is limited (43), but IPNV is considered to be a potential pathogen.

Rhabdoviridae

Rhabdoviruses are enveloped virions, they have two kinds of coat proteins, the outer surface

TABLE 2. Incidence of infectious pancreatic necrosis virus (IPNV) and strains detected on 31 rainbow trout farms during 1988–1991. Results are given as number (or percentage) of sampling events (N)

Years	N	IPNV positive samples	IPNV strain[a]	
			Sp	Ab
1988–1991	236	94 (39.8%)	76 (32.2%)	18 (7.6%)

[a] Strain VR-299 was not detected in any of the samples.

FIG. 2. Some IPNV external signs: darkening, exophthalmia and abdominal distention (arrow).

is covered with projections (peplomers) consisting of trimers of the virus glycoprotein (G). The nucleocapsid consists of RNA and N protein complex (RNP core), associated together with L (for large) and P (for phosphoprotein) (M1) proteins, and it is surrounded by a lipid envelop containing M (M2) protein. The *Rhabdoviridae* family has been classified into the Lyssaviruses (rabies and rabies-like viruses) and the Vesiculoviruses (vesicular stomatitis virus). Taxonomically, fish rhabdoviruses are considered to be "unassigned" virus (29). Three of them are of major importance: viral hemorrhagic septicemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV) and spring viremia of the carp virus (SVCV).

Viral hemorragic septicemia virus (VHSV). Viral hemorragic septicemia is an acute to chronic viscerotropic disease of rhabdoviral etiology that occurs in Europe among certain fish in husbandry. For years, it was known as Egtved disease, for the small village of Egtved, Denmark, where the disease was first detected. The virus has been estimated to cause losses of 40 million pounds of fish per year valued at approximately 80 million US dollars (22). VHSV produce clinical disease in several salmonid species as well as in turbot, seabass, sea bream and pike (*Esox lucius*). VHSV is an economically devasting disease in rainbow trout from aquaculture industry because it may occur in all age groups. However, the susceptibility to disease decreases with age, and occurs in water temperatures below 15°C. The disease is not transmitted vertically. The virus was once confined to continental Europe but has now been described in North America in several salmonid fish and from skin lesions of Pacific cod (*Gadus macrocephalus* L.). The American strains are relatively avirulent in rainbow trout and coho salmon (28). This might suggest that they could have been imported from Europe. As a matter of fact, they are indigenous American strains. In typical outbreaks, afflicted individuals do not feed, their behavior ranges from lethargic to hyperactive, and may have exophthalmia, hemorrhage in the orbits, petechiae in the gills; degenerative changes and necrosis are the most common histopathologic signs, present in many tissues (43).

The mean dimension of VHSV virus is 60×177 nm but defective virus particles are proportionately shorter. The nucleocapsid contains transcriptase activity and is infectious. VHSV have 5 polypeptides, lipids and carbohydrates, and is a serologically distinct pathogen, not related to the other prominent rhabdoviruses infecting fish.

The existence of VHSV in Spain was reported in the past (18), but the outbreaks were restricted to the 1980s in localized sites. The

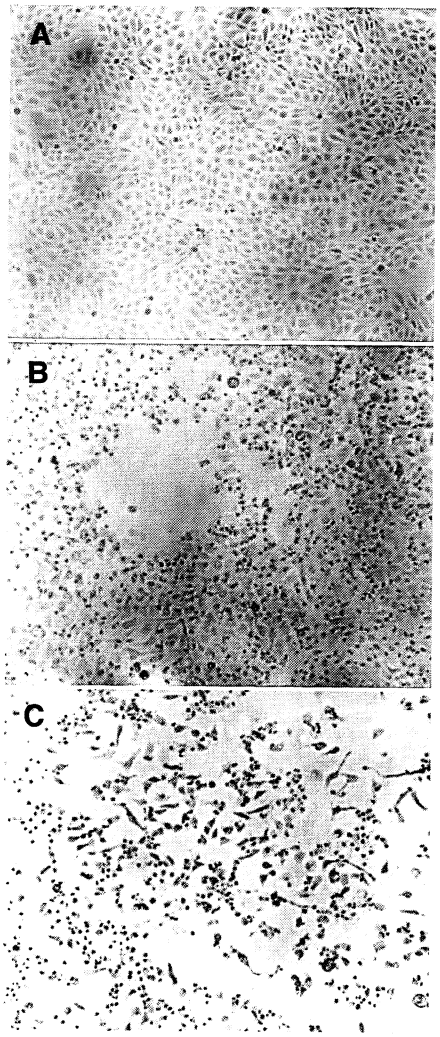

FIG. 3. Cytopathic effects of IPNV virus in CHSE-214 cell monolayers. (A) Uninfected cell control. (B) IPNV-infected cells after 48 h. (C) IPNV-infected cells after 72 h.

disease is considered to be eradicated, since none of the later surveys performed by different laboratories (18, 21, 38) have conducted to isolate the virus again. In Europe, Vestergard Jørgensen implemented a high effective eradication program in progress in Denmark since 1965, that had been extended to North America (42). Steps in the program included removing infected stocks, emptying and disinfecting holding facilities, conducting inspections and repopulating with virus-free stock. Special efforts have been made to eradicate the North American strain of VHSV when detected in live salmonids to prevent its evolution into a more virulent salmonid virus (28).

Infectious hematopoietic necrosis virus (IHNV). IHNV is enzootic in sockeye salmon (*Onchorhynchus nerka*) populations on the west coast of North America, but also causes severe epizootics among stocks of young chinook salmon (*Onchorhynchus tshawytscha*) and rainbow trout. Since its discovery, the worldwide incidence of IHNV has increased dramatically. In 1977 IHNV was introduced into Japan, and since 1987 the virus has been detected also in several European countries. IHNV is a fish rhabdovirus that produces an acute disease (IHN) resulting in destruction of the hematopoietic tissues of the kidney and spleen and in the loss of millions of salmon and trout individuals each year (22, 43).

The mean length of IHNV is 170 nm and the diameter 70 nm. The virion is fragile and, like in VHS virus it was difficult to see its outer spikes. Although IHNV are serologically dissimilar to VHS viruses, structural proteins show similirities. Five polypeptides have been identified. The main target of infection is the hematopoietic organ located in the kidney of fish. The transmission mechanism of the virus in nature is unknown. However, the disease can be transmitted horizontally from infected animals by the water routes and there is evidence that the disease can be transmitted vertically as well.

During an epizootic, infectious virus can be detected in tissue homogenates until approximately 50 days postexposure, after which there is no demonstrable infectious virus. The animals remain apparently virus-free until sexual maturity, when virus can again be detected by plaque assay. Until recently, it was not known whether

infectious viruses isolated from spawning adults are the result of reinfection or whether reactivation of a latent form of IHNVoccurs in the survivor. Drolet et al. (11) have confirmed the presence of IHNV, as an evidence for subclinical persistence of virus in the tissues of IHNV survivors.

Depending on the species and size of the fish, strain of the virus and rearing conditions, an outbreak of IHN may result in explosive losses approaching 100% when infected fish are of small size. Among larger fish, mortality is reduced to 25% or less, but surviving fish are many times unmarketable because of cosmetic problems (scoliosis). Due to the economic significance of IHNV, attempts have been made both to prevent introduction of the virus into new geographic areas and to eradicate the virus by destruction of infected stocks of fish (43).

In Spain, we have isolated a IHN-like virus from rainbow trout, in a dual infection IPNV-IHNV-like occurred in 1992 (39, 40), but our results were not significant enough to declare oficially the disease. Since no more than 30% of the farms have periodic controls, the current spread of this virus is unknown.

Rhabdovirus carpio. Spring viremia of carp virus (SVCV) causes an acute hemorrhagic and contagious viral infection affecting typically cyprinids and more specifically, the common carp *Cyprinus carpio*. Outbreaks of the disease usually occur from April through June, when the water temperatures are commonly between 11 and 17°C, and it causes mortality on both the adults and the young. The disease was first described in 1971 and it is now spread in European countries having carp culture.

The virion is typically bullet shaped, 60 to 90 nm wide and 90 to 180 nm long. SVCV has an RNA-dependent RNA polymerase and the polypeptides are similar to those of the prototype rhabdovirus, VSV. Experimentally, the role of certain parasites as leech or protozoa as potential vectors has been studied, but viral multiplication did not occur. Survivors of the disease are likely to be the major source of virus, although their location in the host and methods of isolation have still to be documented. In that regard, the situation of SVCV is much like that of IHNV.

There is one commercially available vaccine, which has been in use since 1981 (13). The vaccine consists of inactivated viruses of two strains and it is administrated by intraperitoneal injection. Other vaccines, such as an attenuated live vaccine, were tested; they provide excellent protection against lethal effects of a virulent SVCV, but the vaccinated fish apparently became asymptomatic carriers of the virus. The attractive alternative of the development of a subunit vaccine has not yet been undertaken.

In Spain, SVCV was first isolated by Marcotegui et al. (25), from adult carp *Cyprinus carpio* L. The outbreaks occurred in the spring of 1991 in several ponds located in the central part of the country, and high mortalities were recorded in fish 30–60 cm long. No other outbreaks have been described later.

Iridoviridae: lymphocystic disease virus

Lymphocystis is a common chronic disease of several of the higher orders of salt and freshwater fish. The disease is characterized by a distinctive cutaneous hypertrophy forming grossly visible masses (43), and can be transmitted experimentally by exposure to water containing virus, and by feeding or injection of infected cell culture fluid. The earliest reports of the disease were made at the end of the 19th century.

Today is widely distributed geographically and its incidence seems to be more and more increasing (14). The causal agent, fish lymphocystis disease virus (FDLV) belongs to the genus

Lymphocystivirus (29). On the basis of distinct DNA profiles by restriction enzymes, two different types of viruses are recognized: FDLV 1, usually associated with flounder and plaice, and FDLV 2, isolated from dabs (8).

Lymphocystis is the largest of the iridovirus. The virions have icosahedral symmetry, are about 200–250 nm in diameter, and have a complex architecture: a dense core within two unit membranes of the capsid, surface subunits and an outmost fringe of fuzzy material. Genomic DNA is circularly permuted, terminally redundant and is higly methylated at cytosine residues (14, 29). Virions from fish contain as many as 35 polypeptides, their molecular weights ranging from 1400 to 220,000. When culture-grown viruses were similarly processed only 22 polypeptides were obtained, whose molecular weights ranged from 30,000 to 220,000 (14). Viral growth is slow in cell cultures, and incubation should be extended for at least 3 weeks. Cytopathic effects are similar to in vivo lymphocystis cells. The occurrence of lymphocystis in nature or in husbandry indicates that contact transmission is the principal means by which virus is spread.

In Spain, the first report of the viral isolation from cage-reared seabream was described in 1990 (3). The outbreak occurred at the end of 1988 in a farm located on the south shoreline of Spain. Since then some seasonal outbreaks have been observed in different geographic areas (J. J. Borrego, personal communication). A coordinated project between the Dept. of Microbiology, University of Málaga, and our group is in progress to study these viral isolates.

Ungrouped virus: viral erythrocytic infections

The infection is characterized by the presence of cytoplasmic inclusion bodies visible by light microscopy in stained smears of infected erythrocytes. The infection has been found in several species of marine and anadromous fish (14). Attempts to isolate the virus in tissue culture have been unsuccessful.

A group of Spanish researchers described the occurrence of a viral erythrocytic infection (VEI) in the Mediterranean sea bass (*Dicentrarchus labrax* L.) (34). They carried out a complete study and developed a diagnostic procedure that should be useful to establish the differences between similar syndromes, since the in vitro propagation of the virus is not accomplished. The characterization of the etiological agent suggests a retroviral origin of the disease, which is the first retroviral disease having been reported for marine fish (35). An erythrocytic virus infection has been also described recently in cultured turbot (20).

Reoviridae

The International Committee for the Nomenclature of Viruses has recently approved the genus name *Aquareovirus* for the aquatic reovirus (29). Seventeen descriptions of reovirus from different fish species were reported in 1992 (16) from wide geographic areas, and the list of viruses keeps growing (24). The virions are icosahedral in structure, but many appear spherical in shape. They are about 75 nm in diameter (core about 50 nm), and consist of an inner core surrounded by several protein layers. The genome is composed of 11 segments of ds RNA, 3 large, 3 medium and 5 small segments. Cross-hybridization studies indicate that many aquareoviruses are closely related. Virions contain seven structural proteins and five non-structural proteins, and lack a lipid envelope.

In Spain the isolation of aquareovirus from cultured turbot was reported in 1989 (23). Fish

were infected also with a bacterium, and both infectious agents were recovered from the kidneys and livers of affected turbot in a population exhibiting chronic mortality. Another aquareovirus from the same Spanish region (Galicia) has been recently isolated from rainbow trout (10) for the first time.

Control of viral diseases in aquaculture

Health controls have been regularly performed, on stocks of trout and salmon reared in aquaculture in several countries. Protocols include routine checks to identify potential health problems, diagnostic examinations to identify pathogens and pathogen-free certification examinations required to move fish to another location. Diagnosis now requires isolation of the causative viruses in cell culture and their subsequent identification by reaction with specific immune serum (2). This is the most widely used procedure because of its sensitivity and technical simplicity, but it requires prolonged incubation times before the results can be interpreted. It remains a reference standard to evaluate new methods based on the use of monoclonal antibodies, polymerase chain reaction or flow cytometry (4, 12, 26, 33, 41). Those promising techniques, as well as the current methods, represent testing only a few fish and shellfish species. The European Association of Fish Pathologists has recently expressed its concern about revising or implementing fish health regulations to accomodate additional species, since often, in the absence of such protocols, inappropriate testing, or no testing at all, occurs. Before control regulations for imported fish where established in many countries, many of the pathogens associated with serious diseases has already spread. It is therefore critical to establish controls at an early phase in the movements of fish or shellfish.

Successful vaccination should be the ideal measure to control viral diseases. Unfortunately, effective viral vaccines, which are now being developed, are not yet being commercially available. The use of recombinant viral proteins as candidates for both anti-VHSV and IHNV vaccines may be a promising way of producing inexpensive, safe vaccine for fish.

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MICROBIOLOGÍA SEM **13** (1997), 161–172 **MINIREVIEW**

Extracellular bacterial mineralization within the context of geomicrobiology

Nabil Ben Omar, José M. Arias,* María T. González-Muñoz

Department of Microbiology, Faculty of Science, University of Granada, Granada, Spain

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Summary

In the biosphere, bacteria can function as geochemical agents, promoting the dispersion, fractionation and/or concentration of matter. These processes, which are being more and more valued from the point of view of various scientific disciplines, have given rise to the field of geomicrobiology. At the same time, microbial processes resulting in the concentration of matter and thus inducing the formation of minerals, constitute an area of research of growing interest known as biomineralization. In this review a succint summary of various aspects of both disciplines has been offered together with a more detailed review of those aspects related to extracellular bacterial mineralization. The significance of the role played by the metabolism of bacteria is discussed along with the results of recent research on the role of dead bacteria and bacterial remains that act as heterogeneous nuclei of crystallization. The role played by the membranes of bacteria has also been considered to be highly relevant, and a discussion concerning their possible value as models for both the study of more complex biomineralization processes as well as application in the field of biomimetic materials is put forward.

Key words: geomicrobiology, geochemistry, bacterial crystallization, biomineralization, heterogeneous crystallization

Resumen

En la biosfera, las bacterias pueden actuar como agentes geoquímicos favoreciendo la dispersión, el fraccionamiento y/o la concentración de la materia. Estos procesos, cada día más valorados desde

* *Correspondence to*: José M. Arias. Department of Microbiology. Faculty of Science. University of Granada. 18071 Granada. Spain. Tel. and Fax: +34-58-243397. E-mail: jmarias@goliat.ugr.es

distintas disciplinas científicas, han dado lugar a la ciencia de la geomicrobiología. Por otra parte, se estudian los procesos microbianos de concentración de la materia que ocasionan la formación de minerales como una parte de otra área de creciente interés: la biomineralización. En esta revisión se hace un sucinto repaso de varios aspectos de ambas disciplinas, y se revisan con mayor detalle los aspectos relativos a la mineralización bacteriana extracelular. Se pone de manifiesto el papel desempeñado por el metabolismo bacteriano y se destacan resultados de investigaciones recientes referentes a la función de las bacterias muertas o restos bacterianos, que actuarían como núcleos heterogéneos de cristalización. Se ha encontrado que el papel que desempeñan las membranas bacterianas es de gran interés. Se indica además la posible utilidad de esas membranas como modelos para el estudio de procesos de biomineralización más complejos y en el campo de los materiales biomiméticos.

Introduction

The field of geomicrobiology examines the role that microorganisms have played, as much in the past as in the present, over a wide range of fundamental geological processes, such as rock disolution, formation and transformation of soils and sediments, and genesis and degradation of minerals and combustible fuels (12).

Geomicrobiology should not be considered as a new field of study. Although the first researchers to study aquatic and soil microbiology (Ehrenberg, Winogradsky, Harder, Stutzer, Vernadsky, Beijerinck) cannot be regarded as geomicrobiologists as such, their influence on the emergence of this discipline was considerable. Since those early days fundamental discoveries in this field have been continually made.

In the biosphere, bacteria, considered as geochemical agents, can act as agents which disperse, fractionate or concentrate material. When functioning as dispersion agents, they can promote the disolution of insoluble minerals. An example of this would be the disolution of calcium carbonate by respiratory carbon dioxide, or the biochemical reduction of ferric oxide or manganese dioxide to soluble compounds. As fractionation agents, they are understood to act selectively upon mixtures of inorganic compounds and to initiate selective chemical changes within one or more of the compounds in the mixture, causing a selective concentration or dispersion.

As concentration agents, they can accumulate inorganic material via processes such as intracellular deposition, adsorption and fixation at cellular level, as well as precipitation of insoluble extracellular compounds (7, 12, 39).

Nowadays, some aspects of geomicrobiology, especially those related to the behavior of microorganisms as concentration agents, are considered to form part of a wider field of study known as biomineralization. In turn, this subject can be related to other scientific disciplines such as microbial ecology or biogeochemistry (Fig. 1).

Biomineralization

Biomineralization is the process through which organisms are involved in the formation of minerals, as a result of cellular activity that fosters the necessary physical and chemical conditions for such a formation and growth to take place. The study of these complex processes has given rise to the creation of a new field of scientific research, which may be considered as a discipline and is related to other scientific discipli-

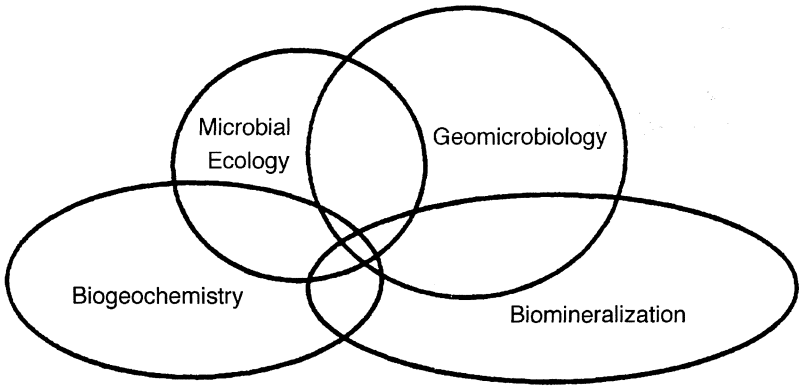

FIG. 1. Interactions among geomicrobiology and related scientific fields.

nes: crystallography, mineralogy, geochemistry, sedimentology, oceanography, microbiology, biochemistry and evolution, among others (21).

Although the study of biomineralization as a science began to emerge slowly during the second half of the XIX century and the begining of the XX century, it was not until 1930 that the modern phase of research in this field began. The introduction of X-ray diffraction techniques, the discovery of advanced histology techniques and the development of the transmission electron microscope played a major role in its development.

The impact of biomineralization in the study of the biosphere is of tremendous consequence. It is known that organisms belonging to 55 phyla form more than 60 biogenically different minerals (21). Biominerals known to exist up to now are shown in Table 1. Organisms which have the capability of precipitating minerals are present in all major groups, from Bacteria to Chordata.

However, the way in which these biominerals are formed can be very different among the different taxon groups. From an evolutionary point of view it can be stated, without any doubt, that biomineralization processes originated from bacterial activity. The majority of the biominerals described contain calcium as their main cation, followed by those containing iron. If these minerals of biological origen were to be grouped in terms of their anions, the most abundant variety would be the phosphates, followed by the oxides and carbonates. Besides, 25% of these biominerals are hydrated, while another 25% are amorphous. Concerning the distribution of minerals of biological origin, calcium and silica are the most widespread among organisms, whereas some given oxides can be precipitated only by bacteria. One outstanding fact is that many of the biogenic minerals may be produced by organisms under conditions where non-biogenic equivalents cannot precipitate minerals spontaneously (38).

The spectrum of biomineralization processes can be easily divided into two different groups. The first is "biologically induced mineralization", a term proposed by Lowenstan and Weiner (21), while the second, proposed by Mann (22), is "biologically controlled mineralization". In the first case, the mineralization process occurs in an open environment, rather than in a closed space designated for this purpose, and there is no specialized cellular or macromolecular mechanism to induce the mineralization process. The

TABLE 1. Biogenic minerals found in biological systems (based on Lowenstan and Weiner [21])

CARBONATES Calcite, aragonite, vaterite, monohydrocalcite, protodolomite, amorph hydrous carbonate, hydrocerussite, strontianite	**IRON OXIDES** Magnetite, goethite, lepidocrocite, ferrihydrite, amorph iron oxide, amorph "ilmenite"
PHOSPHATES Hydroxyapatite, octacalcium phosphate, francolite, dahllite, $Ca_3Mg_3(PO_4)_4$, whitlockite, struvite, brushite, amorph pyrophosphate, amorph calcium phosphate (ACP) indet., ACP (dahllite precursor)[a], ACP (brushite precursor)[a], ACP (whitlockite precursor)[a], ACP (francolite precursor)[a], amorph Mg Ca phosphate, amorph hydrous Fe^{3+} phosphate, $KNa_3(Fe_{15}Mg_{25})\cdot(PO_4)_3(OH_3)_3$, vivianite, bobierrite, schertelite, newberyite	**MANGANESE OXIDES** Todorokite, birnessite **SULFIDES** Pyrite, hydrotroilite, sphalerite, wurtzite, galena, greigite, mackinawite **METALS** Sulfur **CITRATE** Earlandite
HALIDES Fluorite, amorph fluorite, hieratite **SULFATES** Gypsum, celestite, barite, jarosite, taylorite **SILICA** Opal	**OXALATES** Whewelite, weddelite, glushinskite, $Mn_2C_2O_4\cdot 2H_2O$, $CuC_2O_4\cdot nH_2O$, Ca oxalate indet. **OTHER ORGANIC CRYSTALS** Sodium urate, uric acid, paraffin hydrocarbon, wax (long chain), Ca tartrate, Ca malate

[a] The term "precursor" refers to an amorphous phase which, upon heating to 500°C, converts to the designated crystalline phase.

formation of these minerals results in a great variety of sizes and morphologies and are usually random aggregates. The type of mineral produced depends more on the environmental conditions in which the organism lives than on the biological process involved in the formation. In consequence, a given species may produce different minerals under different environmental conditions. As an example, sulfate reducing bacteria release hydrogen sulfide into the environment, where it can react with any metalic ion that is present at a sufficiently high concentration. In laboratory cultures, it has been shown that *Desulfovibrio* can produce up to seven different sulfide minerals. Similar processes can also occur under natural conditions. Biologically induced mineralization seems to be a process in which the prokaryotes and fungi predominate, but it frequently occurs also among some unicellular eukaryotes (21).

A characteristic feature of the second kind of process, biologically controlled mineralization, is that the site where mineral formation takes place is isolated from the external environment

by a barrier through which ions cannot diffuse freely. The lipid bilayers, in both cellular membranes and the localized vesicles, inside or outside the cells, mark the limits for these areas. Other materials which have a similar function are the insoluble macromolecules, such as proteins and/or polysaccharides, which form impermeable sheets. The most characterized example of these macromolecules is the protein periostracin, which is the main constituent of the external membrane of mollusc shells (21).

Note that the organic matrix within the mineral structures may be complex and not to have a uniform composition in the different biological groups. Soluble and insoluble organic fractions can be separated in the case of the exoskeletons of the invertebrates. Besides, the analysis of the soluble fraction of different species of molluscs reveals the existence of many different fractions, with end groups of diverse chemical nature that bind calcium. The anionic groups of the organic matrix can concentrate the calcium ion at various sites and induce the necessary oversaturation for nucleation to take place. Such a concentration is brought about by the presence of a high number of aspartic and glutamic acids with COO^- groups, the ester sulfate groups that provide negative charges on hexosamine residues or specific aminoacid sequences (21).

Related aspects concerning bacterial precipitation of insoluble extracellular compounds will be discussed in the following sections.

Extracellular bacterial mineralization. Bacteria, as a whole, show a great variety of metabolic pathways and frequently release a great amount of metabolic products into the environment. Microorganisms have a geochemical activity which is responsible to a great extent for the deposition of minerals throughout the history of the Earth. Their central role over a wide range of mineralization processes has already been established. The precipitation of calcium carbonate by different bacteria, as we will discuss later, is a relevant exemple.

Redox potential (E_o) also exerts an influence on the metabolism of metals such as iron and manganese, that can exist either in reduced soluble forms (Mn^{2+}, Fe^{2+}) or in insoluble oxidized forms (Mn^{4+}, Fe^{3+}). Consequently, the bacterial activities that affect the pH or redox potential can also affect the distribution of these metals, either indirectly via redox potential, or directly by providing inorganic binding sites (38).

There is relationship between the activity of microorganisms in the sulfur cycle and the elemental sulfur deposits in marine environments. Some authors have also associated elemental sulfur precipitation to the activity of these bacteria in diverse natural environments (40). Furthermore, sulfur reducing bacteria have been responsible for the formation of several types of sulfide metallic ores.

The biosedimentary structures known as stromatolites provide another example of an association between bacterial activity and mineral deposition (15, 38). These structures can be found in fossil records and still form under natural conditions, as in western Australia. Although the stromatolite is produced by a community of microorganisms, it is nevertheless dominated by the cyanobacterium *Entophysalis major*. The filaments of this organism contains organic sheaths that recycle inorganic elements within the community. The mineralization of the community starts in summer. The photosynthetic activity of these oxyphotobacteria result in an increase in pH and facilitates the precipitation of $CaCO_3$ in the sea. Initially, mineral deposition occurs in the polysaccharide layer that envelopes the cyanobacteria, but as time passes by the filament loses its translucid appearance and transforms into an opaque brown structure with a hard interior section. *Entophysalis* starts to deteriorate simultaneously and only a fine film

of live cells remains outside the mineral deposit which, in turn, is populated by a great variety of bacteria and algae. Some of these organisms can destroy the stromatolites (38).

Ecological situations of a much greater complexity exist in which bacteria induce the formation of very large mineral deposits. For example, in laguna Figueroa in Mexico, Margulis and Stolz (25) observed that the flat surface of evaporites seemed to be completely void of any form of life. However, just below the mineral crust, on the surface of the lagoon, a diverse community of microbes was found. The uppermost 0.5 cm layer contains a diverse photosynthetic microbial community which brings about carbon dioxide fixation processes and an increase in aragonite deposition. Several heterotrophic species of bacteria inhabit another layer directly below the first, and they deposit manganese oxides whereas sulfate reducing bacteria, that produce hydrogen sulfide, precipitate several metallic sulfides (38). Other bioconstructions such as oncolites, oolites and microbial bioherms are active mineral deposits in which microorganisms are involved (15).

Bacterial precipitation of calcium carbonate. The calcium carbonate presents three differing polymorphs: calcite, which crystallizes in a trigonal system and produces a highly symmetrical structure; aragonite, in a rhombic system, slightly unstable in comparison with calcite; vaterite, in a hexagonal system of a rather disordered structure. In comparison with the two former types, vaterite is highly unstable and is therefore found to a much lesser extent in the biological systems of all phyla. In fact, most calcium carbonates of organic origin present crystalline aggregates of the first two types of polymorph. The presence of vaterite has been detected only in very few phylogenetic groups (2).

Bacterial $CaCO_3$ precipitation was first described by Murray and Irvine in 1889/90 (28). They observed the formation of this crystalline compound after urine in a state of decomposition and putrefaction was added to a medium of sea water. From this moment on, the role of bacteria in $CaCO_3$ deposition was suggested by several other authors. In 1903, Nadson showed that $CaCO_3$ deposits in lake Veisowe in Karkou, Russia, could have originated from bacterial activity, given that their formation was observed after incubation of lake bed sediments together with non-sterile water from the lake. The following bacterial species were isolated from these sediments: *Proteus vulgaris, Bacillus mycoides,* "Bacillus salinus", "Bacterium alboluteus", "Actinomyces albus" (reclassified as *Streptomyces albus*), "Actinomyces verrucosis" and "Actinomyces roseolus" (reclassified as *Streptomyces roseolus*). The formation of the above mentions compounds was achieved when sterile sediment was incubated with an axenic culture of *P. vulgaris,* and the same result was obtained with *B. mycoides* cultivated in different media (nutrient broth and agar, and gelatine medium).

According to Drew, denitrifying bacteria were responsible for $CaCO_3$ deposition in sediments in Great Bahama, the most significant $CaCO_3$ deposits that exist all over the world. In 1911 and 1913, Drew isolated a denitrifying bacterium from these sediments which he called "Bacillus calcis", and showed that this bacterium could precipitate calcium carbonate in artificial culture media. Later, Kellerman and Smith identified this same bacterium as "Pseudomonas calcis". On the other hand, Lipman indicated that marine bacteria only form $CaCO_3$ when a large quantity of soluble salts are present in a medium, which necessarily included organic and inorganic salts (12).

The photosynthetic removal of carbon dioxide from fresh or sea water containing calcium or bicarbonate ions can lead to the precipitation of calcium carbonate: $Ca^{2+} + 2HCO_3^- \rightarrow CaCO_3$

(prec.) + H_2O + CO_2 (fixed). Carbon dioxide fixation can also occur in the dark by a variety of chemolithotrophic organisms such as nitrifying bacteria, iron-oxidizing bacteria, anoxygenic photosynthetic green and purple bacteria, and by the oxygenic cyanobacteria. Therefore, in an adequate medium, a variety of bacteria can form calcite or, in the presence of magnesium ions, aragonite. The significance of bacteria in these processes has been considered to be a consequence of their different metabolic activities by most authors.

The reduction of nitrate by heterotrophs can induce the production of calcium carbonate deposits: $Ca(NO_3)_2 + 3H_2 + C$ (organic) $\rightarrow CaCO_3 + 3H_2O + N_2$. The reduction of sulfate by *Desulfovibrio* can also precipitate calcium carbonate: $CaSO_4 + 2(CH_2O)$ (organic) $\rightarrow CaCO_3 + H_2S + CO_2 + H_2O$. The presence of ferrous ions leads to the formation of ferrous sulfide and calcium carbonate: $CaSO_4 + 2(CH_2O) + Fe \rightarrow CaCO_3 + FeS + CO_2 + H_2O$.

A similar effect can also be produced by aerobic and anaerobic bacteria that release ammonium after the oxidative deamination of amino-acids: $2NH_4OH + Ca(HCO_3)_2 \rightarrow CaCO_3 + (NH_4)_2CO_3 + 2H_2O$ or $(NH_4)_2CO_3 + CaSO_4 \rightarrow CaCO_3 + (NH_4)_2SO_4$.

Two types of mechanism can cause the bacterial precipitation of calcium carbonate: (i) the presence of bacterial metabolic pathways, and (ii) a change in the redox potential, in this case to pH 7.0 and a redox potential between 250 and 300 mV. Under these conditions, bicarbonate and H^+ combine forming methane and calcium carbonate: $3Ca^{2+} + 4HCO^-_3 + 6H^+ + 8e \rightarrow CH_4 + 3H_2O + 3CaCO_3$.

In an attempt to determine the bacterial groups involved in $CaCO_3$ precipitation, several taxonomic studies were carried out. Bavendamm maintained that $CaCO_3$ precipitation was a major biogeochemical process not restricted to the activity of any specific group of bacteria. Autotrophs and heterotrophs including sulfur bacteria, photosynthetic bacteria, bacteria that hydrolize urea, agarolitic and cellulolytic bacteria, and nitrogen fixing bacteria, are all involved in $CaCO_3$ formation. McCallum and Guhathakurta carried out $CaCO_3$ precipitation studies on twenty four bacteria belonging to the genera *Pseudomonas*, *Bacillus*, *Vibrio* and *Streptomyces*, isolated from the bank of the Bahamas. The experiments were performed by using different liquid culture media and different pH and temperature conditions, achieving mineral precipitation in all the trials (12). In 1973, Boquet et al. (9) concluded that, under favorable conditions, all bacteria can produce calcite crystals. Gerdes et al. (16) have reported a notable structural diversity of biogenic carbonate particles in microbial mats.

The bacterial precipitation of phosphate. In nature, phosphorus exists mainly as soluble and insoluble phosphates. The most abundant insoluble phosphate is apatite [$Ca_5(PO_4)_3$ (F, Cl, OH)], where the free radical may be represented by F, Cl or OH, individually or in any combination of two or three of them. Apatite deposits are usually found in marine environments. The bacterial precipitation of apatite was shown by several authors such as Ennever and Takazoe (13), Ennever et al. (14), McConnell (26) and Piter and Codespoti (32).

In natural environments, phosphates may also appear in the form of calcium, aluminium, iron or magnesium salts. These precipitates are commonly found in soils and sediments. Some researchers attribute phosphate precipitation in soils and sediments to the metabolic activity of microorganisms. The release of iron, calcium and aluminium active ions in organic or inorganic combinations can be a consequence of this activity. Furthermore, its influence upon pH and free energy could also be a determining factor

in the formation of these insoluble phosphates (12, 30).

Struvite ($NH_4MgPO_4 \cdot 6H_2O$) is one variety of the insoluble phosphates that has received special attention from microbiologists. This is due to the fact that many different types of bacteria can produce this mineral under laboratory conditions, and that a relationship has been established between kidney stones and urinary infections (11, 36).

It was Robinson in 1889 who first described the bacterial production of struvite. According to his proposals, such a production could be the consequence of the combination of ammonium ions produced by the metabolism of nitrogenous compounds with phosphate and magnesium present in the environment. Robinson was perhaps the first researcher to hypothesize upon the theory that bacteria were responsible for the presence of struvite mineral in nature. Later on, several researchers showed that bacterial struvite production could occur under laboratory conditions using the following species: *Staphylococcus aureus* (3), "Pseudomonas calciprecipitans" (37), strains of the genera *Pseudomonas, Flavobacterium* and *Arthrobacter* (34, 35), *Ureaplasma urealyticum* (19), *Arthrobacter* sp. and *Pseudomonas* sp. (31), *Bacillus pumilus* (29), *Myxococcus coralloides* (17), *Myxococcus xanthus* (4, 5). In natural environments, struvite has been found in association with various organic materials in a state of decomposition such as guano deposits—for this reason also called guanite—, manure, sediments rich in organic remains and in basaltic caves and marshlands (10).

The possible role of bacterial structures in extracellular mineralization processes. The role of the bacterial structures in the extracellular or intracellular accumulation of inorganic material has been established by experimental work. The formation of manganates by manganese oxidizing bacteria is one of the many studied cases that are of relevance. In this case, the extracellular polysaccharide isolated from *Pedomicrobium* sp. fixes Mn^{2+} and facilitates its oxidation. Another case that may be quoted is that of *Aquaspirillum magnetotacticum,* where magnetite particles (Fe_3O_4) have been detected inside this bacterium, where it may form within the organic membranes.

In apatite formation processes, in the "Bacterionema matruchotii" (reclassified as *Corynebacterium matruchotii*) cell, the lipid-protein complex can induce the formation of the calcium phospholipid-phosphate complex (38). Alper (1) revealed that *Pedomicrobium* sp. is capable of accumulating gold on its cellular wall, when the bacterium lives in a gold rich soil. A major function is performed by bacterial structures that are able to biosorp heavy metals such as uranium, lead, etc., to their cellular walls and/or extracellular polysaccharides (6, 8, 27), or to accumulate mercury (12). However, it has been generally assumed that extracellular precipitation processes of mineral compounds by bacteria are due to metabolic activity that favors the physical, chemical conditions for the formation of biominerals, and that for crystallization to take place the supply of a structure by the bacteria would not be necessary.

Recently, the role played by myxobacteria in biomineralization processes has been studied by González-Muñoz et al. (17). His results suggest that bacterial structures could, at least in some instances, be quite significant in extracellular mineralization processes. In the production of struvite by *M. coralloides,* this was seen to be the case, because (i) the supply of the ammonium ion and (ii) a culture medium of alkaline pH, was not enough to bring about a precipitation. The physical presence of *M. coralloides* in the medium was found to be necessary. Furthermore, a relationship between struvite production in liquid media and the characteristic autolysis of this

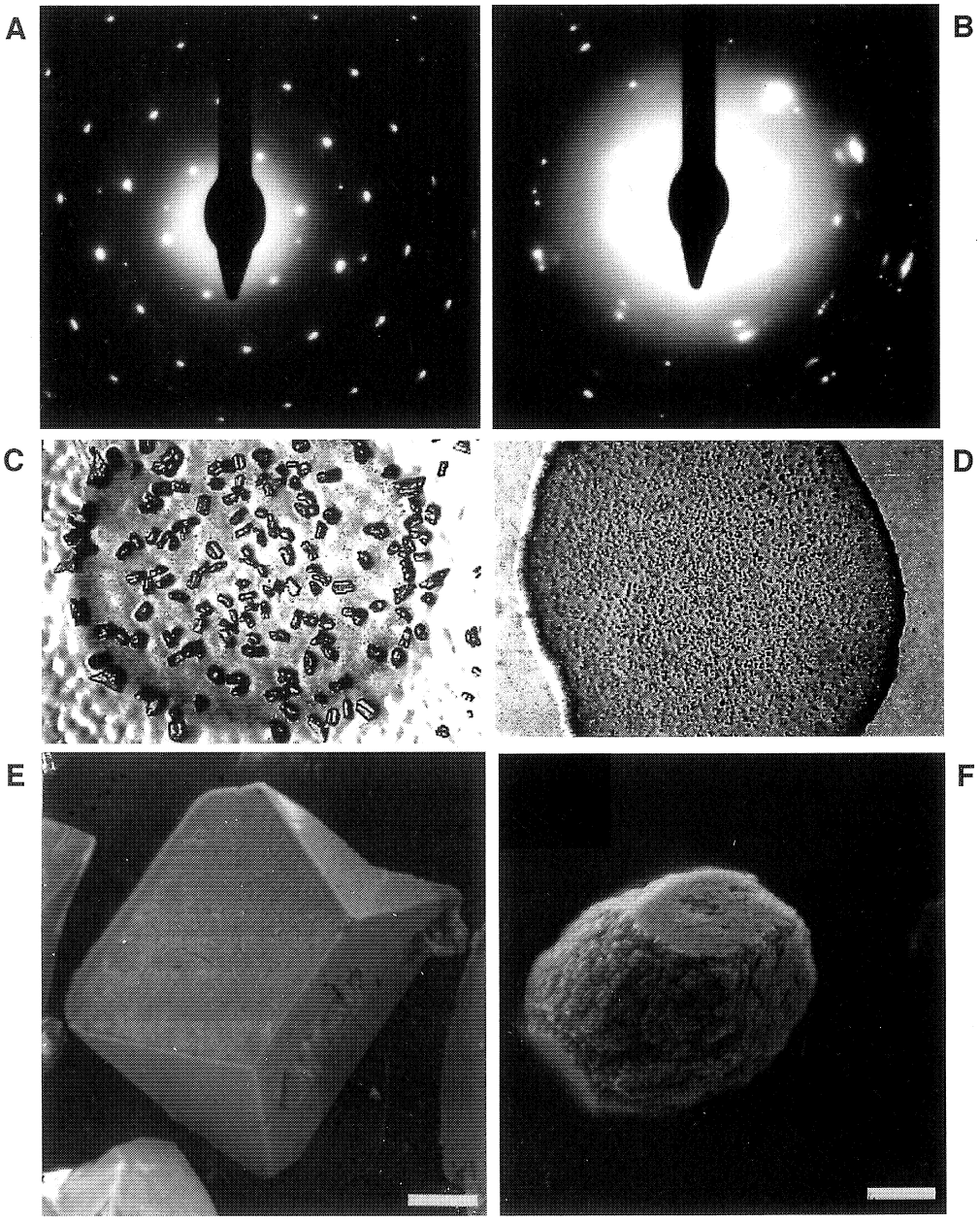

FIG. 2. Struvite and calcite crystallization with *Myxococcus xanthus* membranes. (A) and (B): Electron difraction from crystals after 2 h of incubation, carried out with a high-kilovoltage transmission electron microscope (STEM Philips CM 20). (A): Struvite. (B): Calcite. (C) and (D): Crystals observed with an optical microscope produced on gelified solutions with agar after 8 h incubation. The crystals appear in the inoculated zone along with the bacteria membrane suspension. (C): Struvite (7.7 ×). (D): Calcite (13.4 ×). (E): Struvite crystal. (F): Calcite crystal, observed with a scanning electron microscope (Zeiss, DSM 950) after 8 h incubation. Bars: (E): 50 μm. (F): 10 μm.

bacterium was established, thus indicating that a supply of cellular remains would be necessary for the onset of crystallization to take place (4, 17). Subsequently, Ben Omar et al. (5) showed that dead cells and/or cellular remains from *M. xanthus* could act as heterogeneous crystallization nuclei in the presence of the necessary ions. These authors observed that whereas the concentration of these ions in the medium could not bring about a spontaneous crystallization, this could take place in the presence of dead bacteria. Similar results were also obtained by Rivadeneyra et al. (33), with *Azotobacter* and *Pseudomonas*.

In natural biomineralization processes, bacteria can create oversaturated conditions as a result of their metabolic activity. However, the primordial function in extracellular bacterial biomineralization would be the supply of structures that serve as substrates for heterogeneous nucleation (with lower energy levels than would be necessary in homogeneous nucleation) and for the suitable stereochemical arrangement of the mineral components. Therefore, providing that the suitable conditions and ions are present, the bacterial structures could be sufficient to bring about crystallization.

More recently, González-Muñoz et al. (18) obtained an almost immediate struvite and calcite crystallization by using *M. xanthus* and *Escherichia coli* membrane fractions (Fig. 2). This suggests that these fractions are responsible for a local heterogeneous nucleation or that, alternatively, in oversaturated solutions they could reduce the metastable region for the precipitation of calcite and struvite to take place. It was also observed that *M. xanthus* external membrane produced a more efficient struvite crystallization, whereas the internal membrane was found to be more efficient in the case of calcite. The most convincing evidence that these membranes are directly involved in nucleation is the fact that they are rich in multimolecular complexes, proteolipids and phospholipids, which are capable of absorbing Mg^{2+} and Ca^{2+} ions.

The use of these membranes opens up new perspectives in both the theoretical and practical aspects of biomineralization. Their use could help to clarify biomineralization processes and they can serve as models to study the manufacturing of biomimetic minerals (20, 22, 23, 24). In the last few years, there has been a growing interest in biomimetic minerals with the aim of achieving a higher control of the synthesis of different inorganic compounds. Such new compounds are being designed at present, and others could be designed in the future, for a number of interesting applications including the direct biotechnological exploitation of biogenic materials, the design of new compounds for in vitro precipitation, as well as the mimickins of biological processes in the manufacturing of inorganic materials with technological applications. Such applications could be developed in the fields of magnetic materials, catalytics, optics and polymer ceramic compounds, among others (22).

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Use of Percoll gradient centrifugation for the isolation of diatoms from Wadden Sea sediments; diatom yields, species recoveries and population diversity

Solvejg Sevecke,* Thomas Morawietz, Wolfgang E. Krumbein, Erhard Rhiel

Geomikrobiologie, ICBM, Carl-von-Ossietzky-Universität Oldenburg, Oldenburg, Germany

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Summary

The technique of Percoll gradient centrifugation was applied to samples from Wadden Sea sediments of the shoreside near Dangast (German Bight). Experiments were also performed with Wadden Sea sediment inoculated with diatoms and with axenic and mixed diatom cultures. For axenic and mixed cultures, total recoveries ranged from 6 to 70% of the diatoms loaded onto the gradients, whereas the yields were less than 5% in most of the experiments with Wadden Sea sediments. The extraction of diatoms from mixed cultures and from sediment samples was not quantitative, neither in terms of biodiversity nor of total individuals extracted, because the rates of recovery varied for the different species. Diatom cells extracted by Percoll gradient centrifugation were devoid of sand grains and debris. The obtained material was suitable to be used in biochemical experiments, in cultural studies and for electron microscopy. The overall cell morphology and subcellular organelle organization was excellent and appeared to be unaffected by the purification procedure. The results and advantages and disadvantages of the technique are discussed.

Key words: diatoms, gradient centrifugation, light harvesting complex, Percoll, Wadden Sea sediments

Resumen

Se ha aplicado la técnica de centrifugación en gradiente de Percoll a muestras de sedimento del Mar de Wadden, de una zona costera cerca de Dangast (Alemania). Los sedimentos también se

* *Correspondence to*: Solvejg Sevecke. Geomikrobiologie. ICBM. Carl-von-Ossietzky-Universität Oldenburg. POB 2503. D-26129 Oldenburg. Germany. Tel.: +49-441-7983386. Fax: +49-441-7983384. E-mail: solvejg@africa.geomic.uni-oldenburg.de

inocularon con diatomeas y cultivos axénicos y mixtos de diatomeas. En el caso de los cultivos axénicos y mixtos el porcentaje total de recuperación de diatomeas sobre el gradiente de densidad se situaba entre el 6 y el 70%, mientras que en la mayoría de los experimentos con muestras del Mar de Wadden, la producción fue inferior al 5%. La extracción de diatomeas a partir de cultivos mixtos y de muestras de sedimentos no fue cuantitativa, ni desde el punto de vista de la diversidad, ni del número global de células, ya que la recuperación varía con la especie. Las células de diatomeas extraídas del sedimento mediante centrifugación en gradiente de Percoll quedaban limpias de granos de arena y de restos. El material obtenido era adecuado para experimentos bioquímicos, estudios de crecimiento y observaciones por microscopía electrónica. La morfología global de la célula y la organización subcelular era excelente y no parecía afectada por el procedimiento de purificación. En este trabajo se exponen los resultados y se discuten las ventajas e inconvenientes de esta técnica.

Introduction

Percoll is a colloidal polyvinylpyrrolidon coated silica gel used to separate different cells or subcellular organelles. It is well known and established in medicine mainly for the isolation and purification of blood cells, liver cells, spermatozoa and microorganisms such as bacteria and viruses (16).

Several applications of Percoll gradient centrifugation have been recorded from other fields such as microbiology or environmental microbiology. Thus, Bakken and Olsen (5) determined the buoyant density of bacterial cells and fungal hyphae from soil by isopycnic centrifugation in self-forming gradients of colloidal silica. Guerrero et al. (7) determined the buoyant densities of different procaryotes from pure cultures and natural samples. The authors furthermore reviewed the relevant literature and presented the buoyant densities of several procaryotes, eucaryotes and cell organelles. In a more methodological survey, Alongi (4) described the extraction of protists from aquatic ecosystems by using density gradient centrifugation. The author presented data on the separation procedure, the yields of recovery and different calculation methods. Most of the results were obtained for benthic protozoans, deep sea microbenthos and benthic micro- and meiofauna (1, 2, 3, 23). Price et al. (18) collected dinoflagellates and other marine microalgae such as chrysophytes and diatoms by centrifugation in density gradients, while Oliver et al. (11) measured cell densities of freshwater phytoplankters such as *Cyclotella*, *Chlorella* and *Chlorococcum* with the help of Percoll density gradient centrifugation.

Percoll gradient centrifugation to our knowledge has been never applied to isolate diatoms from Wadden Sea sediments. Wadden Sea sediments differ in several aspects from other ecosystems (marine and terrestrial aquatic habitats). They submerge rhythmically due to tidal activity and exhibit highly ordered oxic and anoxic zones with defined populations of photo- and chemotrophic microorganisms. They are stabilized via exopolymeric substances (EPS) secreted mainly by bacteria and diatoms (14). The diatoms present in these sediments can be categorized according to their motility as epipelic (motile) and epipsammic (less motile or non-motile) forms (15). Motile species are like the unmotile forms tightly attached to the sediment grains via small stalks of EPS secreted by the raphae. In contrast to unmotile forms, however, they show a periodically occuring circadian migration be-

haviour (13). The entire population of Wadden Sea diatoms exhibit rhythmic photosynthetic activities, mainly due to the tides and/or light climate (14, 17, 21).

For the isolation of diatoms from sediments usually their capability to migrate was used. Thus, some species can easily be recovered applying the lens tissue technique (6) or by placing microscope cover slides onto sediment samples. Further methods for culturing diatoms include the isolation of single cells via light microscopy and micromanipulator (12) or streaking sediment samples onto agar plates followed by repetitive substreaking and subculturing steps.

As our group works also on the photosynthetic apparatus of chromophytic algae, we applied the technique of Percoll gradient centrifugation to Wadden Sea sediments in which diatoms were the dominant photoautotrophic microorganisms. Our main interest focused on the following questions: (i) is the Percoll density gradient centrifugation technique useful for the isolation of marine benthic diatoms, which are often tightly bound to the substrate?, (ii) how great is the extraction efficiency?, (iii) can the yield of recovery be optimized?, (iv) are different diatom species extracted at the same rate?, or do some of them stick more firmly to the sediment than others?, and (v) is this technique suitable for the rapid extraction of diatoms for biochemical, molecular, morphological and time kinetic studies?

Material and methods

Cultures and growth conditions. *Cyclotella cryptica* Reimann was obtained from the collection of algal cultures at Göttingen (SAG; strain # 1070-1, Schlösser 21)). *Nitzschia curvilineata* and *Phaeodactylum tricornutum* were obtained from Prof. Dr. Sommer (University of Kiel). Diatom isolates from Wadden Sea sediments of the island Mellum were obtained by one of us (S.S.). *C. cryptica* was grown in Erlenmeyer flasks of 100–2000 ml culture volume without aeration in *Cyclotella* medium according to Werner (27), whereas the other diatoms were cultured on M5 agar plates prepared according to Schlösser (22). Cultures were kept at 18–22°C. The light intensity was 4–5 W/m^2 at a light:dark regime of 16:8 h.

Field sampling. Field samples were withdrawn from muddy sediments located on the North Sea shore near the camping and recreation area of Dangast (Germany). Special care was taken to ensure that only the oxic zone, approximately the uppermost 2 to 6 mm, was collected, as the dark anoxic zone below was completely devoid of diatoms. The presence of diatoms in the upper oxic area was checked by parahistological sediment thin sections (see below).

Percoll density gradient centrifugation. Three modes of Percoll centrifugation were applied, the first one with no gradient of Percoll (i), the second one forming a Percoll gradient during the centrifugation step (ii) and the third one with preformed Percoll gradients (iii).

(i) One gram of sediment was mixed in a Greiner tube with 10 ml of Percoll solution consisting of 9 ml Percoll and 1 ml of 1.5 M NaCl. The tubes were centrifuged for 15 min at room temperature in a Hettich Universal 2S swinging bucket centrifuge at 3000 rpm.

(ii) The centrifugation tubes were loaded as described above and run at room temperature in a Beckman SW40 swinging bucket rotor in a Beckman L8-55 ultracentrifuge for 30 min at 17,000 × *g*. Under these conditions a Percoll gradient was generated.

(iii) Finally, centrifuge tubes containing preformed Percoll gradients (see ii), were loaded with 1–2 ml (equivalent to 1 g sediment) and run as described first (i).

After centrifugation, the band of isolated diatoms was withdrawn from the gradients. The fractions were diluted with seawater and the diatoms were collected by centrifugation (5 min, 5000 rpm, table top centrifuge) and resuspended in small aliquots of seawater.

In another set of experiments, the diatoms *P. tricornutum*, *C. cryptica*, *N. curvilineata* and mixed diatom cultures isolated from Wadden Sea sediments were used instead of sediment samples. Defined amounts of cells were loaded in total volumes of 1 ml each onto the tubes and run as described for the sediment samples.

Gel electrophoresis and immunological procedures. Cells pelleted by centrifugation and defined amounts of sediment samples were subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (9) using 15% or 17.5% separating gels and 5% stacking gels. The samples were either applied without heating or denatured by boiling for 2 min. Special care was taken to ensure that equal amounts of chlorophyll *a* (chl *a*) or protein were loaded onto each lane of the gels. After electrophoresis, gels were blotted onto nitrocellulose membranes (25) and immunodecorized with the antiserum directed against the main light harvesting complex of the diatom *C. cryptica* (20). Its specificity was characterized by Western-immunoblotting according to Towbin et al. (25).

Pigment and protein estimations. Chlorophyll and protein concentrations were measured from sediment samples and from diatom fractions. The chl *a* and *c* were estimated by using the equations of Jeffrey and Humphrey (8). The protein concentrations were determined according to Lowry et al. (10).

Cell countings. Repetitive cell countings of diatoms, isolated by density gradient centrifugation or present within the sediment samples were performed with a 3 ml counting chamber (Hydro-Bios, Kiel, Germany) and a Zeiss Axiovert microscope. The total yields of diatoms per gradient band or per gram of sediment were calculated. Furthermore, the percentage of the five species *Nitzschia sigma*, *Gyrosigma spenceri*, *Pleurosigma angulatum*, *Navicula* sp. and *Scoliopleura tumida* on the total diatom population were estimated.

Parahistological sediment thin sections and microscopy. Parahistological sediment thin sections were performed according to Wachendörfer et al. (26). For light microscopy a Zeiss photomicroscope III or a Zeiss Axiovert inverse microscope were used. Pictures were taken on Kodak EPY64 tungsten film. Electron microscopy followed standard protocols. Diatom cell suspensions, obtained by Percoll density gradient centrifugation, were fixed in 4% (v/v) glutaraldehyde in 100 mM Na-K-phosphate buffer, pH 7.2, post-fixed with 1% (w/v) osmium tetroxid, dehydrated in a graded ethanol series and finally embedded in epoxy resin according to Spurr (24). Ultrathin sections were cut with a Reichard-Jung Ultracut ultramicrotome. Finally, the sections were poststained with aqueous uranyl acetate after Reynolds (19) and examined in a Zeiss EM109 electron microscope operated at 50 kV.

Results

Percoll gradient centrifugations. Defined amounts of unialgal cultures of *C. cryptica*, *N. curvilineata* and *P. tricornutum*, of mixtures thereof and of Wadden Sea sediments were loaded in total volumes of 1 ml each onto the tubes and subjected to Percoll centrifugation. Gradient tubes obtained from these experiments are shown exemplarily for *P. tricornutum* and Wadden Sea sediment (Fig. 1). All three centrifugation modes resulted in a brownish band of about 0.5 to 1 cm width in the upper part of the tube and a dark brown pellet at the bottom. Both, band and pel-

let, contained diatom cells which seemed to be more affected in structure in those experiments, in which the diatoms were subjected to high speed centrifugation. The cells seemed not to be affected in their structure, motility (if present), viability (as judged from light microscopical observations) and further culturing experiments, when they were separated by low speed centrifugation. Thus, low speed centrifugation (3000 rpm) gave best results because distinct bands occurred right on top of the Percoll phase.

The bands were withdrawn quantitatively whereas the pellets were resuspended in 1 ml of artificial seawater. Cell countings of banded diatoms and of the pelleted cells revealed that the total amount of diatom cells loaded could be recovered quantitatively. However, the amounts of banded diatoms varied among the different species investigated. Thus, recoveries ranged from 6% for *C. cryptica* to 67% for *P. tricornutum.* (Table 1.)

The sampling sites chosen for the Wadden Sea experiments were almost exclusively colonized by diatoms as the only oxygenic photoautotrophs (judged from light microscopical observations, Fig. 2A and B). The diatoms were restricted to the uppermost 2 to 6 mm oxic zone, which was deep brownish to green coloured. The anoxic zone underneath was dark brownish to black and completely devoid of diatom cells. This finding was confirmed by parahistological sediment thin sectioning (Fig. 3) demonstrating the presence of diatoms solely in the upper zone.

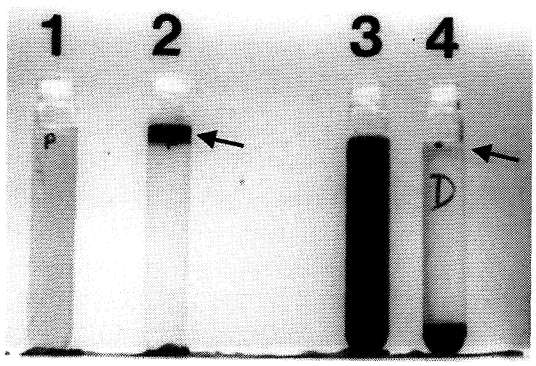

FIG. 1. Sediment samples (tubes 3 and 4) and diatom cultures of *Phaeodactylum tricornutum* (tubes 1 and 2) before (tubes 1 and 3) and after (tubes 2 and 4) centrifugation in Percoll suspension showing a diatom enriched band in the upper part of the tubes.

The low rates of recovery by Percoll gradient centrifugation found for unialgal cultured diatoms were even more pronounced in experiments with these field samples. This is shown in Fig. 1 (tube 4) and Table 1. Maximal recovery rates of 5% of the diatom cells loaded could be calculated. Repetitive, additional centrifugation

TABLE 1. Quantitative enumeration of diatom cells loaded onto the centrifuge tubes (gradient input) and recovered in the Percoll band (Percoll fraction)

	Gradient input [total number of cells]	Percoll fraction [number of cells/ fraction]
Cyclotella cryptica	3 600 000 (100%)[a]	190 000 (6%)
Nitzschia curvilineata	1 600 000 (100%)	340 000 (40%)
Phaeodactylum tricornutum	89 000 000 (100%)	60 000 000 (67%)
Dangast I[b]	7 800 000 (100%)	380 000 (5%)
Dangast II	7 500 000 (100%)	2 000 000 (3%)
Dangast III	4 840 000 (100%)	46 000 (1%)

[a] Values in parenthesis correspond to the percentage of recovery.
[b] The Dangast samples were withdrawn on 28.05.96 (I), 06.06.96 (II) and 21.06.96 (III).

steps of the pelleted diatoms or vigorous mixing, vortexing, mild ultrasonic treatment and addition of minute amounts of detergents before the centrifugation enhanced the total yields only slightly though increasing the appearance of disrupted cells in the samples. As shown in Fig. 2B, the banded diatoms were totally free of particulate detritus and sand grains. Nematodes, however, which were present in these sediments, were recovered quantitatively in the Percoll band.

Quantitative enumerations of chosen diatom species. As the recoveries of the cultured diatom species differed significantly from one another, we investigated whether the different diatom species present in field samples were extracted to various degrees. In Fig. 2 sediment samples before the Percoll centrifugation step (Fig. 2A) and banded diatoms afterwards (Fig. 2B) are shown. The overall composition of the mixed diatom populations in sediment samples and Percoll fractions was checked by comparing the occurrence of five distinct species before and after centrifugation. The five exemplarily chosen diatoms were *Nitzschia sigma*, *Gyrosigma spenceri*, *Pleurosigma angulatum*, *Navicula* sp. and *Scoliopleura tumida*, respectively. All of them are known to live in/on Wadden Sea sediments. They seemed to represent the dominating species in the muddy sediments of Dangast during the period studied. The rates of recovery calculated for the five species are given in Table 2. All but one species could be recovered in the fraction. The ratios of the different species, however, changed. Proportions of 0 to 2.75% of the initially loaded amounts of diatom cells were recovered afterwards, demonstrating, that the ratios of different diatoms present in the Percoll fraction do not reflect the ratios of these diatoms in the field.

Chlorophyll, protein and Western blot analyses. The low recoveries of diatoms out of

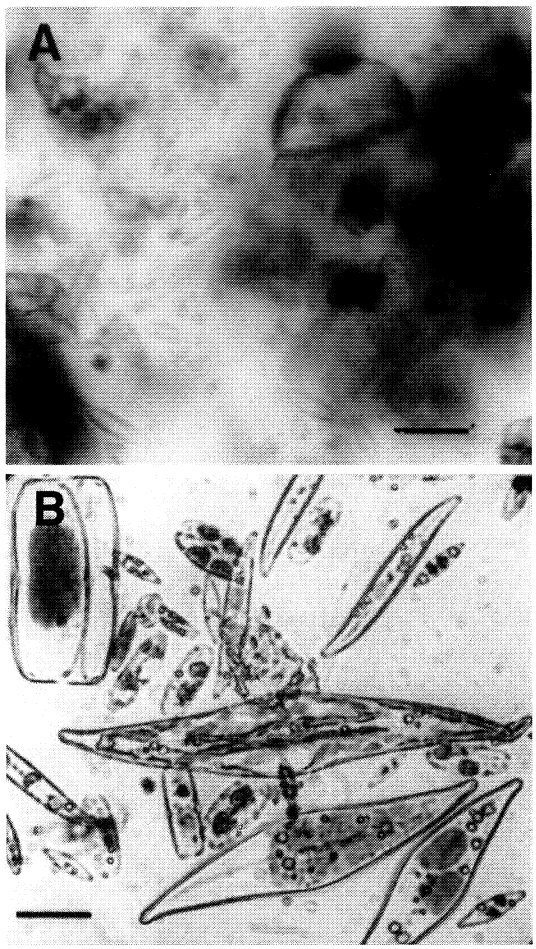

FIG. 2. Microscopical view of untreated field samples withdrawn from the sediment suface at the Dangast site (A), and of diatoms obtained by Percoll gradient centrifugation and resuspended in artificial sea water (B). Bars equal 25 μm.

Wadden Sea sediments and the altered species compositions after Percoll centrifugation were further investigated with biochemical methods. For this, sediment samples and diatom fractions obtained from Percoll gradients were subjected to chlorophyll and protein estimations and to protein analyses. The results of one representative experiment are compiled in Table 3. Approximately 300 μg chl *a*, 45 μg chl *c* and 30 mg protein were measured per 1 g of sediment of

FIG. 3. Parahistological sediment section of Dangast Wadden Sea sediment demonstrating the location of diatom cells exclusively in the uppermost zone. Bar equals 150 μm.

Dangast, while less than 1% of the initial load of chl *a* and chl *c* and less than 7% of the protein loaded onto the tube could be recovered in the banded diatom fraction. The low chl *a* and chl *c* contents in the Percoll fraction are consistent with the low yields of less than 1% of the diatoms loaded onto the tubes. The chl *a* to chl *c* ratio was 6.7 in the field samples and decreased to approximately 5.7 in the diatom fractions.

Defined amounts of sediment samples and banded diatom fractions were subjected to SDS-PAGE. Here, care was taken to ensure that either equal amounts of protein or equal amounts of chl *a* were loaded onto the gels. The gels were blotted onto nitrocellulose and immunodecorized with an antiserum directed against the dominant chl *a*/*c* light harvesting complex of the diatom *C. cryptica*. This antiserum was shown to immunodecorize the subunits of the light harvesting complexes of several marine and limnic diatom species (20). As can be seen in Fig. 4 lanes A' and A, the antiserum selectively decorized protein bands in the relative molecular mass (*M*r) range of 18,000 to 24,000 in sediment samples. No smear of proteins of lower *M*r could be detected, which demonstrated that no major amounts of degraded light harvesting complex subunits were present in sediment samples. When diatoms obtained from Percoll gradients were subjected to SDS-PAGE a similar banding profile was obtained. However, the staining intensities of the immunodecorized subunits differed from those of sediment samples (Fig. 4 lanes B and B'). This finding is consistent with the altered chl *a* to chl *c* ratio and with the changed species compositions in Wadden Sea sediments and Percoll fractions.

Electron microscopical investigations. Transmission electron microscopy of diatoms in sediment samples is not possible due to the sand grains, which hinder ultrathin sectioning with diamond or glass knives. Thus Percoll gradient

TABLE 2. Quantitative enumeration of five exemplarily chosen diatom species present in the Wadden Sea sediment of Dangast; gradient input, total amounts of cell loaded; Percoll fraction, amount of cells banded during the centrifugation step. In the last line the ratios of the different species are given

	Gradient input [total number of cells]	Percoll fraction [number of cells/fraction]
Nitzschia sigma (A)	118 000	ND[a]
Gyrosigma spenceri (B)	944 000	7 080 (0.75%)[b]
Pleurosigma angulatum (C)	472 000	12 980 (2.75%)
Navicula sp. (D)	944 000	14 160 (1.50%)
Scoliopleura tumida (E)	1 890 000	10 620 (0.56%)
A : B : C : D : E	0.25 : 2 : 1 : 2 : 4	0 : 0.6 : 1 : 1.1 : 0.8

[a] ND, not detectable.
[b] The values in brackets correspond to the percentage of recovery.

centrifugation was applied for the isolation of diatoms out of Wadden Sea sediments. The banded cells were pelleted by centrifugation, washed in artificial sea water, fixed and finally embedded in resin. The electron microscopical investigations revealed that the overall ultrastructure of the cells was not affected by the isolation procedure. The cells seemed to be intact and the cellular organization generally described for chromophytes could be seen. The chloroplasts exhibited the typical morphological features with girdle lamellae encircling the rim of these organelles and thylakoids organized in triplicate layers (Fig. 5).

Discussion

Three modes of Percoll gradient centrifugation were applied to Wadden Sea sediments and diatom cell cultures and gave rise to different results. High speed centrifugation with preformed and generating Percoll gradients resulted in diffuse bands and a smear of diatom cells in the upper part of the centrifugation tubes. The cells seemed to be damaged as judged from light microscopical observations of the overall cell morphology. On the contrary, the best results were obtained by low speed centrifugation in which the Percoll solution did not generate a

TABLE 3. Quantitative chlorophyll and protein estimations from one representative experiment with a sediment sample of Dangast and the corresponding diatom fraction

	Sediment	Diatom fraction
chl *a*	300 µg/g sediment	1.3 µg/fraction
chl *c*	44.6 µg/g sediment	0.228 µg/fraction
chl *a* : chl *c*	6.7	5.7
protein	30.43 mg/g sediment	1.86 mg/fraction
chl *a* : protein	9.86	0.7
chl *c* : protein	1.47	0.122
chl *a* + *c* : protein	11.32	0.822

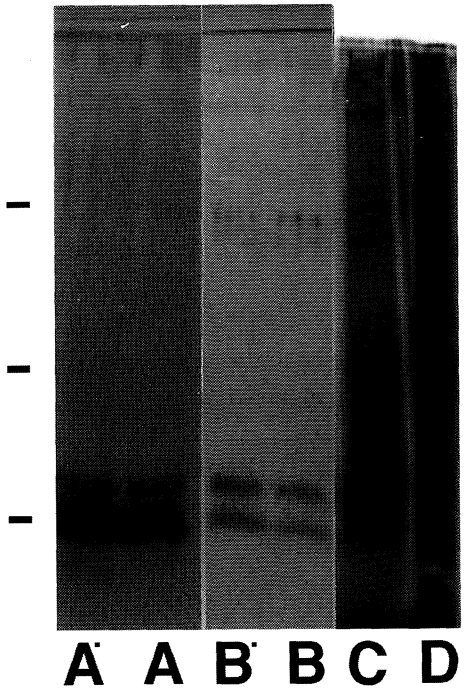

FIG. 4. Immunochemical screening of Dangast Wadden Sea sediment (lanes A' and A) and the corresponding diatom enriched Percoll fraction (lanes B' and B). Equal amounts of chlorophyll *a* were loaded onto the gels, electrophoresed, blotted onto nitrocellulose and immunoscreened with an antiserum directed against the main chlorophyll *a/c* light harvesting complex of the diatom *Cyclotella cryptica*. A silver stained gel on which Wadden Sea sediment protein and the corresponding Percoll fraction were electrophoresed are given in lanes C and D.

gradient, but was rather used as a cushion. Defined fractions of diatom cells out of cell cultures or released from sediments banded as sharp zone immediately on top of the Percoll cushion. The cells seemed to be unaffected by this procedure, stayed viable afterwards and could be used for isolation and culture experiments, for biochemical analysis and for electron microscopical investigations. Low speed density gradient centrifugation with mixtures of Percoll and artificial seawater has already been used for the collection of dinoflagellates and other marine microalgae from sea water. Light microscopical investigation on cell motility and oxygen evolution measurements confirmed the viability of the cells (18). As the entire procedure is not time consuming and restricted to a few prerequisites such as Percoll stock solution, centrifugation tubes and a table top centrifuge, this method seems suitable to be performed rapidly even in the field.

The application of the described method to Wadden Sea sediment samples allowed the rapid extraction of diatoms and was easy to handle. The time-saving practicability compared to other methods, most of them depending on the diatom movement towards light, is very useful especially in time scale experiments, e.g. sampling every hour during a tidal cycle. However, the overall recoveries, which ranged from 6% to 70% in experiments with diatom cultures, ranged from 3% to 0.5% for diatoms of Wadden Sea sediments. The yields are thus moderate to low and seemed to be species specific. Price et al. (18) also found certain amounts of cells pelleted while the rest banded above the Percoll solution. Thus, a quantitative recovery and isolation via Percoll gradient centrifugation which seems to work for micro- and meiofauna and for planktic photoautotrophs (4), is not possible at present for benthic diatoms of Wadden Sea sediments. Guerrero et al. (7) showed that several microorganisms modify their densities by synthesizing storage compounds, inclusion bodies, gas vesicles or exoplasmatic polymeric substances. Thus, cell densities may vary drastically depending on the physiological state of the individual cells. This is supported by the results obtained for the axenic cultures and by the quantitative enumeration of the five exemplarily chosen species from Wadden Sea sediments. Up to 67% of the cells of *P. tricornutum* banded, whereas most of the cells of *C. cryptica* sedimented during the centrifugation step. In the field experiments, the five different species ext-

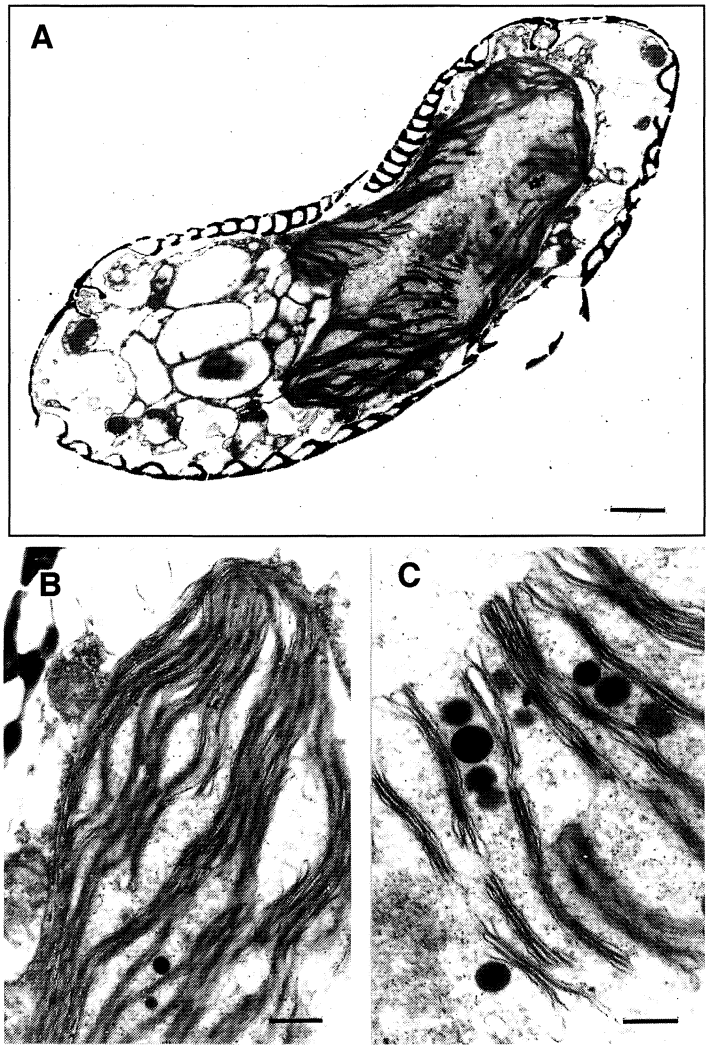

FIG. 5. Electron microscopical micrographs of diatom cells obtained from a diatom enriched Percoll fraction (A). Micrographs B and C show part of the ultrastructure of the chloroplasts. Bars: in A, 1.6 μm; in B, 400 nm; in C 250 nm.

racted and banded in different ratios. Here, the physiological state might be influenced by additional factors, such as circadian and annual rhythms, temperature, light and salinity or nutrient availability. All of these factors might have a great impact on the rate of release from the sediment grains and on the recovery after Percoll gradient centrifugation. Further experiments such as the use of exopolysaccharide degrading enzymes or the application of chemical or physical treatments or agents and time scaled field experiments have to be tried in order to enhance the recoveries.

Defined amounts of Wadden Sea sediment samples were subjected to chlorophyll and protein concentration measurements. The values recalculated on a wet weight basis are in the range of what one can expect in sediments. Thus, Yallop

and Paterson (28) measured up to 45 μg chl *a* and up to 19 mg protein per g dry weight sediment during a seasonal field study at the river Severn estuary. Our somewhat higher chlorophyll estimations might be explained by the extremely large amounts of diatoms present in the sediments of Dangast during spring and summer 1996. Paterson et al. (15) for example, recorded up to 3.4×10^6 epipelic and epipsammic diatoms in the Texel microbial mat system.

The low recoveries of diatom cells after Percoll gradient centrifugation was confirmed by the estimation of the concentrations of the chl *a* and *c*. In the experiment shown, recoveries of less than 1% were calculated for both pigments and also counted for the yield of diatom cells. The protein concentrations measured were higher than expected. This finding is most probably caused by the meiofauna and bacteria, which also banded during Percoll gradient centrifugation. The altered species composition of diatoms present in the sediments and isolated by Percoll might be responsible for the changed chl *a* to chl *c* ratios and is reflected in the results obtained from Western-immunoblotting experiments. Various different diatom species inhabit Wadden Sea sediments. Thus, the signal obtained from the antiserum against the main chl *a/c* light harvesting complex of the diatom *C. cryptica* is caused by the sum of many different light harvesting subunits of different species of this community. The staining intensities of the immunodecorizable subunits, however, differ in samples of Wadden Sea sediments and those of Percoll fractions, demonstrating the accumulation or loss of distinct species relative to others.

Currently, Percoll gradient centrifugation serves as a first step for the purification of diatoms. The procedure eliminates sand grains and debris but does not work in a quantitative manner. Subsequently, however, the diatom cells can be used for biochemical analysis, electron microscopical preparations and further isolation and cultivation experiments.

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Production of carpophores of *Lentinus edodes* and *Ganoderma lucidum* grown on cork residues

Héctor Riu, Guillem Roig, Josep Sancho*

Departament d'Indústries Agroalimentàries, Escola Superior d'Agricultura de Barcelona, Universitat Politècnica de Catalunya, Barcelona, España

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Summary

Cork, being widely used in industry, generates high amounts of waste of difficult elimination because of its complex biological degradation, and the high pollutant smokes from its burning. Similarities between suberin (major component of cork) and lignin suggest that fungi with high lignin degrading capacity could colonize cork residues. Basidiomycetes such as *Lentinus edodes* and *Ganoderma lucidum*, besides their capacity for degrading, are edible. Thus, while using them to degrade cork, it is also possible to obtain a food product. In this study, dry matter was reduced 40%, suberin was degraded 45%, oxidizable carbon was increased 35%, and *Lentinus* showed a high rate of growth. These results indicate that there is an environmental alternative to the elimination of residues from the cork industry.

Key words: *Lentinus edodes*, *Ganoderma lucidum,* cork, suberin, biological degradation

Resumen

El corcho es un material cuya extensa aplicación industrial genera un gran volumen de residuos de difícil eliminación, ya que su degradación biológica es muy compleja y su incineración produce humos altamente contaminantes. La semejanza entre la estructura de la suberina, componente mayoritario del corcho, y de la lignina, sugiere que los hongos con alta capacidad degradativa de la lignina podrían también colonizar los residuos del corcho. Los basidiomicetos *Lentinus edodes* y *Ganoderma lucidum*, además de presentar esta capacidad, son comestibles, con lo cual se

* *Correspondence to*: Josep Sancho. Departament de Microbiologia. Facultat de Biologia. Universitat de Barcelona. Av. Diagonal, 645. 08028 Barcelona. España. Tel.: +34-3-4021484. Fax: +34-3-4110592. E-mail: jsancho@porthos.bio.ub.es

puede conseguir el doble objetivo de eliminar un residuo industrial recalcitrante y de obtener un complemento alimentario. En nuestros experimentos se ha conseguido un 40% de reducción de materia seca y un 45% de degradación de la suberina del corcho, así como aumentar un 35% el carbono oxidable, con un rendimiento considerable en la cosecha de *Lentinus*. Estos resultados indican que existe una alternativa ecológica eficaz para la eliminación de los residuos de la industria del corcho.

Introduction

Cork is a parenchymatous tissue made of dead cells forming the bark of the cork-oak (*Quercus suber*). Its peculiar cellular structure and complex chemical composition, which is characterized for its basic component, suberin, produces a highly stable and permanent material that allows it to perform its biological function of protecting living tissues (waterproofing, thermal insulation, defense against pathogens, etc.). Suberin makes cork suitable for many industrial applications. However, its chemical characteristics make its biological degradation difficult.

Industries that make agglomerate cork (for instance, bottle-stoppers) are among those that generate a major amount of residues; 60% of the total treated cork is wasted away in dust of different grain sizes. A medium size factory produces around 1000 Tm of dust cork per year. In Catalonia (NE Spain), where most of the Spanish industries that elaborate agglomerate cork are located, around 18,000 Tm of dust cork are produced yearly.

In the past, those residues were used as a fuel in brick factories, but the utilization of more convenient and effective fuels has made it necessary to find alternative systems to eliminate cork residues. Since the degradation of cork is extremely difficult, most companies have decided to burn it in their own incinerators or in waste-treatment plants. However, those methods involve a high cost and the production of pollutant smokes loaded with large amounts of carbon monoxide and particles in suspension. Consequently, it seems obvious that the cork industries still need a more suitable method for treating cork residues, which should result in both lower costs and lesser environmental impact.

Cork degradation. Difficulties in the degradation of cork are due to its own complexity. Cork is a biopolymer composed of 45% fatty acids and resins, 20% soluble acids, 27% woody substances and 7% tannins, pigments and mineral salts. Some of these molecules are joined in a complex structure that form suberin, the characteristic cork biopolymer. A hypothetical structure has been proposed for suberin (7). A phenolic matrix similar to lignin is linked by means of a covalent bond to cellular wall glucides and to a long-chained aliphatic fraction (C_{18} to C_{30}). This aliphatic section makes the structure hydrophobic because of its interaction with the wall waxes. The chemical structural complexity of suberin makes cork biodegradation difficult (6–8). The biological degradation of these polymers is carried out by heterotrophic bacteria and lisotrophic fungi through external digestion.

The white rot fungi. Among the fungi which grow in the Catalan oak forests (NE Spain) (24), those that produce the white rot have the highest degrading ability. They are homobasidiomycetes from the group of the Aphillophorales Hymenomycetes, and have a high amount of enzymes: manganese peroxidase (MnP) and lignin peroxidase (LP), both able to depolymerize lignin in vitro (16, 25); hydrogen peroxide generating enzymes, such as glyoxalate-oxidase (GLO);

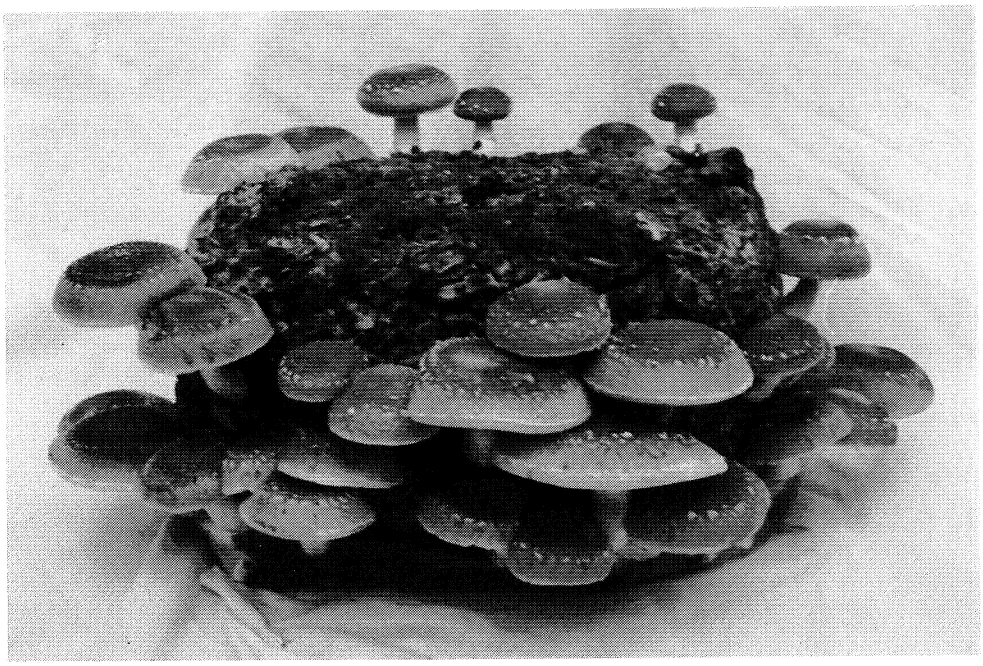

FIG. 1. Mushrooms of *Lentinus edodes* grown on a pile of cork residues.

arylalcohol-oxidase and other peroxidases, such as manganese-independent peroxidase (MIP) and laccase (LAC) (18). Other common enzymes in these fungi are: cellulases, hemicellulases, phosphatases and acid proteinases, which allow them to feed on complex macromolecules which other microorganisms cannot degrade.

Lentinus edodes (Fig. 1) and *Ganoderma lucidum* (Fig. 2) are two fungi that produce the white rot. They have high lignocellulolitic ability and grow spontaneously on *Quercus* spp. (1, 9, 10). *L. edodes* has a lignocellulolitic activity similar to *Pleurotus* during its vegetative growth (21). Two kinds of lignocellulose degradation have been described on *G. lucidum,* both on *Quercus* spp. and on other trees: (i) a global degradation characterized by a uniform change in all wood components, and (ii) a selective degradation acting on lignin and hemicellulose.

L. edodes, also called "shiitake" or "Chinese mushroom", originating in Japan (13) and in other Eastern Asian countries, has been cultivated on barks of different species since ancient times. Nowadays it is grown on other materials, such as sawdust, wastes from the wood industry, straw and remains from prunings. As a result, its cultivation and consumption have greatly increased, because of its exceptional organoleptic qualities and its remarkable aptitude for preservation (4).

L. edodes is marketed both as a foodstuff (fresh, dried or pickled) and also is a dietetic product of shiitake extract, obtained from mycelium grown in liquid medium.

G. lucidum, or "reishi", which means "mushroom of the Endless Youth" in Japanese, is a cosmopolitan fungus and is native to Europe, North America and Asia. Although it is not edible because of its hardness, it has been cultivated in China and Japan, where it has been used for therapeutic purposes since antiquity according to written references dating from 300 BC (4). During the last few decades, research on this species has increased in China and Japan (2).

FIG. 2. Carpophore of *Ganoderma lucidum* in different phases of development on cork residues.

Modern analytical techniques have allowed for the identification of several polysaccharides (26), steroids (3, 11) and terpenoids (5, 12, 14, 19, 23) from *G. lucidum* and *L. edodes*. Those molecules have high antitumoral, therapeutic, cardiotonic and cholesterol reducing effects and they highly stimulate the immunitary system. As a consequence, reishi and shiitake are being gradually marketed in western countries as a dietetic product with many applications for human health. They are currently marketed in capsules, tablets and vials made of hydroalcoholic extracts from mycelium or carpophore solid preparations. Solid preparations can also contain dried mushroom dust. These two species of fungi were chosen for this study for their lignocellulolitic ability and their marketing prospective, in the hope of finding an environmental and economical solution to the cork industry wastes.

Materials and methods

Lentinus edodes ABM1 strain and *Ganoderma lucidum* ABM strain, which had already been grown on barks of different species of trees, were used.

Two products were tested as growth substratum: 'S', a mixture of cork residues as they are produced and stored in the cork industry; and 'SE', a mixture of 70% S and 30% wheat bran (in volume), to enrich the nitrogen concentration which in turn favors lignocellulolitic activity (9).

The two substrata were humidified with 60% water and were sterilized for 45 min at 120°C. The two species of fungi were cultivated on both substrata, as a result, four types of culture were obtained: *Lentinus* (LS and LSE) and *Ganoderma* (GS and GSE).

Culture was carried out in chambers where light, temperature and humidity were controlled. All these factors were adapted to the special characteristics of each species and its growing stages (colonization and fructification). During the colonization stage, the mycellium acts on the cork dust and transforms it into a compact block structure. This process is carried out in the absence of light and at a constant temperature (25°C for *L. edodes* and 30°C for *G. lucidum*). In

order to promote carpophores production, temperature is then reduced (18°C for *L. edodes* and 25°C for *G. lucidum*), and humidity is increased to 95%. Both are provided with white light from a fluorescent lamp with a high proportion of radiation in the blue-violet spectrum. Finally, they are left in the dark for 14 h.

The cork residue was analyzed both physically and chemically before, during and after the fungus culture, using the recommended methods for fodder by the Association of Official Analytical Chemists (AOAC, 973.18) and the modified method of Zetzsche (17).

Results

Table 1 shows time (in days) of every culture stage for each combination of fungus and substratum. Yield obtained in each case is also showed. The duration of the culture is long, as it was intended to deplete the substratum. However, results on degradation in the colonization stage show that it is not necessary to extend the time that long to obtain satisfactory results as far as cork degradation is concerned. Moreover, this time might be reduced even more, optimizing culture conditions. Yield was low for *Ganoderma*, but we must take into account that this fungus has a very low water content (around 10% compared to 90% in *Lentinus*).

In all the treatments, the substrata were acidified as a consequence of fungic metabolism. This agrees with previously published results on physical and chemical characterizations of substrata, before and after cultures take place (20). An increase in electrical conductivity due to the rise in the number of NH_4^+ and NO_3^- ions can be observed, both throughout the colonization stage and at the end of the cultivation. Those ions appear as a result of the solubilization of their organic salts by the fungic action, which also induces an increase in the total nitrogen proportion.

The mineralization of the substrata in all treatments is obvious from both the increase of the ash rate and the decrease in the total organic matter. The rate of mineralization is higher in the enriched substrata and *Lentinus* is, in this respect, the most efficient fungus. A clear advance in cork decomposition is reflected both by the mineralization and the readily oxidized carbon.

At the end of the cultures a significant loss of dry matter and large suberin degradation in all substrata was observed. Rate of loss of dry matter has been high in all cases, *Lentinus* being superior to *Ganoderma* again. However, there are significant differences in the specific molecules attacked. Table 2 shows results on degrada-

TABLE 1. Types of cultivation and substrata yields of *Lentinus edodes* and *Ganoderma lucidum* growing on cork residues

Treatment*	Time (days) Colonization	Fructification	Production (% fresh weight / dry weight)
LS	67	90	24.78
GS	60	105	14
LSE	34	70	8
GSE	30	115	2

* Abbreviations: L, *L. edodes*; G, *G. lucidum*; S, cork; SE, enriched cork. (See text for explanation.)

TABLE 2. Fiber and organic matter degradation of substrata during the cultivation of *Lentinus edodes* and *Ganoderma lucidum* growing on cork residues

Treatment	Total organic matter	Neuter fiber detergent	Acid fiber detergent
LS	39.89	47.51	41.32
GS	34.97	35.57	31.90
LSE	44.59	46.84	36.46
GSE	41.91	37.90	25.91

* Abbreviations: L, *L. edodes*; G, *G. lucidum*; S, cork; SE, enriched cork. (See text for explanation.)

tion of fibres and organic matter of substrata during the culture.

Discussion

The colonization stage developed normally in both fungi, and no contamination of other pathogenic fungi occurred. *L. edodes* and *G. lucidum* behaved similarly. In both cases, cork and enriched cork substrata produced different results. On the cork substratum, the pale colored mycelium spread quickly over the surface, probably in search of easily assimilable nutrients. However, in the enriched substratum the mycelium had a more intensive color and spread slowly. This can be explained because of the high availability of nutrients due to the presence of wheat bran. Although both substrata were satisfactorily colonized, it took twice as long for the unenriched substratum to complete this stage.

Throughout the fructification stage, the culture in enriched substrata showed a higher sensitivity to contamination by other fungi. The opposite occurred in the cork substrata: although both substrata were located together, the enriched substratum was contaminated, while the other was not. This suggests the great difficulty in degrading cork and the highly specialization of fungi able to grow on it.

Suberin is attacked by both species, but *Ganoderma* is more efficient. This could be expected, since this species grows on *Quercus* stumps, which it colonizes totally both internally and externally. On the other hand, when *Lentinus* grows on cork trees (*Quercus suber*), evergreen oaks (*Quercus ilex*), or oak trees (*Quercus robur*), it settles on the inner areas which are more lignified and have less suberin. The contrary happens in the case of ceroids.

A different situation, depending on the substrata, has been observed in fibers. During the colonization stage, when the substrate is cork, the degradation rate is very high. If the substrate is enriched cork, lower rates result. The same happens for total organic matter. This suggests that whenever nutrients from the enrichment are available, molecules of more difficult degradation are not necessary.

Studies on the utilization of residues from the cork industry as a germinative substratum, and for rooting and growth of plants in containers, have shown obvious potential for being used on a large scale. However, a depressive action (15, 22) of cork on plants grown on it has been observed. It is mainly due to the abundance of tannins and other phenolic compounds in cork. These substances are also metabolized by the fungi. The cork, being digested by fungi in the production of edible mushrooms, can then be used as a substratum growth of plants and, after a compost process, can be used as a fertilizer or organic corrector of soil structure. These aspects have not been studied in the research described here.

Conclusions

The growth of *L. edodes* and *G. lucidum* on cork industry residues is viable for these two species; they can develop well and complete their biological cycles. The positive results in the case of the cork industry indicate that no enrichment, neither organic nor mineral, is needed for an efficient growth of mushrooms. The tested enrichment favors contamination by undesirable fungi, thus reducing mushroom production.

The two tested species degrading cork residues cause a loss of dry matter, around 40% in *L. edodes* and 35% in *G. lucidum*. Major transformations in the chemical composition and physical characteristics of the residue have also been observed, among which we must point out the degradation of suberin by *G. lucidum*.

In cultures of *L. edodes*, mushrooms of marketable interest have been obtained with a production around 25% dry matter. These mushrooms are highly valued; they maintain all morphological and organoleptic characteristics of the species, and they have no taste of cork. The final aspect—the possibility of recycling the residues of fungic culture substrates in agriculture—could offer added value to this material, whose disposal represents high expenses to the cork industries. In conclusion, the growth of *L. edodes* and *G. lucidum* on cork residues is suggested as a feasible and economical environmental alternative to the disposal of cork .

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Implementation of conditions of the inoculum stage for *Streptosporangium* cultures

Gonzalo Platas,* Javier Collado, Herminia Martínez, Marta Arrese, Fernando Peláez, María T. Díez

Centro de Investigación Básica, Merck, Sharp and Dohme de España, Madrid, España

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Summary

Different factors concerning the inoculum stage of cultures classified within the genus *Streptosporangium* have been studied. These factors include a comparison among several inoculum media and sources, the influence of aeration and volume of medium in the growth of the cultures, and the effect of the inoculum size on the course of the production of two antibacterial substances. The results suggest that a slight variation in the status of the seed culture (age, biomass concentration, etc.) affect the course of the batch culture during its initial exponential phase (3–5 days), whereas, at longer times, the behavior of the culture does not depend greatly on the characteristics of the inoculum. The results obtained have allowed to define a set of conditions producing the maximum increase of biomass in the minimum time.

Key words: *Streptosporangium,* inoculum implementation, culture aeration, antimicrobial activities, biomass

Resumen

Se han estudiado distintos factores relativos al inóculo de cultivos de producción en organismos clasificados dentro del género *Streptosporangium.* El estudio consiste en la comparación entre fuentes y medios de inóculo, la influencia de la aireación y el volumen de medio en el crecimiento de estos cultivos, y el efecto de la cantidad de inóculo en la producción de dos compuestos antibacterianos. Los resultados sugieren que pequeñas variaciones en el inóculo (edad, concentración

* *Correspondence to*: Gonzalo Platas. Centro de Investigación Básica. Merck, Sharp and Dohme de España. Josefa Valcárcel, 38. 28027 Madrid. España. Tel.: +34-1-3210719. Fax: +34-1-3210614. E-mail: gonzalo_platas@merck.com

de la biomasa, etc.) afectan el curso del cultivo en el inicio de la fase exponencial (3 a 5 días), mientras que con tiempos más largos el comportamiento del cultivo es independiente del inóculo. Los resultados del estudio han permitido establecer una serie de condiciones para producir la mayor cantidad de biomasa en el mínimo tiempo.

Introduction

The genus *Streptosporangium* is an Actinomycete isolated mainly from soil. Its most relevant morphological feature is that its non-motile spores are contained inside spore-vesicles or sporangia, borne on aerial mycelium.

The ability of members of this genus to produce useful secondary metabolites has been reported by several research groups. Examples of their capabilities as antibiotic producers are: chloramphenicol (10), platomycin (9), 1,6-dihydroxy-2-chlorophenazide (7) and dehydrosinefungin (1). Some other potentially useful substances have also been described: endothelin converting enzyme inhibitors (11), inhibitors of the lactic acid production by the oral microbiota (3), or insecticide compounds (6). The diverse chemical nature of these natural products makes *Streptosporangium* an interesting genus to be studied in a natural products screening program.

One of the most significant aspects for the production of all the microbial products is the seed stage, that can be defined as the preparation of a population of microorganisms from a dormant stock culture to a state useful to be inoculated to get a final productive state. There are several purposes for this step: to minimize the loss of microorganisms during recovery from dormancy, to obtain genetically identical copies of the initial stored population, to increase biomass and to develop the culture into a physiological state suitable for the performance in the final production stage (2).

The goal of this study was to identify the conditions needed to improve the inoculum stage for members of the genus *Streptosporangium*, in relation to the production of a non characterized antibiotic. For this purpose, several conditions were analyzed, including: (i) The influence of three culture preservation methods (spores, slants tubes and frozen agar plugs) in the seed culture development. (ii) The composition of the seed medium on biomass yield and growth rate. (iii) The influence of factors such as aeration or concentration of the initial inoculum on the development of the seed culture. (iv) The influence of the concentration and the inoculum growth phase for the appearance of antibiotic activities during the production stage.

An additional benefit from this study has been the possibility of collecting some basic information about the physiology of some strains of this poorly understood genus, not extensively reported on the literature.

Materials and methods

Strains. Five wild type cultures (from now on, referred as S-1 to S-5) isolated by Dr. O. Genilloud (CIBE, MSD Spain) were used in the experiments performed to select the best inoculum medium and source of inoculum. These strains were chosen as representative of some of the different phenotypes exhibited by members of this genus, regarding to pigmentation, morphology of the sporangia and ability to sporulate in different media. All the remaining experiments were performed with three out of these five cultures. Data from several cultures were averaged (Figs. 1, 4 and Table 1).

Incubation conditions. *Solid cultures*: Stock cultures were routinely maintained on Bennett sucrose medium slants; only for special purposes OYGG slants were used. The incubation was performed at 28°C. Media formulation (per liter) is as follow: Bennet medium (yeast extract 1 g, beef extract 1 g, casein hydrolysate 2 g, glucose 10 g), OYGG medium (ISP medium #3 [Difco] 22 g, yeast extract 1 g, glucose 2 g, glycerol 1.7 g). *Liquid cultures*: As a rule, the cultures were incubated in 250 ml Erlenmeyer non-baffled flasks, at 28°C and 220 rev./min. Any difference from this set of conditions is indicated in the text. Three seed media formulations were tested (per liter): A (glucose 10 g, soluble starch 20 g, beef extract 3 g, N-Z amine E 5 g, yeast extract 5 g, Bactopeptone 5 g, $CaCO_3$ 1 g, pH 7) (5), B (glucose 2 g, soluble starch 10 g, beef extract 3 g, N-Z amine E 5 g, pressed yeast 0.5 g, ardamine 1 g, $CaCO_3$ 0.5 g, pH 7) and C (glucose 10 g, dextrin 10 g, yeast extract 5 g, Bactopeptone 5 g, $CaCO_3$ 1 g, pH 7) (4). The course of the fermentation was followed measuring biomass as dry weight, glucose depletion (glucose Trinder reagent [Sigma]) and pH.

Production stage. Forty five ml of the production medium were delivered into 250 ml non-baffled flasks, inoculated with 1 ml of the seed culture and incubated at 28°C and 220 rev./min. The formulation of the production medium, per liter, is: dextrin 30 g, primary yeast 10 g, soybean meal 5 g, tomato paste 7.5 g, $CoCl_2 6H_2O$ 5 mg (8).

Antibiotic activity. The antibiotic activity was measured using *Bacillus subtilis* as test strain. *B. subtilis* seeded plates were prepared by pouring 10 ml of a suspension (0.5 ml /l) of *B. subtilis* spores (Difco) in nutrient agar (Difco) into the Petri dishes. These plates were incubated overnight at 37°C, and the activities against *B. subtilis* were measured as diameter of the inhibition zone (mm). The sensitivity tests were performed using 12 mm diameter filter paper disks (Schleicher & Schuell) that were dipped in the whole broth of the culture and placed on to the surface of a confluent lawn of *B. subtilis*.

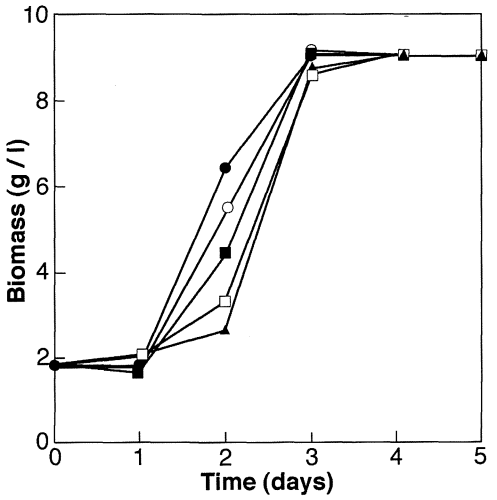

FIG. 1. Influence of the concentration of inoculum in the biomass yield: inoculation of different spore concentrations. The experiment follows the development of cultures S-1, S-2 and S-3 along five days when they were started from spores. Five spore concentrations were inoculated in 50 ml of medium A. The figure shows the average dry weight of the three cultures. Symbols: (●) 5×10^7 spores, (○) 2.5×10^7 spores, (■) 1.25×10^7 spores, (□) 6×10^6 spores, (▲) 3×10^6 spores.

Results

Influence of the concentration of the pre-inoculum in the inoculum stage culture. Due to the differences in the concentration of viable cells that different preservation methods might yield, before comparing the performance of these methods as the source of pre-inoculum of *Streptosporangium* cultures, it was essential to test the influence that the concentration of the initial inoculum could have in the course and timing of the seed stage. With this goal, five spore amounts (5×10^7, 2.5×10^7, 1.25×10^7, 6×10^6 and 3×10^6 spores), as well as four densities of inoculum gathered after diluting 1/10, 1/50 and 1/100 a

TABLE 1. Comparison of inoculum media and source of inoculum

Source[a]	Seed media					
	Medium A		Medium B		Medium C	
	Maximum biomass[b] (g/l)	Stationary phase first day (range)[c]	Maximum biomass (g/l)	Stationary phase first day (range)	Maximum biomass (g/l)	Stationary phase first day (range)
Spores	8.0 ± 1.40	4–10	4.1 ± 0.90	4–9	5.5 ± 0.30	4–9
Slant	7.4 ± 1.10	3–6	4.4 ± 0.80	3–6	5.3 ± 1.05	3–6
Cryopreserved agar plug	7.5 ± 1.05	3–9	4.1 ± 0.30	3–9	4.6 ± 0.75	4–9
Average	7.6 ± 1.06		4.2 ± 0.66		5.1 ± 0.92	

[a] The concentration of the culture inoculated in every flask varies depending on the source: 5×10^6 spores, 1/10 of a homogenate suspension obtained from a Bennett sucrose agar slant or one agar plug also homogenized. All the suspensions were performed in 0.9% NaCl to avoid interference with the seed media. The final volume added in all the cases was 1 ml to 50 ml inoculum medium.

[b] The results of biomass correspond to the average value of the maximum dry weight measure of the five *Streptosporangium* cultures.

[c] Stationary phase first day reflects the range observed among these five cultures. The growth of the cultures was followed for 14 days.

homogenized cell suspension collected from cultures grown in slant transfer tubes with an initial CFU of 10^4, were inoculated separately into seed flasks (Fig. 1).

The cultures that started their inoculum state as spores behaved homogeneously. During the first day of incubation the dry cell weight remained constant, regardless of the concentration of the spores inoculated, what probably reflects the lag period corresponding to their germination. In the second day, the biomass reached by the five concentrations of spores studied was different. The value of the biomass observed on the second day, plotted against the initial concentration of spores seeded, followed a logarithmic distribution (data not shown). This function approached asymptotically to the value of maximum biomass, which was reached in the third day in all cases. This observation suggests that the lowest concentration of spores used (close to 6×10^6 spores) was the most appropriate to inoculate seed flasks, as the maximum biomass was achieved at the same time, independent of the spore concentration inoculated.

The effect of four different inoculum densities in the development of the seed cultures of three *Streptosporangium* strains was also studied. The growth profiles observed in the three seed cultures inoculated with the most concentrated cell suspensions were homogeneous, reaching the stationary phase at the third day. The seed cultures, inoculated with the less concentrated suspensions, reached the stationary phase with a time delay that increased with the dilution and varied with the culture. For example, strain S-2 inoculated with the most concentrated cell density, reached its stationary phase in 3 days, whereas the same culture inoculated with 1/10, 1/50 and 1/100 dilutions reached the same growth phase at 4, 4 and 6 days respectively.

In all the cultures tested, when the same concentration of biomass was reached, regardless of the initial dilution of the preinoculum seeded or the time required to reach that biomass, the values observed for other variables studied, such as glucose concentration or pH, were simi-

lar (Fig. 2). In both experiments, the maximum of biomass (about 8.5–9 g dry weight/l) was reached independently of the concentration of culture inoculated.

Influence of inoculum source and seed medium on seed culture biomass. To define the most appropriate sources of inoculum and seed medium for *Streptosporangium,* three usual sources of inoculum (spore suspensions, Bennett medium transfer slants, and cryopreserved agar plugs) were tested and the biomass yield obtained by each preservation source was evaluated in three seed media.

Table 1 shows that the maximum biomass for the three inoculum sources was achieved in medium A, the richest one. As was expected, the maximum level of biomass was not dependent on the inoculum source, because their variations in the biomass yield were not significant. However, the seed flasks inoculated from slant transfer tubes reached the stationary phase sooner than the cultures seeded from spores or agar plugs. This observation was reproducible in the three seed media studied.

Influence of the aeration in the inoculum stage. To ensure the maximum aeration of a seed culture, several strategies have been traditionally used, such as the use of baffled flasks or variations in the volume of seed media. The influence of aeration in the growth of *Streptosporangium* was studied following two approaches: (i) comparing the profiles of biomass, glucose depletion and pH obtained from cultures grown in baffled flasks with the same cultures grown in ordinary flasks, and (ii) testing three series of 250 ml flasks containing different volumes of seed medium.

As it can be observed in Fig. 3, baffled flasks appear to produce a slightly higher biomass yield than in the non-baffled ones (around 10% increase). This difference, although modest, was observed in all the cultures tested.

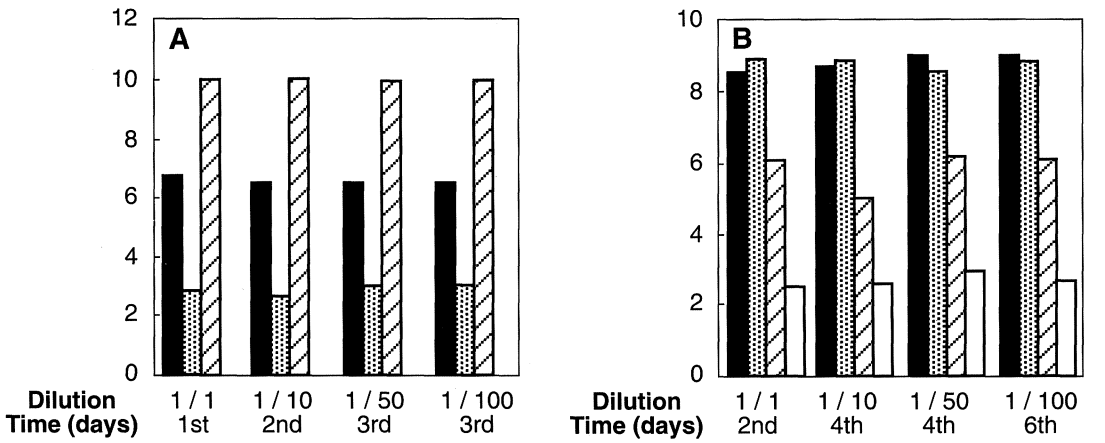

FIG. 2. Influence of the amount of pre-inoculum in the production stage. Bennett sucrose agar slants from cultures S-1, S-2 and S-3 were used to inoculate glass tubes containing 10 ml of medium A. After 3 days of incubation the stationary phase was reached, as revealed by the stabilization of dry weight. A late exponential phase culture was converted into a thin suspension with a glass homogenizer. One ml of this suspension was diluted to 1/10, 1/50 and 1/100 in medium A. One ml of each dilution was inoculated in one flask containing 50 ml of the same medium. As representative of the three cultures, the figure shows the values of several physiological variables (pH, glucose concentration and antibiotic activity), in the different dilutions tested, at a certain value of biomass. Panel A presents the results attained from culture S-2 in its initial exponential phase, and panel B shows the data gathered by S-1 at its late exponential phase. Symbols: (■) pH, (□) biomass (g/l), (▨) glucose concentration (g/l), (□) antibiotic activity inhibition zone.

Fig. 4 shows the result attained growing *Streptosporangium* strains at different volumes of seed medium. It can be observed that the increase of the volume of medium correlated with an increase in biomass density. This suggests that a decrease in the aeration may cause an enhancement of *Streptosporangium* biomass, until a certain value of biomass was reached (around 12 g/l).

Effect of the concentration and growth phase of the inoculum stage in the production of antimicrobial activity in batch culture. To check the influence of inoculum concentration on some production stage variables (dry weight and antibiotic activity yield), production flasks were inoculated with seed cultures grown in 50 or 100 ml. It was observed that cultures reached the highest values of biomass at the same day. In terms of antibiotic activity yield, it could be observed that, in strain S-1, the inoculation from 50 ml seed flasks gave higher levels of activity at earlier times than inoculation from 100 ml seed flasks. However, the effect was just the opposite for culture S-2. In both cases, the level of activity detected reached the same degree after the fourth or fifth day of incubation (data not shown).

To check the influence of the growth phase of the inoculum in the release of antimicrobial activities at the production stage, cultures in exponential or stationary phase of growth (3 and 5 days of incubation, respectively), were inoculated into production flasks. The production of the antibiotic activity by culture S-2 was not affected by the growth phase of the seed culture. However, when the production flask was seeded

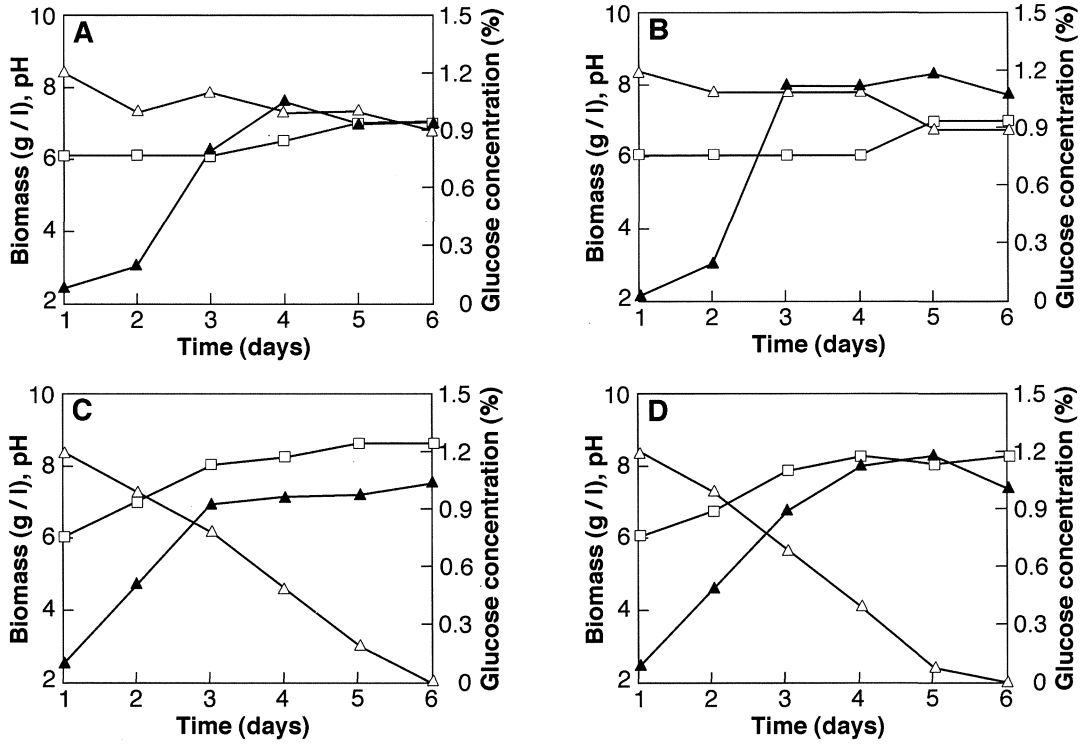

FIG. 3. Influence of aeration: baffled versus non-baffled flasks. The experiment was performed in 250 ml baffled or non-baffled flasks containing 50 ml of medium A. The growth profile of S-1 and S-2 were followed for six days. Panel A: culture S-1, non-baffled flask. Panel B: culture S-1, baffled flask. Panel C: culture S-2, non-baffled flask. Panel D: culture S-2, baffled flask. Symbols: (□) pH, (▲) biomass (g/l), (△) glucose concentration (%).

from an exponential stage inoculum, the activity produced by culture S-3 was detected with two days of delay. As in the previous example, both cultures showed comparable levels of antibiotic activity after eight days of incubation (data not shown).

Discussion

The results suggest that the concentration of a culture inoculated into a seed or production stage affects the time course of the cultivation, but not the relations established among the physiological variables: biomass, pH, glucose depletion or quantification of antibiotic activities, measured at a certain point of its development.

Medium A was the most adequate medium for growing *Streptosporangium* strains, among the seed media studied. Medium B has been successfully used as inoculum medium for *Streptomyces* strains, and medium C has been used as seed medium for the production of victomycin by *Streptosporangium violaceochromogenes* (4). Medium A is the most nutrient enriched and produces an increase of almost 50% of biomass when compared with the second best medium.

Although the three culture maintenance sources analyzed yield comparable results in the final biomass concentration, slant transfer tubes were selected as the most suitable ones, based on the fact that the seed cultures originated showed a more uniform behavior regarding to the time required to reach maximum biomass. This result, as well as the easiness of handling for the considerable number of cultures to be in a large-scale screening program, suggests that slant transfer tubes are the most appropriate source of inoculum for moderately long time storage of the culture. The slant medium selected was Bennett sucrose agar, but similar results have been obtained with different media, such as OYGG (data not shown).

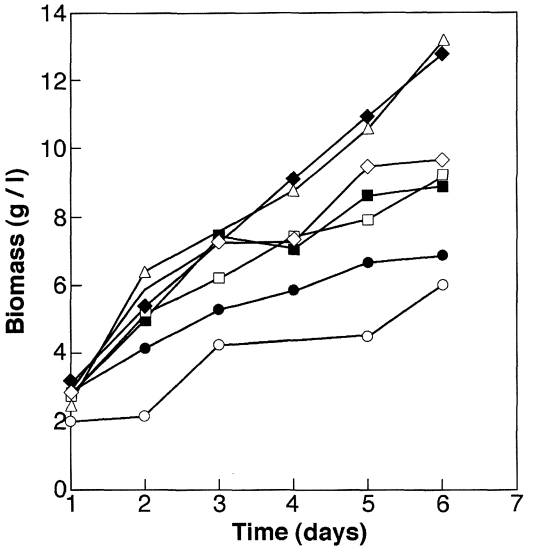

FIG. 4. Influence of aeration: effect of seed medium volume. *Streptosporangium* strains were grown to stationary phase in medium A, and 10 ml of this culture were inoculated into 1 liter of medium A (1% inoculum). Different volumes: (△) 125 ml, (◆) 100 ml, (◇) 75 ml, (■) 62.5 ml, (□) 50 ml, (●) 25 ml, (○) 12.5 ml, of this stock were cultured into 250 ml flasks, inoculating equal density of cells per ml in every flask. The figure shows the average increase in biomass over the course of six days for *Streptosporangium* cultures S-1, S-2 and S-3.

The influence of aeration on *Streptosporangium* seed cultures was not clearly established by our experiments. Baffled flasks, which are expected to increase aeration, induced a slight but reproducible increase of biomass (10%) compared with non-baffled flasks. On the other hand, experiments performed culturing these microorganisms at different volumes, suggest that they may prefer moderate rather than high aeration, as they reach higher biomass densities when growing in larger volumes. One possible explanation for this apparent contradiction is that baffled flasks produce better aeration in the culture and, also, higher fragmentation of the mycelium, increasing the number of actively growing mycelial units. The moderate enhancement in the biomass yield may have occurred because, in these conditions, the dissolved nutrients were

accessible to a higher proportion of actively growing mycelial units than in those larger mycelial pellets generated in non-baffled flasks.

It may be of interest to point out the differences observed in the rate of glucose consumption among the two cultures presented in Fig. 3. In some cultures, glucose does not seem to be a source consumed preferentially, exhibiting a predilection for nitrogen enriched sources. Both patterns of glucose utilization were observed to be similarly spread among a group of 24 *Streptosporangium* strains (data not shown). All the cultures tested degrade efficiently starch and dextrin (data not shown).

The results attained in this series of experiments with members of the genus *Streptosporangium*, also indicate that the different conditions in which the inoculum stage was performed, like aeration or growth phase of the inoculum, may affect the time course of the production stage, especially in the performance of short-term incubations. However, longer incubation times are not apparently affected by the status of the inoculum.

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Characterization and selection of lactobacilli isolated from Spanish fermented sausages

Arancha Rovira, Juan C. Nieto, Ana Rodríguez, Juan I. Reguera,*
Zulema González

Área de Microbiología, Facultad de Ciencia y Tecnología de los Alimentos y Ciencias Químicas, Universidad de Burgos, Burgos, España

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Summary

The use of starter cultures to control and run the fermentative process is a usual way of manufacturing sausages in meat industries. The first stage in the starter culture designing process is to characterize the lactic acid bacteria isolated from these meat products, in order to select the best strains. The strains used for this study were isolated from different dry fermented sausages, obtained during the manufacturing process. The main tests used to identify the isolated bacteria were: microscopic-morphologic characteristics, catalase activity, production of gas, growth at 8, 15 and 45°C, fermentation of carbohydrates and production of lactic acid isomers. A total of 194 strains were identified. *Lactobacillus sake* and *Lactobacillus plantarum* were the most frequent species. Other microbiological tests were also performed, and three strains of *Lactobacillus sake* were found which did not produce dextran from sucrose.

Key words: lactobacilli, starter culture, fermented sausages, biochemical characterization, lactic acid isomers

Resumen

La utilización de cultivos iniciadores que controlen y dirijan el proceso fermentativo es una práctica habitual de elaboración de embutidos en las industrias cárnicas. La primera etapa del diseño

* *Correspondence to*: Juan I. Reguera. Área de Microbiología. Facultad de Ciencia y Tecnología de los Alimentos y Ciencias Químicas. Universidad de Burgos. Plaza Misael Bañuelos, s/n. 09001 Burgos. España. Tel.: +34-47-258812. Fax: +34-47-258831.

de un cultivo iniciador consiste en caracterizar las bacterias lácticas aisladas de los productos cárnicos, con objeto de seleccionar las cepas más ventajosas. Las cepas se aislaron de distintos embutidos fermentados crudos-curados obtenidos durante su proceso de elaboración. Las pruebas de identificación más importantes fueron: características morfológicas-microscópicas, actividad catalasa, producción de gas, crecimiento a 8, 15 y 45°C, fermentación de carbohidratos e isómero de ácido láctico producido. Se identificaron 194 cepas. *Lactobacillus sake* y *Lactobacillus plantarum* fueron las especies que se encontraron con más frecuencia. Se realizaron también otras pruebas microbiológicas y se hallaron tres cepas de *Lactobacillus sake* que no producen dextrano a partir de sacarosa.

Introduction

Microbiology fermentation technology not only contributes to healthier food but it also allows for the development of new processes of production (21). Microbial metabolic activity produces expected changes which determine the product's characteristics, such as flavor, taste, texture and shelf life. This activity depends also on the microorganism used to start the fermentation (4, 21).

Lactobacilli, among other lactic acid bacteria, mainly contribute to the production of cured, dried and fermented meat products (5). Acid lactic fermentation is necessary to achieve the flavor and stability characteristic of the product (5). Secondary fermentations and other chemical reactions play major roles in the singular organoleptic properties of these products (1).

At present, the use of starter cultures is commonly used in the meat industry for the production of dried fermented sausages. Lactobacilli, pediococci and non-toxigenic staphylococci (9) are the most commonly used microorganisms for these processes. Lactic acid bacteria behave as preservative cultures, mainly due to the acidification of the product, as a consequence of the production of lactic acid. Some species of *Lactobacillus* and *Pediococcus* are able to synthesize substances with antimicrobial activity (3, 19).

Micrococci play a significant role in giving the product a red color, thanks to its nitrate-reductase activity (4). Micrococci and staphylococci (13) might also be involved in flavor formation.

Generally, two groups of starter cultures can be distinguished: (i) natural starters, and (ii) commercial starters, which are directly inoculated into the meat. Marketed products can be obtained from supplier companies, which produce and sell them. Natural starter cultures are not sold by specialized companies, but develop naturally on the products (Garriga, M., 1993. Ph. D. Thesis, Universitat Autònoma de Barcelona). There are two techniques of proceeding with natural starter cultures. The most common technique consists of having the meat set for at least 24 h at a low temperature (16). Thus, besides improving the organoleptic properties, there is an increase and selection of homofermentative lactobacilli and micrococci. Therefore, in retarding the sausage stage, the growth of these bacteria is favoured (16).

Lactobacillus sake, *Lactobacillus curvatus* and *Lactobacillus plantarum* (2, 11, 12, 18, 20) are the most common lactobacilli species found in meat and meat products, including dried fermented sausages. The accurate identification of *L. sake* and *L. curvatus* (atypical streptobacteria) is difficult (7, 15). In Spain, Hugas et al. (6) were the first to characterize dried fermented sausage lactobacilli to the level of species.

In this study, results of the identification and characterization of lactic acid bacteria, isolated from dried fermented sausages, are shown. The purpose of the study was to select the strains of higher technological value for their use as starter cultures.

The mass composition of the sausages studied was approximately: (lean pork/fresh bacon sides) 75 kg/15 kg, water 5 kg/100 kg, NaCl 3 kg/100 kg, lactose 1 kg/100 kg, glucose 1 kg/100 kg, powdered milk 500 g/kg, ascorbate 0.5 g/kg, nitrate 0.3 g/kg and nitrite 0.15 g/kg. The diameter for all produced products was 80 mm. During the heating process, the temperature ranged from 18 to 22°C, and the relative humidity from 90 to 95%, to get a pH 5.2 in 24–48 h. Posterior curing took place between 14–16°C, and relative humidity values were 70–80% for 21–23 days. When the curing process had finished, pH was 4.8–5 and water activity (A_w) 0.90.

Materials and methods

Strains were isolated from three kinds of dried fermented sausages (*chorizo*, salami, and *salchichón*—Spanish salami—), produced industrially with natural starter cultures, in three stages of production: end of heating process, middle of the drying, and end product.

The isolation was made in MRS agar plates (Oxoid), pH 5.4, according to Millière et al. (10), in microaerophilic conditions, at 30°C. Isolated strains were maintained at 4°C in Litmus Milk (Oxoid) medium and were frozen at –40°C in 0.1 M phosphate buffer to pH 7.2, using monosodium glutamate as a cryoprotector.

Counting ranged from 10^7 cfu/g at the end of drying to 10^8 cfu/g in the first steps of fermentation and curing of the products.

Identification was based on lactobacilli, as these are the bacteria which predominate on this kind of product. A morphological study was initially performed for all the strains, through phase-contrast microscope and Gram stain. Five colonies from every isolation in MRS agar for every product were chosen at random, obtaining 194 isolates. Catalase activity was later examined.

Gram-positive, catalase-negative and long-shaped strains, were chosen to follow the identification. Lactobacilli can be differentiated from other lactic acid bacteria by their morphology, which is long instead of coccoid, as is the case of most bacteria. Gas production was determined in MRS tubes with ammonium sulfate instead of ammonium citrate, in Durham's tubes.

Lactobacilli were identified to the species level through the following tests of identification, following Schillinger and Lücke (18): (i) arginine hydrolysis, (ii) acetoin production, (iii) determination of the lactic acid isomer, (iv) growth over a week at different temperatures (8, 15 and 45°C), (v) salt tolerance through growth in MRS culture medium with a 5% and 7% NaCl supplement, (vi) growth at pH 3.9 in MRS culture medium, and (vii) carbohydrate fermentation, using API systems 50 CHL (bioMérieux).

Other tests were performed with some of the strains to determine suitability: (i) growth at different nitrite and nitrate concentrations in supplemented MRS culture medium, (ii) bacteriocin production and, (iii) dextran production from saccharose in MRS agar, in which glucose is replaced by 5% saccharose.

To evaluate organoleptic characteristics conferred to the product by strains which produce a bacteriocin, both strains producing and strains not producing bacteriocin were inoculated in the same set of products, which had been divided up into parts. In each mass one of the strains was inoculated. At the end of the process, a group of expert tasters evaluated taste, color, smell and flavor of the products.

Detection of inhibitory activity. Culture media for strains were MRS broth, low in sugars. Two consecutive cultures were performed at 30°C for 16–18 h. Media, inoculated at 1%, were incubated in microaerophilic conditions. Cell concentration at the end of the incubation stage was around 10^8 cfu/g. Cells were collected by centrifugation at 5000 × *g* for 15 min, maintaining a temperature of 4°C. The pellet was washed twice with sterile physiological serum, and was resuspended in 5 ml of the same medium.

The culture supernatants of some strains that showed bacteriocinogenic activity from the pellet were filtered with Millipore filters of 0.22 µm pore size. They were neutralized with NaOH 1 N at pH 6.5, in order to exclude a possible inhibitory effect caused by a decrease of pH by lactic acid bacteria. To also exclude an inhibitory effect from the possible production of hydrogen peroxide during the development of the cultures, supernatants were treated with catalase (Sigma) dissolved in a phosphate buffer at pH 7.0, to a final concentration of 1 mg/ml, and incubated during 30 min at room temperature. Supernatants were then concentrated ten times in a rotary evaporator at 80°C.

Pellets and supernatants obtained in this way were utilized to detect inhibitory substances using two procedures: the agar spot test, and well diffusion assay, described by Schillinger and Lücke (17).

Agar spot test. Each pellet was resuspended in 5 ml of physiological serum, and was placed with an inoculation loop on the surface of MRS agar low in sugar (0.2% glucose), leaving it to dry for 2 h at room temperature. On these plaque surfaces, 5 ml of semisolid agar (0.75% agar) was placed, inoculating 50 µl of a 16–18 h incubated culture of every stater strain. Concentration of indicator microorganisms in each inoculum was approximately 10^5 cfu/ml. Plaques were dried for 2 h at room temperature, and later incubated under anaerobic conditions at 30°C for 24–48 h. The results were considered positive if an inhibition halo larger than 1 mm appeared around the colonies.

Well diffusion assay method. To perform this assay, 50 µl neutralized, filtered and concentrated supernatants of the cultures of each strain that gave a positive result in the agar-spot test were placed in Petri dishes with MRS agar. Then, semisolid agar inoculated with the strain was poured over it, in the same way described for the agar spot test. Inoculated plaques were dried at room temperature for 2 h, and later incubated for 24–48 h at 30°C in microaerophilic or anaerobic conditions, depending on the strain used. Petri dishes with inhibition haloes equal to or larger than 2 mm, were considered positive.

Results and discussion

Results refer to isolated strains after leaving the heater. Their proportion was maintained constant at both the middle and the end of the curing process, since the most interesting ones are mainly lactobacilli, implanted and grown after heating and feasible during the maturing stage. This shows that selected strains have had a good implantation as starter cultures.

From 194 isolated strains, 98.5% [191] belonged to the genus *Lactobacillus*. Most of them were *L. sake* (61.3% [119]), followed by *L. plantarum* (20.1% [39]), *L. curvatus* (8.7% [17]), *L. viridescens* (6.1% [12]), *L. casei* subsp. *casei* (2.0% [4]), and *Carnobacterium piscicola* (1.5% [3]). The fact that *L. sake* was the predominant species is in agreement with the results obtained by other authors (5, 11, 15, 18). Although Hugas (5) found that the second most predominant species was *L. curvatus*, we found it to be *L. plantarum*. In our case, the higher temperatures of the heating (18–22°C) would

explain in part that *L. plantarum* is the second predominant species. *L. sake* and *L. plantarum* have been species generally described as components of lactic acid bacteria in meat and meat products (1). The other species, especially *L. curvatus*,are also frequently isolated in meat products (6). Independently of the species, all lactobacilli observed through the microscope had a typical morphology: short chains composed by bent rods, forming horseshoe-shaped semicircles. No significant differences were found among different lactobacilli isolated from *chorizo*, salami and Spanish salami. Only a variation in percentages was observed, but it was not related to the kind of product.

Table 1 and 2 show the phenotypic characters studied, and the results obtained. Most of the lactobacilli strains isolated in this study grew at 8°C and 15°C. However, growth at 45°C was variable in the classified strains. As for tolerance to NaCl, all the strains grew at concentrations of 5% and 7%. Tolerance to NaCl is a major factor when choosing a given strain as a starter culture in dried fermented products. Another major factor in the selection of a strain as a starter culture is the most abundant lactic acid isomer obtained. The production of the D-isomer of the lactic acid is highly related to the acid taste of the sausages, because at the same pH value products with microorganisms that mainly produce D-lactic acid are not well accepted by the consumer because their taste is considerably more acid. On the other hand, sausages that contains microorganisms producing D and L forms, or mainly L-lactic acid (14), have not the characteristics mentioned above. Most of the 194 selected strains produced D- and L-lactic, but four strains produced only L-lactic.

All the isolated lactobacilli strains grew properly at a nitrite concentration of 150 ppm, or lower, and 300 ppm nitrate (which are the maximum concentrations legally permitted in sausages production). Only concentrations higher than 500 ppm nitrite or nitrate, were able to negatively influence the growth of the isolated lactobacilli.

From all the isolated lactobacilli strains, three *L. sake* produced "bacteriocin-like" substances active against *Listeria monocytogenes*. Two strains of *L. plantarum* also produced inhibitory substances active against *L. monocytogenes* and *Clostridium perfringens*. Besides, two strains of *L. curvatus* inhibited *L. monocytogenes* and *Clostridium sporogenes*, as well as other

TABLE 1. Phenotypic traits investigated (I)

				Growth (°C)[d]			NaCl(%)[e]			
	Arginine[a]	Acetoin[b]	Lactic acid[c]	45	15	8	7	5	pH ≤3.9[f]	Total strains
L. sake	113	44	DL	36	96	101	119	119	6	119
L. plantarum	18	23	DL	14	39	36	39	39	22	39
L. curvatus	0	6	DL	0	9	9	17	17	0	17
L. viridescens	0	0	DL	12	12	12	12	12	12	12
L. casei subsp. *casei*	1	4	L	3	4	3	4	4	3	4
C. piscicola	2	3	DL	1	3	3	3	3	2	3

[a] arginine hydrolysis; [b] acetoin production; [c] lactic acid isomer produced; [d] growth at 45, 15 and 8°C; [e] growth at 5% and 7% Na Cl; [f] growth at pH ≤3.9.

TABLE 2. Phenotypic traits investigated (II): fermentation of carbohydrates

	L. sake	*L. plantarum*	*L. curvatus*	*L. viridescens*	*L. casei* subsp. *casei*	*C. piscicola*
Cellobiose	96[a]	37	5	0	3	2
Lactose	30	35	10	0	2	1
Maltose	34	38	8	12	3	2
Mannitol	3	38	1	0	3	2
Melibiose	119	37	2	0	1	2
Melezitose	2	25	1	0	3	1
D-Raffinose	8	37	1	0	0	0
Rhamnose	0	7	0	0	0	0
Ribose	119	38	17	0	3	2
Saccharose	119	38	7	12	3	2
D-Trehalose	119	37	5	12	3	2
Total strains	119	39	17	12	4	3

[a] Figures are number of strains which are positive in the test.

Lactobacillus species. "Bacteriocin-like" substances have an inhibitory action, and are sensitive to proteases. Because these inhibitory substances have neither been isolated nor purified, they are called "bacteriocin-like", not bacteriocin. None of the producer strains of "bacteriocin-like" components was selected as starter culture, because they did not have the adequate technological characteristics, and did not confer the best organoleptic properties to the product.

The taxonomic classification of lactobacilli isolated from meat and meat products has been under debate ever since Reuter (15) and Laban et al. (8) showed, in their experiences with Spanish salami, that most of the microbiota in these products differed considerably from any of the three taxonomic groups classically defined. The atypical streptobacteria show a lower resistance to acid (minimum pH: 3.9–4.1) than characteristic streptobacteria (minimum pH: 3.7–3.8); a minimum growth temperature lower than the rest of streptobacteria, and they are coccoid-, or rod-shaped. According to Kagermeier (Ph. D. Thesis, Technical University, Munich, 1981), most of the atypical streptobacteria could belong to *L. sake* and *L. curvatus*.

In 1987, Schillinger and Lücke (18) published some schemes for a fast and simple characterization of lactobacilli of meat and meat products, based on the fermentation of some carbohydrates and other physiological characteristics, easy to detect, such as the production of different lactic acid isomers.

Carbohydrates fermentation has been the method used traditionally for the characterization of lactic acid bacteria, and the most suitable method when a high number of bacterial strains has to be characterized. However, a wide diversity of fermentation patterns is detected when trying to classify the isolated strains from meat. Thus, it is difficult to compare information from different authors that establish "clusters" according to carbohydrate fermentation.

The taxonomic position of *L. sake* and *L. curvatus* is uncertain, because they have a certain grade of genetic homology. The differentiation between the two species is based on the fermentation patterns of five sugars (Kagermeier, 1981, cited), among which melibiose and maltose are the most significant. However, there exist discrepancies between the descriptions of the standard strains and the pro-

perties of the species recovered from a meat habitat.

In the phenotypic study of the isolated strains in this work, fermentation patterns of the sugars were analyzed: cellobiose, melezitose, D-raffinose, rhamnose, ribose, saccharose and D-trehalose. Those are the least variable in the phenotypic evaluation of lactobacilli from meat products.

One of the main differences found between *L. sake* and *L. curvatus* was melibiose fermentation; in all cases, *L. sake* fermented it, whereas *L. curvatus* rarely did. In the taxonomic keys published by Schillinger and Lücke (18), production of ammonia from arginine has no taxonomic value. Nevertheless, according to Montel et al. (Montel, M. C. 1989. Proc. 35th Congress of Meat Science and Technol., p. 229), this character can be of great taxonomic value to differentiate *L. sake* from *L. curvatus*, since *L. sake* can hydrolyze arginine and *L. curvatus* cannot. Therefore, it could be useful to include this characterization to distinguish lactobacilli, together with melibiose fermentation, which is positive for *L. sake* and negative for *L. curvatus*.

Although *L. plantarum* does not usually deaminate arginine, it is well known that while isolating lactobacilli strains from meat, strains from different species as *L. plantarum* are identified, and these do not always respond to the standard pattern. So, it is quite difficult to compare information coming from different works (5). Certain physiological characterizations (growth in extreme conditions, NaCl sensitivity, resistance to biliary salts, etc.) might be more discriminatory than other biotypes.

Finally, we concluded that, from the 194 strains isolated, only three strains of *L. sake* do not ferment mannitol nor do they produce dextran from saccharose, but showed good qualities to be selected for the manufacturing of the starter cultures. This made clear the importance of control in the development of lactic acid strains, which naturally contaminates meat, to avoid problems from its metabolism in meat products.

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Growth of methylaminotrophic, acetotrophic and hydrogenotrophic methanogenic bacteria on artificial supports

Homero Urrutia,*[1] Roberto Vidal,[1] Marcelo Baeza,[1] Juan E. Reyes,[1] Estrella Aspe[2]

[1] *Laboratorio de Microbiología Ambiental, Departamento de Microbiología, Facultad de Ciencias Biológicas, Universidad de Concepción, Concepción, Chile*
[2] *Laboratorio de Bioingeniería, Departamento de Ingeniería Química, Facultad de Ingeniería, Universidad de Concepción, Concepción, Chile*

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Summary

The efficiency of organic matter degradation in attached biomass reactors depends on the suitable selection of artificial support for the retention of bacterial communities. We have studied the growth on glass and clay beads of methylaminotrophic, acetotrophic and hydrogenotrophic methanogenic bacterial communities isolated from anaerobic reactors. Bacterial counts were performed by the standard MPN technique. Experiments were performed in 50 ml vials for 12 days at 35°C. Increase in the counts of methylaminotrophic and hydrogenotrophic methanogens occurred on both glass and clay beads. The latter support material also stimulated the growth rate of methylaminotrophic methanogens.

Key words: methanogenesis, bacterial colonization, methanogenic trophic groups, artificial supports, anaerobic degradation

Resumen

La eficiencia en la degradación de materia orgánica en fermentadores de biomasa adherida depende de la adecuada selección de soportes inertes para la retención de comunidades bacterianas.

* *Correspondence to*: Homero Urrutia. Departamento de Microbiología. Facultad de Ciencias Biológicas. Universidad de Concepción. Casilla 152-c. Concepción. Chile. Tel.: +56-41204118. Fax: +56-41245975. E-mail: hurrutia@buho.dpi.udec.cl

En este trabajo se estudió el crecimiento sobre esferas de vidrio y de arcilla de bacterias metanogénicas acetotróficas e hidrogenotróficas aisladas de reactores anaeróbicos. Los recuentos de cada grupo se realizaron mediante la técnica del NMP, en viales de 50 ml durante 12 días a 35°C. Los resultados muestran un aumento en los recuentos de metilaminotróficos e hidrogenotróficos sobre esferas de vidrio y de arcilla. Este último material de soporte estimula significativamente la velocidad de crecimiento de bacterias metanogénicas metilaminotróficas.

Introduction

Anaerobic treatment seems to be the best technique to clean effluents produced by the fishing industry due to its low operational cost, low amount of sludge produced and because of the methane generated in the process (11). However, high sulfate concentrations in those effluents restrict the efficiency of the process (3). Besides, the fact that sulfate reducing bacteria (SRB) have a higher affinity for acetate and H_2 (7, 8) favors a sulfidogenic environment in which the activity of methane producing bacteria (MPB) decreases (5, 6, 15, 18, 22).

To promote methanogenic activity in anaerobic reactors, inert support materials favoring MPB immobilization have been used. In these systems, competition between SRB and MPB communities is regulated by their growth kinetics and adhesion characteristics (6, 7, 8, 11). However, the colonization of any support material is also influenced by the material's physical features, such as porosity and hydrophobicity, and by its chemical composition (4). Thus, knowing the methanogenic communities growth capacity on different support materials would allow to select a suitable material which favored methanogenesis in the treatment of the fishing industry effluents.

We studied the kinetics of growth on glass and on clay for different trophic groups of methanogenic bacteria that had been isolated from communities in marine sediments (VIII Region, Chile).

Materials and methods

Support material and growth substrate. Clay and glass beads 0.5 cm in diameter were used. The necessary amount of beads (support material) equivalent to 10 ml was added to 50 ml vials that contained 25 ml liquid substrate. The vials were closed with butyryl stoppers, and sealed with metallic rings. The vials were sterilized at 121°C for 15 min.The content of each vial was later reduced with cysteine-HCl and Na_2S in an atmosphere of 80% N_2 and 20% CO_2.

Inocula for the anaerobic discontinous reactor. Inocula, obtained from the sludge of an anaerobic reactor, was fed with wastewater from the fishing industry every 4 days for 12 months. Samples were obtained with a 20 ml sterile syringe, connected to the feeding pipe, through a perystalthic pump. Air bubbles in the syringe were eliminated, and needles were sealed with rubber caps. To disgregate sludge granules, samples were sonicated for 10 min at 35 kHz (Brasonic Ultrasonic Cleaner-80W). The experimental design consisted of series of 25 discontinous reactors for each support material and the same number of reactors without support material (control). Each of them was inoculated with an aliquot from the sludge (with a bacterial content of about 10^4 cel/ml). Reactors were incubated at 30°C for 40 days, shaking them constantly (120 rpm). Counts of every trophic group were made twice every 48 h.

Counting the adhered methanogenic bacteria. Countings of MPB were made by the

standard most probable number (MPN) method (1). A reactor from each experimental series (clay, glass, and control) was selected from each sampling. From each system, 25 ml of substrate material was extracted. To eliminate cells not adhered, the samples were cleaned up by injecting 10 ml of the following solution twice: $(NH_4)_2HPO_4$, 1.0 g/l; KH_2PO_4, 0.2 g/l; K_2HPO_4, 1.6 g/l; $MgSO_4 \cdot 7H_2O$, 0.2 g/l; NaCl, 0.1 g/l; $CaCl_2 \cdot 2H_2O$, 0.02 g/l; $FeSO_4 \cdot 7H_2O$, 0.01 g/l; $Na_2MoO_4 \cdot 2H_2O$, 0.005 g/l; resazurin, 0.001 g/l; cysteine–HCl, 0.25 g/l, pH 7 (10). Support materials were then resuspended in 10 ml of that solution, and sonicated for 10 min to remove the adhered bacteria (Brasonic Ultrasonic Cleaner-80W) (9). Subsequently, the samples were shaked in a Vortex for 1 min, and serial dilutions were made. The culture medium for counting MPB contained 0.1% of the specific substrata of each nutritional group (acetate, trimethylamine), 0.01% yeast extract, mineral salts, mineral traces, vitamin traces plus the reductor agent and a gas mixture containing 80% N_2 and 20% CO_2 (2). To quantify hydrogenotrophic strains, the gas phase in each tube was replaced by a gas mixture containing 80% H_2 and 20% CO_2. Countings were expressed by the MPN of resuspended cells per total volume of the reactor, with the aim of comparing the suspended cell counts (from reactors without support material) and resuspended cell counts (from reactors with clay and glass beads).

Samples incubation. Samples were processed in a controlled atmosphere chamber (Glove Box-Labonco) containing 80% N_2 and 20% CO_2. They were incubated at 37°C for 30 days. The detection of methane in the gas phase of the flasks was performed with a Hach-Carle Serie 100 chromatograph, and was used for counting methanogenic bacteria.

Growth kinetics of biofilms. Growth kinetics of each trophic group on glass, on clay and in the absence of support was determined by using the Gompertz growth model. The Neperian logarithm of the relative growth (N/N_0) was used as a variable, N_0 being the initial counting (MPN/ml) and N being the count at a time t expressed in hours (25). The effect of the support material on the specific growth rate (μ) and the maximum counting on the support material at the beginning of the stationary phase were studied by the variance analysis (17). All experiments were duplicated and a Systat 5.0 program was used to process the data (23).

Results and discussion

Methane production. Methanogenic activity increased rapidly at the beginning of incubation. It stabilized after ten days, when it reached 75% of the total production over the period studied (Fig. 1). In systems with clay as the support material, this methane concentration was reached in the first ten days, but it took twenty days to reach the same concentration in the control series and in those with glass support. The

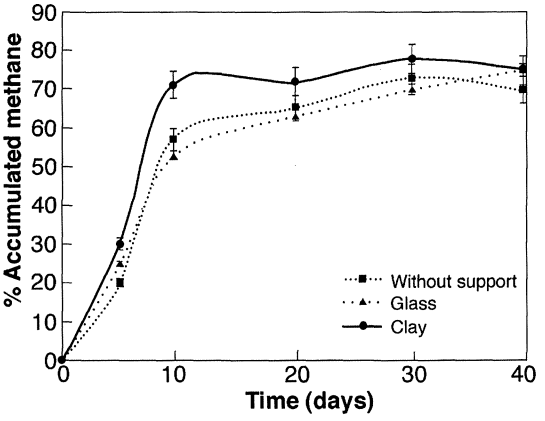

FIG. 1. Methane production during growth of methanogenic community on glass and clay spheres (bars indicate standard deviation, n=3).

TABLE 1. Specific growth rate (μ ± standard deviation, n=2) of methanogenic acetotrophic, methylaminotrophic and hydrogenotrophic bacteria on glass and clay beads

Support	Trophic group	Specific growth rate, μ (h^{-1})
Control (without support)	Acetotrophic	0.019 ± 0.0045
	Methylaminotrophic	0.045 ± 0.0089
	Hydrogenotrophic	0.046 ± 0.0091
Glass	Acetotrophic	0.022 ± 0.0038
	Methylaminotrophic	0.074 ± 0.0090 (*)
	Hydrogenotrophic	0.029 ± 0.0042
Clay	Acetotrophic	0.040 ± 0.008
	Methylaminotrophic	0.110 ± 0.015 (*)
	Hydrogenotrophic	0.090 ± 0.012 (*)

(*) $p \leq 0.05$.

slope of the curves of accumulated methane production in time suggests that clay fosters methanogenesis more than the systems with glass or without support.

The results agree with those of Muñoz et al. (13). These authors studied the effect of inert support material on methane production in reactors. However, few studies have been made on the kinetic parameters that characterize colonization and growth of different methanogenic trophic groups on inert support materials (16).

Growth on support materials. Growth rates ranged from 0.019 h^{-1} (acetotrophes growth without support material) to 0.11 h^{-1} (methylaminotrophs on clay). The highest growth rate occurred on clay, which showed a significant increase ($p \leq 0.05$) in the growth of methylotrophs and hydrogenothrophs. On glass beads, only methylaminotrophs showed an increase in their growth rate. However, it was lower than the growth rate of the same group on clay (Table 1).

Maximum counts at the beginning of the stationary phase are showed in Table 2. Hydrogenotrophs predominate on clay beads, followed by acetotrophs and methylaminotrophs, which represent 10% of total counts. Predominant

TABLE 2. Countings of adhered methanogenic bacteria, at the beginning of stationary phase during the growth of different trophic groups, on clay and glass beads

Trophic group	Resuspended cells survey (MPN/ml)		
	Without support	Glass	Clay
Acetotrophic	3.9×10^4	6.2×10^4(*)	3.4×10^6
	SD = 5.3×10^3	SD = 4×10^3	SD = 5.1×10^5
Methylaminotrophic	2.9×10^7(*)	2.3×10^5	1.2×10^6
	SD = 4.9×10^4	SD = 5.6×10^4	SD = 3.9×10^5
Hydrogenotrophic	2.2×10^8(*)	2.2×10^5	2.3×10^7(*)
	SD = 5.6×10^6	SD = 6.1×10^4	SD = 5.6×10^6

(*) significant increase (Anova, $p \leq 0.05$). SD: standard deviation.

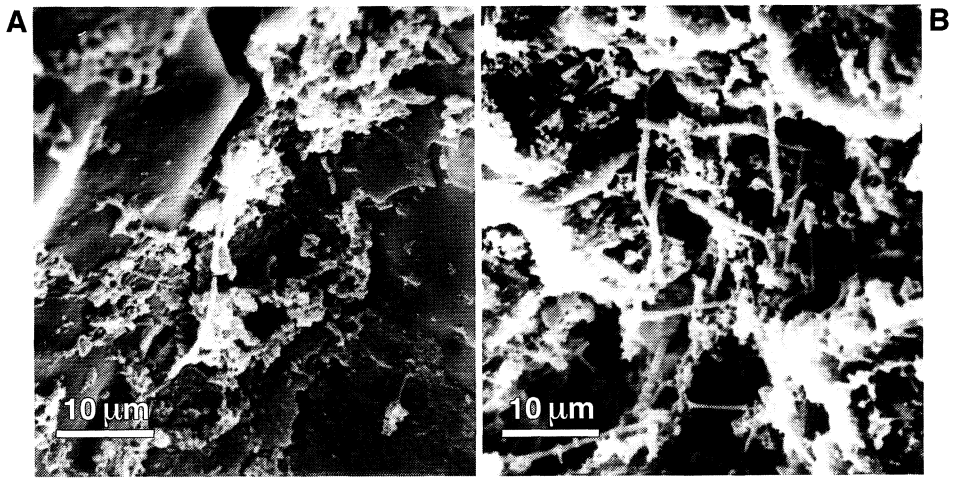

FIG. 2. Scanning electronic micrographs showing: (A) glass beads colonized by the methanogenic community, and (B) clay beads colonized by the methanogenic community, which presents *Methanosaeta* morphotypes (filaments). After 10-day incubation. Bars equal 10 µm.

groups on glass beads were methylaminotrophic and hydrogenotrophs, ten times higher than acetotrophs.The colonization of methanogenic bacteria depends on the support material characteristics (4, 9), on the adherence of the cell surface, and on the specific growth rates of each trophic group (19, 20, 21). For example, predominance of hydrogenotrophs and methylaminotrophs on glass and clay could be explained by their higher growth rate on those support materials (Table 1), which agrees with the results obtained previously for the same groups in liquid medium (24).

Methanogenic acetotrophic bacteria showed lower growth rates and lower relative abundance in biofilm (Tables 1 and 2). *Methanosaeta* morphotypes found were hydrophobic and had a filamentous morphology (Fig. 2), which helped to stabilize the biofilm (19, 21).

In systems with no support material, the relative distribution of methanogenic communities was similar to that found in systems with support. The absolute counts, however, were higher. This fact, along with the lower methanogenic activity in the control series, and results obtained on clay support, suggests that clay promotes specific methanogenic activity of the community.

Some authors have studied the efficiency of clay used as a support material for microorganisms in anaerobic digestion processes (13). Clay releases trace elements in the medium (14) and has a high specific surface (10). In previous studies using the same experimental system, SRB showed growth rate higher than methanogenic bacteria in systems without support materials. This was probably a consequence of their lower generation time and their higher affinity for acetate and hydrogen (lower K_m) (12). However, by using a support material its abundance decreases, especially in systems with clay beads. We can conclude that the use of clay beads as support material in discontinous systems favors the adherence of methylaminotrophic and acetotrophic methanogenic bacteria.

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MICROBIOLOGÍA SEM **13** (1997), 215–220 **PERSPECTIVES**

Gonzalo Vidal (1943–1997). A life for micropaleontology and Precambrian research

Malgorzata Moczydlowska-Vidal

Earth Sciences, Micropalaeontology, Uppsala University, Uppsala, Sweden

Gonzalo Vidal, Professor of Micropaleontology at Uppsala University, died on 10 January 1997 in Uppsala of a heart attack. His scientific interest over 25 years of research was devoted to Precambrian and Cambrian organic-walled protoctists. These ancient unicellular algal, cyanobacterial, bacterial mats, and other microfossils of unknown biologic affinities were collected from sedimentary rocks, ca. 1700–500 million-year old, in worldwide occurrences. His paleontological discoveries and interpretations helped to elucidate the paleobiology of microbiota, the early evolution of life, environmental changes and development of early ecosystems, and to understand the succession of bio- and geo-events by inferring the relative age and correlation of strata from widespread geologic sections.

Gonzalo Vidal was born on 29 May 1943 in Madrid, his native Spain. He was an engineering student at Madrid University for two years before moving to Sweden, where he graduated with the highest honours as Master of Science at the Geology Department, Lund University, in 1969. The academic degrees of "Filosofie licentiat" (former Swedish Ph. D.) in 1973 and "Filosofie doktorexamen" in 1979 were awarded to Gonzalo at Lund University for his dissertations on late Precambrian micropaleontology and biostratigraphy in Sweden and the North Atlantic region. The search for organic-walled Precambrian microfossils was a challenge at that time, not only in Scandinavia, and a real turn in biostratigraphy and in the study of the history of life. Research on Precambrian biota was still in its infancy, being pioneered by B.V. Timofeev, S.N. Naumova and N. A. Volkova (the "Russian school"), and E. S. Barghoorn, S. A. Tyler and J. W. Schopf (the "American school"). Not yet 20 papers had been published on the subject when Gonzalo defended his thesis in 1973. His strong geologic background and experience in geology, gained through mapping and prospecting in various areas, was advantageous in comparison to his predecessors. Soon he achieved also expertise in palynology and natural systematics by carrying out several projects on Mesozoic to recent pollen, and spore analyses. Due to

Correspondence to: Malgorzata Moczydlowska-Vidal. Earth Sciences. Micropalaeontology. Uppsala University. Norbyvägen, 22. S-752 36 Uppsala. Sweden. Tel.: +46-18-182743. Fax: +46-18-182749. E-mail: Malgo.vidal@pal.uu.se

FIG. 1. Gonzalo Vidal (1943–1997).

these experiences, Gonzalo undertook in his research, in the doctoral thesis and later on, two previous approaches to studying Precambrian microfossils: the application of informal taxonomy for biostratigraphy (Russian school), and an attempt of recognizing the natural systematic and biological affinities of microfossils based on morphological similarities to Modern biota (American school). His unconventional interpretations, which may have seemed controversial at the beginning, are in most cases up to date and are still inspiring. Gonzalo was a modest researcher who never followed the establishment nor sought recognition, but he enjoyed seeing that his ideas were accepted and responded. He had artistic abilities and always prepared his own drawings and photographs. Besides, he was technically innovative and designed and built his own devices to extract microfossils. Gonzalo combined his dynamic and comprehensive research, based on new findings from materials he collected himself, with academic activities: he lectured for undergraduate courses and supervised doctoral studies. He was appointed Professor of Micropaleontology by the Swedish Natural Science Research Council in 1988, and became head of the Micropaleontological Laboratory. In 1991 the laboratory moved to Uppsala University, and in 1994 Gonzalo became Professor at this university.

Gonzalo acquired new and fascinating insight into the natural world in the geologic past by meticulous microscopic studies in the lab and by observations in the field. Throughout the years he participated in field-work and expeditions in Australia, New Zealand, Antarctica, North America, Canada, Scandinavia, Siberia, China, Southern Urals, almost all European countries, and especially in Spain. He organized and led expeditions to Arctic Norway, Greenland, Siberia, and the Arctic Kola and Kanin Peninsulas. Gonzalo actively contributed to several international projects on bio- and chemostratigraphy, geological correlations, paleoecology and paleoenvironments. His wit and his profound knowledge, enabled him to make sense of apparently contradictory observations by proper reinterpretation. He spent a number of years as post-doctoral Research Fellow in New Zealand, Research Fellow and visiting professor of the University of California, Los Angeles (UCLA), exchange Research Fellow of the Royal Swedish Academy of Sciences in the former USSR, China and Poland, and Fulbright Senior Research Scholar and visiting professor at Harvard University. Since 1992 he was appointed "Catedrático del Programa Propio" by the Ministry of Education in Spain. Gonzalo proposed and co-organized the Nobel Symposium on "Early Life on Earth" in 1992. He was, however, most satis-

fied to be able to devote his professional time to research and teaching.

A close partnership with co-workers, guest-researchers and doctoral students and a friendly working atmosphere was felt in his laboratory. He was an enthusiast scientist and supportive colleague. Gonzalo is cordially remembered for his kindness, encouragement, and love for science. It was most rewarding to work with him.

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MICROBIOLOGÍA SEM **13** (1997), 221–226 **PERSPECTIVAS**

Hongos y biodiversidad: Iniciativas internacionales

David L. Hawksworth

International Mycological Institute, Egham, Surrey, United Kingdom

Abstract

Interest in biodiversity has spawned international actions both political and scientific, many of which are relevant to mycologists. The political initiatives include the Convention on Biological Diversity and Biodiversity Action Plans, and the scientific: the Global Biodiversity Assessment, Species 2000, Systematics Agenda 2000 International, BioNet International, Diversitas, All Taxa Biodiversity Inventory, and Biodiversity Methods Manuals. Mycologists need to contribute to such programmes, maintain an enhance profile, and remain flexible to respond to new challenges.

Key words: fungi, biodiversity, science, policy, research

Resumen

El interés por la biodiversidad ha producido iniciativas internacionales, políticas y científicas, que en muchos casos atañen a los micólogos. Entre las iniciativas políticas están la Convención sobre Diversidad Biológica y los Planes de Acción en Biodiversidad. Entre las científicas, proyectos como Evaluación de la Biodiversidad Global, Especies 2000, Agenda Sistemática Internacional 2000, BioNet Internacional, Diversitas, Inventario de Biodiversidad de Todos los Taxones y Manual de Métodos de Biodiversidad. Para los micólogos es esencial conocer las múltiples posibilidades de estos programas y participar en ellos.

Correspondencia: David L. Hawksworth. Internat. Mycological Institute. Bakeham Lane. Egham. Surrey TW20 9TY. U.K. Tel.: +44-1784-470111. Fax: +44-1784-470909. E-mail: D.HAWKSWORTH@CABI.ORG

Información previa

La importancia de la diversidad biológica en cuanto a especies, y en los aspectos genéticos y de ecosistemas, fue advertida por Elliot R. Norse y sus colegas (10, 24), a la vez que Thomas E. Lovejoy se refería a la *diversidad biológica* en el sentido más restrictivo de riqueza de especies (22, 23). El término *biodiversidad* apareció en 1985, cuando se dio un nombre al "National Forum on BioDiversity", que se celebraría en Washington, en septiembre de 1986 (30). Apareció por primera vez impreso en un informe sobre la exposición "Biodiversidad en peligro" (2), y en un trabajo de divulgación de Wilson (29), como equivalente a "sistemática en sentido amplio". Hoy, alrededor de 250 artículos por año llevan el término biodiversidad en el título (7).

En 1992 la Convención sobre Diversidad Biológica adoptó una definición, haciéndose eco de la propuesta por Norse: "variabilidad entre organismos vivos de cualquier procedencia, incluyendo *inter alia*, terrestres, marinos y otros sistemas acuáticos y los complejos ecológicos de los que forman parte; esto incluye diversidad intraespecífica, interespecífica y de ecosistemas".

Los tres niveles, designados biodiversidad genética, de organismos y ecológica por Harper y Hawksworth (10), incluyen la variación de todos los organismos de la jerarquía taxonómica y fue aceptado y elaborado en el programa Evaluación de la Biodiversidad Global (16).

La biodiversidad incluye todos los grupos de organismos, por lo tanto, aunque no se indique de forma explícita, los hongos están incluidos.

Iniciativas políticas

Convención sobre Diversidad Biológica. Se desarrolló en la Cumbre de Río en 1992 y fue ratificada por 152 países en julio de 1996, que adoptaron una serie de compromisos respecto a "la conservación de la diversidad biológica, el uso sostenible de sus componentes y el justo y equitativo reparto de los beneficios surgidos del uso de su biodiversidad indígena". La comunidad científica internacional está representada en los organismos que revisan y planifican su ejecución a través del International Council of Scientific Unions (ICSU), que incluye la International Mycological Association (IMA) y la International Union of Biological Sciences (IUBS).

La Convención, un complejo documento de 42 Artículos y dos Anexos, interesa a los micólogos ya que sus disposiciones se aplican a todos los organismos. El acceso a la información es esencial en el asesoramiento y seguimiento de la biodiversidad, especialmente en países menos desarrollados. Como parte de su mandato y para cumplir sus objetivos se ha creado un mecanismo cuya estructura se está discutiendo que comprenderá una serie de nodos de acceso cada uno de los cuales unirá las bases de datos de determinados temas, por ejemplo colecciones de recursos genéticos o bases de datos taxonómicos (p.e. Especies 2000, véase más adelante). Cada país comprometido con la Convención fija un nodo nacional que será el centro que recogerá y difundirá los datos. Las limitaciones al acceso de los recursos genéticos de un país, preocupa a los poseedores de las colecciones, y a las empresas farmacéuticas y de biotecnología, encargadas de la prospección (20, 21, 25). Hay que saber cómo estas restricciones pueden llegar a influir en la investigación biosistemática, incluyendo el acceso a colecciones tipo y cepas (13).

Se han establecido mecanismos para financiar el aumento de los costes de los diferentes países para cumplir las obligaciones contraídas en ésta y otras convenciones. La principal fuente de fondos, dotada en 1995 con 2100 millones de US$, es el Global Environment Facility (GEF),

bajo administración y control del Banco Mundial, y de las diferentes organizaciones de Naciones Unidas. Se destina a programas de países en vías de desarrollo y a un número limitado de iniciativas de beneficio global. La necesidad de que los proyectos del GEF sean interdisciplinarios asegura la incorporación de los hongos.

En muchos países desarrollados, agencias gubernamentales y voluntarias están respondiendo a las necesidades financieras. En el reino Unido podemos citar el Programa de Recursos Naturales Renovables, que depende de la Overseas Development Administrativa, y el proyecto Iniciativa Darwin, que depende del Departamento de Medio Ambiente.

Planes de Acción para la Biodiversidad. Son los que algunos países desarrollan como parte de sus compromisos con la Convención. El UK Biodiversity Action Plan fue fundado en1994 (3), y el Steering Group, constituido por recomendación del Plan, cuyo informe se publicó en 1995 (4). Este documento, detalla las acciones para preservar siete líquenes (*Buellia asterella*, *Caloplaca luteoalba*, *Collema dichotomum*, *Gyalecta ulmi*, *Pseudocyphellaria aurata*, *P. norvegica* y *Schismatomma graphidioides*) y otros cuatro hongos (*Battarraea phalloides*, *Boletus satanus*, *Poronia punctata* y *Tulostoma niveum*). Otros 35 hongos (incluyendo 30 líquenes) están previstos en planes de los próximos tres años. En 96 (incluyendo 77 líquenes) no se ha observado que estén globalmente amenazados o en declive. Los hongos (incluidos los líquenes) constituyen un 8.5% de las 1666 especies de todos los grupos de organismos catalogados por su especial interés.

En 1994 la Office of Science and Technology (OST) estableció la UK Systematics Forum para mejorar la coordinación de asuntos relacionados con la biosistemática de la nación, en referencia a las diferentes colecciones tipo. En su seno, sistemáticos ingleses han recopilado una detallada base de datos (27) y se ha encargado una revisión paritaria de las colecciones microbiológicas nacionales (31).

Iniciativas científicas

Evaluación de la Biodiversidad Global (GBA). Para disponer de una visión autorizada del estado actual de los conocimientos de todos los aspectos de la biodiversidad, se destinaron 3,3 millones de dólares de la GEF en el período 1993–95 (16). En el proyecto participaron 1500 científicos, de ellos 300 especialistas de 50 países, en sesiones de trabajo y revisiones. Los datos sobre hongos se han ido incorporando, dando lugar en algunos casos a otros textos generales. Dos hallazgos merecen especial mención:

(i) El número de especies aceptadas en la GBA es de 72.000, para las conocidas, y 1,5 millones para las estimadas en toda la Tierra. Estas cifras están en la misma línea que las del *Dictionary of the Fungi* (14) y en extrapolaciones de diferentes grupos de datos (11).

(ii) El número de científicos que describieron nuevas especies de diferentes grupos de organismos en 1992 era de 6989, de los cuales 498 describieron 1600 especies de hongos. A este ritmo, no se completará el inventario de los hongos del planeta hasta el año 2888, contando que ninguna especie se describa más de una vez. Suponiendo una tasa de sinonimia del 2,5 (14) la cifra sería de 4227. La GBA aporta valiosa documentación sobre biodiversidad para iniciativas políticas y científicas.

Especies 2000. Se encarga de elaborar un catálogo autorizado de los organismos conocidos del mundo (5). Servirá para unir electrónicamente listas de especies de diferentes grupos, con un localizador de nombres que incluirá todos los nombres científicos. Los datos

de nombres de hongos publicados están coordinados por IMI, y por el Laboratorio de Micología y Botánica Sistemática que depende del gobierno estadounidense, pero la producción de especies "master" para familias y órdenes particulares de hongos será un reto de mayor envergadura.

Agenda Sistemática Internacional 2000. Este programa complementa el de Especies 2000. Su objetivo es descubrir, describir y clasificar las especies del (26) mundo mediante tres misiones interrelacionadas:

(i) Descubrir, describir e inventariar la diversidad específica global. (ii) Analizar y sintetizar la información en un sistema de clasificación predictiva, que refleje la historia de la vida. (iii) Organizar la información de este programa global en una forma eficientemente recuperable.

BioNet-Internacional. Para paliar la escasez de expertos en biosistemas en países poco desarrollados, especialmente en los grupos con mayor riqueza en especies (bacterias, hongos, insectos y nematodos), se desarrollaron en 1992 redes regionales de especialistas y colecciones con ayuda de instituciones fuera de la región (19). Establecido oficialmente en 1993, tiene un secretariado técnico apoyado por el CAB International. Las redes se han ido estableciendo en el Caribe (CARINET), Europa (EuroLOOP), el Pacífico (PACINET), y Sudáfrica (SAFRINET). Está previsto realizar sesiones de trabajo en Kuala Lumpur (Malasia; ASEANET, para el suroeste de Asia), Benin (WAFRINET, para el oeste de África), Kampala (Uganda; EAFRINET, para el este de África) y Calcuta (SAARCNET, para el sur de Asia).

Diversitas. Marco conceptual de programas de biodiversidad (6), fue desarrollado por IUBS y el Scientific Committee on Problems of the Environment (SCOPE), y UNESCO en 1989. La primera fase de su actividad contribuyó a la creación del GBA, y a la creación de lo que sería el Comité IUBS/IUMS para la Diversidad Microbiana, y el concepto ATBI's (véase más adelante). El plan de trabajo incluye cinco programas principales y cuatro áreas especiales:

(i) Principales programas: Biodiversidad en el funcionamiento de los ecosistemas; Orígenes, mantenimiento y cambio de biodiversidad; Sistemática: Inventario y clasificación; Seguimiento de la biodiversidad; Conservación, restablecimiento y uso sostenible de la biodiversidad.

(ii) Áreas especiales de Investigación (STARS): Biodiversidad del suelo y del sedimento; Biodiversidad marina; Biodiversidad microbiana; Dimensiones humanas.

Los elementos sobre micología se incluirán en los programas apropiados, especialmente en los relacionados con la microbiología de suelos y sedimentos. El Comité IUBS/IUMS para la Diversidad Microbiana controla que el tratamiento que se da a los microorganismos en el programa Diversitas y en otros programas multidisciplinarios sea el adecuado. Aunque formalmente se estableció en 1994, tuvo sus orígenes en dos reuniones, en Amsterdam en 1991 con participación de D. Hawksworth y R. Colwell, y en Egham en 1993 (1); las actas de aquellos encuentros inciden en la importancia de los hongos para la biodiversidad.

Evaluar la extensión y papel del suelo y de la biodiversidad del sedimento presenta problemas específicos, por lo que se han establecido dos áreas STAR complementarias: (i) Desarrollo de metodologías integrativas para el tratamiento de los inventarios de una única muestra. Ya se ha completado una primera fase con una sinopsis de las técnicas en uso (9). (ii) Evaluación de los conocimientos sobre la diversidad en suelo y sedimento, en relación al funcionamiento de los ecosistemas.

Inventario de Biodiversidad de Todos los Taxones (ATBI). Un componente clave en la primera fase de Diversitas consistía en estudiar intensivamente un número limitado de emplaza-

mientos para lograr un inventario completo, en tanto que otros lugares se estudiarían extensivamente con métodos de extrapolación, calibrados con los datos de los lugares de estudios intensivos. A este respecto, y para tratar de la dificultad y costo de los estudios intensivos, sobre todo en las zonas tropicales, se celebró un encuentro de trabajo en Filadelfia en 1993 (18), y posteriormente otros en Costa Rica, donde también se incluyó uno para hongos. Se considera sobre todo su gran potencial en términos de gestión científica de la conservación y explotación (17).

Manual de Métodos de Biodiversidad. A medida que los diferentes países desarrollan programas de asesoramiento sobre los recursos propios se hace necesario disponer de manuales con claves y otros requisitos de identificación, adecuados para no especialistas, serán instrumentos muy necesarios. La propia Smithsonian Institution, de Washington, reconoció la necesidad de un conjunto de manuales que incluyan métodos estándar de medición y seguimiento de la diversidad biológica para todos los grupos. Un primer volumen, sobre reptiles, apareció en 1995; y hay otros en preparación. El número de hábitats donde es posible encontrar hongos es enorme, especialmente en bosques tropicales (15). Muchos de ellos requieren técnicas específicas para estudiarlos en profundidad, por lo que dos volúmenes de la serie de Smithsonian, a cargo de micólogos de todo el mundo, estarán dedicados a los hongos.

Contribución de los micólogos

Las aportaciones de los micólogos a estas iniciativas han permitido valorar la diversidad de los hongos y su importancia en el mantenimiento de los ecosistemas. Así se reconoce en el resumen de Evaluación Global de la Biodiversidad (28); que proporciona una nueva visión útil para los científicos y gestores de la política científica que no se tuvo en la década de 1980. Aunque lo conseguido es satisfactorio, es preciso seguir avanzando sobre lo ya edificado para mantener y aumentar los niveles de concienciación y dedicación a esta tarea (12).

Es necesario igualmente mantener una actitud flexible para responder a nuevos retos científicos y políticos, lo cual significa operar cada vez más de forma interdisciplinaria. Además, hay que ser sensibles hacia la labor de las agencias financiadoras y a los países menos desarrollados. Es de desear que se adapten las prioridades y prácticas de trabajo a fin de que se tienda más a colaborar que a competir. La exploración de la biodiversidad en los hongos en sus tres niveles contribuirá tanto a la creación de riqueza como a la salud del medio ambiente, pilares de la actual política científica (8).

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MICROBIOLOGÍA SEM **13** (1997), 227–228 **OPINION**

Macrolife and beyond

Robert D. Richards

International Space University (ISU), Founder and Trustee

Everywhere we look we find the building blocks of life. From the hearts of ancient meteorites to the vast interstellar nebulae, the tendency of the universe towards intelligence is manifest. The evidence of extraterrestrial life discovered recently in an ancient Martian meteorite grabbed headlines the world over and stirred up my long-held interest in the universality of life. The evolution of life is a powerful idea, one that has been the basis of conflicts between science and religion since the Beagle set sail. We know that, in at least one case, life has evolved the faculty of reason, the ability to ponder itself and the universe from which it evolved. If under the right conditions life is pandemic and intelligence is a universal survival skill, perhaps we humans are not alone, perhaps there are others. People in general like to believe this—at least long enough to get a thrill from beating back the attacking aliens in the latest sci-fi thriller.

But if life is actually a property of nature, a fundamental but unquantified force in the universe, is it possible that we humans, the apex of the terrestrial evolutionary chain, are part of a larger process that by its enormity evades our awareness? Could this perspective help us understand why we venture into space?

Life is typically defined as something that has molecular complexity, metabolizes, and reproduces. A rock is not alive. A plant is. Humans are obviously alive; we eat, give off objectionable waste products, and make baby humans. But the human body itself is a collection of symbiotic living organisms, not all of them alike, each with a purpose that serves both the individual organism and the body as a whole. From this perspective the replication of human life can be viewed as the proliferation of much simpler life-forms, all imbued with an innate desire to survive.

Beneath the awareness of our consciousness, our bodies host a universe of competition, cooperation and survival among inde-

Correspondence to: Robert D. Richards. c/o "Severn Oaks". South River Road. P. O. Box 353. Washago, Ontario. Canada L0K 2B0. Tel. & Fax: +1-416-4102300. E-mail: richards@isu.isunet.edu

pendent but interconnected life-forms. An advanced electronic network enables communication between the life forms and a central processor coordinates the activities. As a cooperative group, these life-forms are able to manipulate their environment and effect change in the world around them to the benefit of the overall organism, which itself is considered "alive". As communication technology advances, so does the ability of the human species to coordinate its activities. Large groups communicating instantaneously across the planet can affect large changes. The evolution of the telephone, the Internet and other advanced communications system could be viewed as analogous to the evolution of the human nervous system, creating a super-organism, one capable of manipulating its environment with planetary, or extra-planetary, repercussions.

Of course human beings do not live in a vacuum or independently from other life-forms. Humans are dependent on a complex biosphere and interactions with other planetary organisms. In this sense could the Earth be viewed as a symbiotic superorganism in and of itself? Could the Earth be considered "alive"? This is sometimes referred to as the Gaia Theory. From this perspective, the Earth is a self-contained complex organism that eats solar energy and gives off waste products, so it metabolizes. But does it reproduce? Aha! Space colonies. Little Earth's created in its own image by interconnected organisms evolved from its biosphere. Or is it the solar system that is actualy "alive"...?

Well, the idea is clear. It is called Macrolife —a term coined in the 1960's by Dandridge Cole and popularized by George Zebrowski's novel of the same name. Within this stratum of thinking, space exploration is a natural instinct of planetary life to reproduce, intelligence the faculty that enables it, and us humans the contemporary lucky ones met with the challenge. With space colonization we may certainly be perpetuating our species, but we are also perpetuating every species that we are dependent on to live. In this respect space colonies are really the seeds of mother Earth; the propagation of the planetary DNA library. And we thought we were so smart...

If we look at humanity today with a set of Macrolife eyes our record would be dubious. Are we symbiotic organisms or contaminants? The degree of bio-degradation on Earth is a subject of hot debate, but certainly we can all agree that the planet is not happy. There are open wounds that can be seen from space. Physical evidence of humanity was not easily observable from orbit until recently, now any untrained eye can see the effects of devastated rain forests, desertification and large-scale industrial and civilian pollution. The industrialization of third-world countries is sure to magnify these problems dramatically, perhaps catastrophically, as they strive to emulate the economies of industrialized consumer societies.

But nature always balances its books. Life itself seems to be anti-entropy: organize, procreate, survive. Nature's way of balancing the tendency of non-life to follow the second law of thermodynamics: slow down, give up, die. If we are contaminants you can be sure the planetary anti-bodies will kick in. Perhaps the anti-bodies are microscopic, pre-wired viral countermeasures that attack the uncontrolled proliferation of any one species, dooming it to plague and pestilence. From a macroscopic perspective, these anti-bodies may well be extraterrestrial in nature —a 10 kilometer asteroid impacting the Earth at many kilometers per second can be an effective biological cleanser. Ask any dinosaur.

Space colonization is not a bad job to be entrusted with, if we humans do it properly. Most of us at the ISU like the idea immensely. But what if we screw up? Is it possible to get fired in the Macrolife universe?

MICROBIOLOGÍA SEM **13** (1997), 229–236 **OPINIÓN**

Internet, nueva fuente de información

Mercè Piqueras

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Introducción histórica

Internet es un medio de comunicación global surgido de la aplicación conjunta de la informática, la electrónica y de otros medios de comunicación existentes con anterioridad. Aunque su gran desarrollo es bastante reciente, Internet nació hace más de treinta años en los Estados Unidos a partir de un proyecto estratégico del Departamento de Defensa de aquel país. En plena guerra fría, el gobierno estadounidense necesitaba disponer de una red de comunicación que permitiese establecer contacto entre el ejército y las autoridades locales, de cada uno de los estados, y federales. Su control no estaría centralizado en un lugar concreto, para que no pudiese ser el blanco de un misil enemigo; debería seguir funcionando aunque fallase una parte de sus componentes. Su principal artífice fue J. C. R. Licklider, ingeniero del Departamento de Defensa, que vio la posibilidad de los computadores como aparatos de comunicación, además de su función como máquinas de calcular (7). En 1964 se estableció la primera red de ese tipo (17). En el desarrollo inicial del proyecto intervinieron el Massachusetts Institute for Technology (MIT) y la Universidad de California en Los Angeles (UCLA) (19). Posteriormente se decidió que los nodos de la red fuesen supercomputadores de alta velocidad, conocidos como IMP ("interface message processors"). El primero quedó establecido en septiembre de 1969, en la UCLA. Este acontecimiento se considera el nacimiento de Internet, aunque no se le diese este nombre hasta unos diez años más tarde (2). A finales de aquel mismo año se habían constituido cuatro nodos de una red a la que se denominó ARPANET (Defense **A**dvanced **R**esearch **P**rojects **A**gency **Net**). De su función estratégica inicial, pasó a ser un conjunto de superordenadores que podían ser utilizados a distancia por investigadores de diferentes centros. Y empezó su crecimiento; de los cuatro nodos iniciales pasó a tener quince en 1971, y treinta y siete en 1972.

Pronto se dio una nueva orientación a la red. Muchos de sus usuarios empezaron a uti-

Correspondencia: Mercè Piqueras. Revista *Microbiología SEM*. Apartado 16009. 08080 Barcelona. España. Tel.: +34-3-4482373. Fax.: +34-3-3341079. E-mail: mpiq@bcn.servicom.es

lizarla como un medio de comunicación personal mucho más rápido que el correo. ARPANET evolucionó siguiendo los pasos a los avances tecnológicos. En 1983 se segregó el sector militar de la red, que creó MILNET para usos exclusivamente estratégicos. Basadas en el mismo principio, habían surgido otras redes en el seno de diferentes grupos sociales y económicos. Por ejemplo, USENET (UNIX Users Group Network), que empezó a funcionar en 1979, o BITNET (aunque quizás sea una broma, M. Hauben y R. Hauben (8) dicen que es el acrónimo de "**b**ecause **i**t's **t**ime **net**work"), establecida en 1981 para conectar ordenadores de IBM con centros de datos de universidades (14). En 1984 la National Science Foundation (NSF) entró en escena, añadiendo más cables y conexiones, que incorporaron a la red ordenadores cada vez más potentes y rápidos. La NSF utilizaba un "software" de conexión de dominio público que se conoce por las siglas TCP/IP. Este "software" convierte los mensajes en "paquetes" de información en el nodo de origen (TCP, "Transmission Control Protocol") y reúne los distintos paquetes que componen un mensaje en el nodo de destino (IP, "Internet Protocol"). La adopción de una misma tecnología básica por las distintas redes telemáticas establecidas permitió su interconexión.

El sistema de red se estableció para evitar el gasto innecesario de llamadas telefónicas de larga distancia que hubiese representado la distribución radial, con todos los usuarios conectándose directamente a los supercomputadores. De ese modo nacieron las redes regionales, en las que los usuarios se conectaban al nodo más próximo de la red, que a su vez estaba conectado con los nodos vecinos. Cada cadena así establecida enlazaba con un centro de supercomputación y los distintos centros estaban conectados entre sí. Con esa configuración era posible la comunicación entre dos ordenadores muy alejados, pasando la información a través de otros intermedios. Esta solución tuvo tanto éxito que terminó por colapsar el sistema. En 1987 empezó a reemplazarse la red por líneas telefónicas unas veinte veces más rápidas y se instalaron ordenadores también más potentes.

El aspecto más positivo de la entrada de la NSF en Internet fue la posibilidad de acceso para muchas personas. Esa entidad creó subvenciones para establecer la conexión a la red en los campus universitarios que presentasen proyectos para expandir el acceso a la misma. Terminó lo que había sido un privilegio del sector informático, del funcionariado y de empresas que establecían contratos con el gobierno (9). Por otra parte, el mínimo o nulo costo de conexión animó a muchas personas a conectarse a un nodo u otro de la red. Internet se comercializó, especialmente a partir de la difusión de la "Web" o WWW (World Wide Web), inventada en 1989 por Tim Berners-Lee, físico del CERN de Ginebra (1). La "Web" permite el acceso a páginas multimedia, es decir, documentos que pueden incorporar gráficos, sonido y vídeo al texto, y que enlazan dichas páginas con otras localizadas en otros ordenadores por medio del llamado "hipertexto". El tipo de personas que utilizan Internet se diversificó con la integración al sistema de las redes comerciales, cada vez más abundantes.

Paulatinamente otras instituciones gubernamentales estadounidenses se adhirieron al proyecto: NASA, los National Institutes of Health (NIH), el Departamento de Energía, etc. En 1989, ARPANET, como tal, desapareció engullida por el monstruo de la comunicación que ella misma había creado. Se inició entonces la gran expansión internacional de la red, conocida ya como Internet. A finales de 1991 se había extendido por más de 30 países, integrando unas 5000 redes, que eran utilizadas por unos cuatro millones de personas. Ese número ha crecido vertigi-

nosamente y en 1996 se calculaba que el número de usuarios superaba los veinte millones, con un crecimiento aproximado del 10% mensual.

Internet como fuente de información

Aunque Internet ofrece diferentes tipos de servicios, vamos a considerarla únicamente como fuente de información científica, a través de los documentos y bases de datos a los que permite acceder. Las principales revistas científicas tradicionales, sin abandonar su versión impresa en papel, están adoptando gradualmente la versión digital. En algunos casos, incluyen sólo una parte de la información que contiene la publicación y los índices o resúmenes de los artículos de cada número. Entre aquellas cuya publicación no parte de un planteamiento comercial, las hay que ofrecen el mismo contenido que en su versión en papel. *Microbiología SEM*, además de la tradicional versión impresa, publica desde hace un año la versión "on line", que incluye todos los artículos completos.

En los últimos años han surgido revistas que no disponen de otra versión que la digital, "publicada" en Internet. Mencionaremos *Neuroscience-Net*, que apareció en enero de 1996 y cuenta con prestigiosos investigadores en su comité editorial, y *Conservation Ecology*, que depende de la Ecological Society of America (1). Esta última iniciará su publicación el 15 de junio de 1997, y dispone ya de página web, que el 6 de mayo de 1997 había recibido 62.182 "visitas" (consultas). En la misma fecha, el número de "suscripciones" era de 3399. *Conservation Ecology* acepta artículos de ecología teórica y aplicada, tales como fundamentos ecológicos para el mantenimiento de ecosistemas, paisajes, especies, poblaciones y diversidad genética, así como la administración de recursos naturales. Según escribía su director-coordinador, C. S. Holling, en la conocida revista *Science* (de la American Association for the Advancement of Sciences, AAAS), unos meses antes de lanzar al ciberespacio el primer número de *Conservation Ecology*, se había conseguido la semiautomatización de la presentación y revisión de artículos, mediante el empleo de un sistema electrónico que realiza muchas de las funciones de secretaría. Según Holling, el sistema es genérico y puede ser empleado por cualquier persona que desee crear una publicación electrónica (10).

Universidades e instituciones científicas ofrecen también información sobre el trabajo de sus departamentos o de sus miembros. Bastantes científicos tienen incluso "páginas" propias en la red, en las cuales incluyen el resultado de su propia investigación o, por medio del hipertexto, remiten a otras páginas de Internet que contienen información relacionada con la que ellos exponen. De todo ello resulta un ingente caudal de información disponible para cualquier persona que tenga acceso a Internet.

Excepto en los casos en que se conoce la "dirección" exacta o URL ("Uniform Resource Locator") del lugar que se quiere consultar, la mejor manera de obtener información sobre un tema determinado es recurriendo a los programas buscadores ("browsers"), que suelen ser de varios tipos. En algunos, la búsqueda es temática; a partir de un listado general se accede a sublistados cada vez más específicos. Otros, ofrecen la posibilidad de realizar la búsqueda a partir de algunas palabras clave sobre el tema del cual se desea obtener información. Sin embargo, la introducción de unas pocas palabras sobre el tema en el buscador puede conducir a la obtención de varios miles de posibles puntos de consulta en la red, que en su mayor parte serán irrelevantes para nuestros fines. La experiencia enseña a reducir esas posibilidades y a delimitar cada vez más el campo de búsqueda.Un buscador muy práctico es Altavista:

<http://altavista.digital.com>, para usuarios en América.

<http://altavista.telia.com>, para usuarios europeos. Desde febrero de 1997, España cuenta con un "mirror" en castellano:

<http://altavista.telia.com/es>.

Altavista construye índices, buscando la información a medida del usuario (12), y destaca por su rapidez. Además, el listado de resultados que ofrece, lleva incorporado el URL de cada punto de información. Ello permite poder conservarlo (imprimiéndolo o guardándolo en el propio ordenador) para acceder a la página donde se encuentra la información en el momento más oportuno.

Fiabilidad de la información

Una vez obtenida la información deseada, se plantea un problema: cómo saber si es fiable. Cuando se trata de una publicación impresa, existen maneras de determinar su fiabilidad. Su inclusión en listados internacionales suele ser una garantía, lo cual no significa que haya que restar credibilidad a las publicaciones que no están en los mencionados índices. También garantiza la calidad de un artículo científico la revisión paritaria ("peer review"). Actualmente existen en Internet publicaciones digitales, como la mencionada *Conservation Ecology,* que han incorporado el proceso de revisión previa a su "publicación". De todos modos, y dada la complejidad de la red, es más frecuente hallar información que no corresponde a ningún tipo de publicación. Habrá que evaluar la credibilidad de la fuente en cada caso, ya que la propia estructura de la red hace de ella un arma de doble filo; cualquier persona con acceso a Internet puede crear su propia página e introducir allí cualquier dato, sea cierto o no.

Si la información se ha localizado en el servidor de una institución, es conveniente acudir a la página principal ("home page") y comprobar de qué entidad se trata. El que sea una universidad no es, sin embargo, una garantía. En Estados Unidos se encuentran las mejores universidades de los países occidentales, pero también las peores. Tampoco hay que fiarse de entidades que en su nombre incluyan palabras como "investigación", "instituto", "centro de estudios", etc. Por ejemplo, en El Cajón (California) radica un "Institute for Creation Research" que realiza "investigación" para demostrar que el origen de la vida fue el resultado de la creación divina, tal como narra la Biblia. Esta institución tiene una web en Internet, en la que pueden consultarse muchos artículos pretendidamente científicos. Los impulsores y los acólitos de las paraciencias han encontrado en la red un medio ideal para difundir sus ideas y productos. La publicación electrónica *Homeopathy Online* es otro ejemplo de ello. Incluye enlaces con numerosas páginas sobre homeopatía y otras "medicinas" alternativas, tanto humanas como veterinarias, información bibliográfica, listas de distribución, grupos de discusión, servicios de suscripción a organizaciones, etc. Refiere también a páginas comerciales, donde pueden encontrarse anuncios de curanderos y adquirirse "medicamentos" homeopáticos. Cabe destacar que la página principal de *Homeopathy Online* ha recibido 54.463 visitas desde el 16 de mayo de 1996 hasta el 1 de marzo de 1997, y que uno de los recursos (MedWeb) a los que puede llegarse por el hipertexto permite a su vez el acceso a otros recursos localizados en un centenar de países, aproximadamente. El buscador Olé, en castellano, tiene un listado de las mencionadas medicinas alternativas que comprende desde profesionales inscritos en colegios oficiales hasta curanderos y adivinadores; incluso enlaza con la web de una secta, como es la iglesia de la cienciología.

Cuando la búsqueda de información científica lleve a una página individual, hay que extre-

mar la cautela. En primer lugar, es conveniente comprobar si la página principal proporciona información sobre el autor. Si así fuera, deberá ponderarse si aquella información es suficiente. Puede utilizarse la conexión con otras páginas de la misma persona, para ver si demuestran conocimientos y experiencia en el tema. En determinados casos será aconsejable buscar referencias sobre el investigador incluso fuera de la red. Naturalmente, si se trata de un tema muy general, es posible que el nombe del autor no nos sea familiar. En cambio, cuando se trate de un área científica en la que trabaja quien realiza la búsqueda, si el nombre no resulta conocido, es aconsejable pedir la opinión de terceras personas, tanto sobre el investigador como sobre el trabajo publicado en Internet.

En un momento en que la revisión paritaria está en entredicho (15), quizá la publicación de los resultados de la propia investigación en Internet, sin revisión previa, sea una manera de probar la sensatez de las voces que se alzan contra esta selección, tan poco natural. Sería la propia comunidad científica la que, con sus citaciones, daría validez a los resultados distribuidos en la red.

Propiedad intelectual e Internet

Actualmente el concepto de propiedad intelectual parece estar desprestigiado, especialmente cuando se trata de reproducir información disponible en formato electrónico. Por una parte, hay quien propugna que el conocimiento, al ser un concepto abstracto, no puede convertirse en propiedad de nadie. Cuando alguien aumenta sus conocimientos incorporando información a su bagaje intelectual, dicha información sigue estando a disposición de cualquier persona que acuda a la misma fuente, a diferencia de un bien físico que cuando pasa a ser propiedad de alguien deja de estar disponible para otras personas. Sin embargo, el conocimiento suele ser el resultado de un trabajo de investigación, que puede haber resultado laborioso, e incluso costoso desde el punto de vista económico. Parece, pues, natural considerarlo una propiedad e intentar protegerlo poniendo un precio al acceso al mismo. Según Harry H. Chartrand (3), hay dos maneras de proteger el conocimiento. Una, que suele encontrarse en todas las culturas, es mediante el secreto. En sociedades primitivas el secreto guarda conocimientos sólo accesibles a unas pocas personas privilegiadas en cada generación, y con frecuencia está relacionado con la religión. En las sociedades modernas, el secreto está más bien relacionado con actividades comerciales o políticas. La otra manera de preservar el conocimiento se basa en leyes que protegen la propiedad intelectual; es más propia de sociedades avanzadas y comprende, entre otras, el "copyright", patentes, registros, y acuerdos entre distintas partes.

Las características de los documentos digitales les hacen muy vulnerables. Cualquier cosa que pueda ser reducida a bits puede ser copiada (16). Se ha propuesto que la red sea un banco de datos accesible a todo el mundo, pero la dificultad en controlar dónde va a parar la información ha provocado reticencias en autores y editores, que prefieren reservar su mejor producción para el soporte tradicional en papel. Sin embargo, la tecnología nos brinda ya "software" y "hardware" que permiten especificar los términos y condiciones legales del copiado de trabajos digitales, así como controlar el uso que se haga del material. A la larga esto repercutirá en beneficio del usuario, ya que una mayor protección de los documentos estimulará a los autores a publicar en Internet. Y aunque terminemos pagando una pequeña cantidad por "apoderarnos" de la información procedente de la red, siempre costará menos que obtenerla en cualquier otro soporte.

Separatas electrónicas

Un primer paso hacia la publicación exclusivamente en Internet, que hay quien prevé para un futuro no muy lejano, lo constituyen las separatas electrónicas, que ya han sido incorporadas por algunas revistas. La posibilidad de distribuir las separatas a través de Internet ahorra tiempo y dinero; evita la impresión de un determinado número de ejemplares y la persona que las solicita las recibe de manera inmediata. Actualmente se dispone del "software" necesario para montar las separatas en Internet a un coste reducido. De la misma manera que algunas publicaciones obligan a los autores a comprar un determinado número de separatas impresas, podrían "venderles" el montaje de las mismas en Internet. Una de las primeras revistas científicas que han introducido esta modalidad es *Florida Entomologica*, que se publica, sobre papel, desde 1917. Sus artículos se encuentran en la WWW como archivos PDF ("portable document format"), que reproducen las páginas de la revista con el mismo formato que la versión impresa. Dichos archivos pueden verse, copiarse o imprimirse con una aplicación de lectura (Adobe Acrobat) que es de distribución gratuita, y que también puede obtenerse en Internet. La calidad del producto final es la de una buena fotocopia del artículo original (18).

La publicación electrónica se ve como una solución a los costos cada vez mayores de edición y distribución de las revistas tradicionales. De todos modos, y aunque los gastos de publicación sean más reducidos, alguien deberá pagarlos. Habrá que hallar la manera de compensar la falta de suscripciones institucionales y de bibliotecas. Una posibilidad sería la creación de suscripciones virtuales, que, por otra parte, quizás ya existan. Otro recurso podría ser cobrar una cantidad determinada por cada separata electrónica que se solicite.

Recursos sobre microbiología en Internet

Internet es la mayor base de datos que haya existido nunca. En la WWW puede encontrarse información casi sobre cualquier tema, desde el más intrascendente hasta el más elevado. Al tiempo que crece el número de personas conectadas a la red, aumenta también el número de webs que se crean. Según datos de la revista *CompuServe*, publicada por el mayor proveedor comercial de Internet, el número de páginas Web aumenta mensualmente un 20% (11). En cuanto a la microbiología, los investigadores pueden hallar archivos de bibliotecas virtuales, con los índices de centenares de revistas e información sobre millares de libros; accesos a bancos de datos genéticos; descripciones de colecciones de cultivos tipo (13); páginas de sociedades microbiológicas con información de sus actividades; páginas creadas por departamentos universitarios o por los propios investigadores, describiendo la investigación que llevan a cabo; información sobre congresos, cursos y conferencias, o sobre becas y subvenciones; tiendas virtuales donde es posible comprar todo tipo de material y aparatos de laboratorio; grupos de distribución donde se puede discutir con colegas de todo el mundo temas de microbiología. Creemos innecesario referir el URL de las principales páginas cuya consulta puede interesar en un momento u otro a un microbiólogo. Es posible que, cuando se necesite acudir a ellas, hayan cambiado de localización. De todos modos, la propia interactividad de la WWW y la eficiencia y rapidez de los buscadores permiten hallar fácilmente lo que interesa si quien busca aplica el criterio adecuado en cada caso.

Los recursos "online" han llegado también a los libros de texto de microbiología. La 8ª edición del ya clásico *Brock Biology of Microorganisms* (véase la revisión en *Microbiología SEM* **12**, 671–672), incluye la posibilidad de

obtener información adicional a cada capítulo; para ello hay que acudir a la web del propio libro, y desde allí el hipertexto remite a las páginas del WWW adecuadas en cada caso.

Referencias en Internet

Todo artículo científico requiere la documentación necesaria que acredite las fuentes de información utilizadas para escribirlo. En general, hay que documentar la información procedente del trabajo de otra persona. Deben indicarse las citas directas de otros autores contenidas en el texto, resúmenes, información e ideas que proceden de textos publicados anteriormente. Además, debe incluirse la referencia que permita a quien lea el artículo localizar la fuente original (4). Las revistas científicas incluyen en las instrucciones a los autores de artículos unas normas sobre la disposición de las referencias, que sirven para mantener una uniformidad en la publicación. Pero dichas normas no pueden aplicarse de la misma manera en el caso de información conseguida en otros medios. La mayoría de publicaciones están incorporando instrucciones que permitan uniformizar el listado de referencias procedentes de documentos de Internet, así como de publicaciones en otros soportes electrónicos, como CD-ROM. Las diferentes asociaciones de editores trabajan también en ello y algunas (Council of Biological Editors [CBE], Modern Languages Association [MLA], Alliance for Computer and Writing [ACW], etc.) le han prestado al tema especial atención (6).

Hay que tener en cuenta que la publicación en Internet presenta algunas diferencias en relación a la publicación impresa, además de hacerse en un soporte distinto. En primer lugar, cabe la posibilidad de introducir cambios. Es posible actualizar un texto y corregir posibles errores, ya sean tipográficos, ya de concepto o comprensión. En muchos casos los artículos hacen constar visiblemente la fecha de creación y la fecha de actualización, si ésta se ha producido. Al anotar la referencia será necesario tenerlas ambas en cuenta. Si no constasen, deberá tomarse como referencia la fecha en que se ha encontrado el documento. Como ha ocurrido con las referencias de publicaciones impresas, es posible que no se alcance un acuerdo sobre la manera de citar documentos electrónicos. Sin embargo, tanto en un caso como en el otro existe un consenso sobre los datos básicos que debe contener una referencia. En el caso de la citas de documentos obtenidos en Internet habrá que hacer constar: *a*) el autor y el título del texto referenciado; *b*) el nombre o la descripción de dicho texto; *c*) el tipo de documento (WWW, Gopher, etc.); *d*) ciudad de publicación, si es posible determinarla; *e*) editor ("publisher"); *f*) fecha de publicación o, en su defecto, de acceso a la información; *g*) señas de identidad del documento (número de identificación o camino de acceso para llegar hasta el material) (5). Cuando el documento corresponda a una publicación que existe también en versión impresa, conviene obtener una separata, o consultar la revista en una biblioteca y citar la referencia del artículo en su versión "real", en vez de la "virtual". En cualquier caso, el URL no debe reemplazar nunca el nombre de la publicación y del editor, ya que los archivos que se encuentran en Internet pueden cambiar de lugar, en cuyo caso el URL deja de tener sentido. Por ello hay quien cuestiona el valor de las referencias en Internet. Si la página referida no pertenece a una entidad oficial, o de solvencia reconocida, es muy difícil probar la falsedad de la referencia después de un cierto tiempo.

En general, las revistas de mayor impacto aún no incluyen en sus normas para la preparación de artículos ninguna que haga referencia a la cita de material publicado en soporte electrónico. Es probable que, a medida que vaya afian-

zándose la publicación "online", se fijen unos criterios para citar dichas referencias. Ya que es un terreno aún nuevo, esperamos que las diferentes asociaciones de editores y las propias revistas aprueben unas normas uniformes. De todos modos, en tanto no se alcance un acuerdo, las publicaciones deberían adoptar normas provisionales que orientasen a los autores para conseguir, como mínimo, una uniformidad interna en ese tipo de referencias.

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El contenido completo de los últimos números de *Microbiología SEM* puede encontrarse en Internet en la siguiente dirección (URL):

http://morgat.udg.es/microbsem

MICROBIOLOGÍA SEM **13** (1997), 237–242 **OPINIÓN**

Una vela en la oscuridad*

Ricard Guerrero

Departamento de Microbiología, Universidad de Barcelona, Barcelona, España

El 20 de diciembre de 1996 fallecía en Seattle Carl Sagan, astrónomo, profesor de universidad, escritor y uno de los más famosos divulgadores científicos del siglo xx. Sagan, cuyo mantra de "billones y billones de estrellas" se ha convertido en el símbolo de la vastedad del universo, murió en el solsticio de invierno, cuando en el hemisferio norte podemos ver durante más tiempo el pálido brillo de los astros. Pocos meses antes de su muerte había publicado *The Demon-Haunted World: Science as a Candle in the Dark*, su último libro (4).La segunda parte del título está tomada de un proverbio inglés que dice que, cuando se está a oscuras, es mejor encender una vela que proferir una maldición. Cabe preguntarse si la ciencia es realmente una luz que nos permite distinguir lo que es real de la confusión reinante en las tinieblas que dominan nuestra sociedad.

La presión por la información

Los medios de comunicación tienen como fuente de información, para difundir el conocimiento científico entre la población, a los propios investigadores que publican en revistas especializadas. *Science* y *Nature* son punto de referencia para divulgadores y periodistas. Esas revistas facilitan los "press releases", o comunicados de prensa (5). Son resúmenes de las principales noticias de cada número, que se envían a los periodistas abonados a ese servicio con antelación a la aparición de la revista, con el compromiso de no difundir el contenido hasta que haya salido la revista. La información está "embargada" en el tiempo.

Hay periodistas que antes de publicar una noticia científica acuden al especialista en busca de asesoramiento. Pero es difícil opinar sobre una investigación que se desconoce, especialmente cuando los resultados se apartan de los obtenidos anteriormente, o cuando suponen un descubrimiento o innovación. Siendo estricto, cualquier artículo que revoluciona un aspecto de la ciencia será rechazado inicialmente por las revistas a donde se envíe. Así ha ocurrido en muchas ocasiones.

La publicación de una noticia científica depende más de decisiones dentro de los medios de comunicación que del significado de la investigación o el hallazgo. El periodista ve limitado su campo de acción para determinar

* Resumen de un artículo publicado en la revista *Quark. Ciencia, Medicina, Comunicación y Cultura*, Barcelona, abril-junio 1997, **7**, 29–40.

Correspondencia: Ricard Guerrero. Departamento de Microbiología. Universidad de Barcelona. 08028 Barcelona. España. Tel.: +34-3-4021484. Fax: +34-3-4110592. E-mail: guerrero@bcn.servicom.es

qué noticia científica debe ser publicada, principalmente porque en la prensa o en la televisión el sensacionalismo prima sobre la rigurosidad. Un ejemplo de esto fue el anuncio de la posible existencia de vida en Marte, a partir del examen de un meteorito caído en la Tierra hace 13.000 años. La noticia fue difundida por un periódico que no respetó el embargo de *Science* varios días antes de que se pudiera leer el artículo completo, que fue publicado el 16 de agosto (3). No fue la única vez que un medio de comunciación rompía su compromiso. En junio de 1995, *USA Today* tampoco respetó el acuerdo y publicó antes de tiempo el descubrimiento de las mutaciones en un gen que podrían ser la causa de la aparición prematura de la enfermedad de Alzheimer. No hace mucho, un periódico británico publicó la famosa noticia de la obtención de una oveja clónica, anticipándose a la aparición del artículo (6).

Valores éticos en la investigación, en la información y en la aplicación

Según Noëlle Lenoir, presidenta del Comité de Bioética de UNESCO, la práctica científica debe regirse por cuatro principios: respeto a la dignidad y a la libertad; prevención de los riesgos tecnológicos de los que depende el futuro de la humanidad; preservación de la libertad de creación científica; y solidaridad intelectual y moral, que permita que las ventajas del progreso beneficien a toda la humanidad.

Las posibilidades de las nuevas tecnologías hace que, por delante de la ética individual, se pida la creación de leyes que proporcionen un marco legal de actuación. Habrá que decidir sobre la conservación y selección de embriones para concepciones posteriores, el control de la descendencia, la corrección de alteraciones genéticas, etc. Todo esto recuerda demasiado el infeliz *Mundo Feliz* de Huxley, en la ficción, y las desgraciadamente auténticas prácticas de la época nazi. La polémica continuará y aumentará ante un tema con tantas connotaciones éticas, morales, políticas y económicas, pero tan retadora para la inteligencia y la capacidad humanas.

Pero, además, hay una conducta ética que es razonable exigir de los organismos e instituciones, y es la de creación de un sistema de reconocimiento fidedigno de la calidad de la investigación, de integridad y correcta ejecución de los resultados. Faltar a esta actuación ocasiona pérdida de recursos públicos, pérdida de prestigio para la comunidad científica y pérdida de confianza en la capacidad de gobierno y en la actuación —que debería ser inteligente e inteligible— de las organizaciones responsables. Por otro lado, disfrazar la demagogia de ética es una característica de los políticos que puede perjudicar a la ciencia cuando, en nombre del bien comunitario, intentan limitarla al estudio de aquello que puede tener aplicación inmediata. Ese razonamiento, aparentemente ético, sugiere que por delante de las necesidades de la sociedad se derrochan los recursos en investigaciones a las que nadie ve el beneficio. Para evitar una práctica demagógica semejante, que prende fácilmente en la opinión ciudadana, se requieren formación, conocimiento e información veraz.

Ante los grandes avances del conocimiento y la tecnología, no hay que olvidar dos grandes instrumentos de nuestra especie: el ordenador de mayor capacidad conocida, más barato, y a veces menos usado, que tenemos, que es el cerebro; y la aplicación práctica de su función a la mejora de la sociedad, que es la ética.

Los cambios de orientación en la ética

No es fácil decidir qué es ético y qué no lo es. Los valores, que dependen de la actitud de la

sociedad en un lugar y en un tiempo concretos, determinan el conjunto de directrices que, desde una esfera sociopolítica, se consideran adecuadas. La ética incide en todas las actividades profesionales, y es exigible a los responsables de la información, y a los investigadores (1). Cuando se habla de ética es probable que se haya producido una falta de ésta. En Estados Unidos se crearon comités de investigación de comportamientos y productos antiéticos (fraude, plagio, y otras formas de conducta inapropiada), cuya actuación no fue satisfactoria, y algunos tuvieron una existencia fugaz (2). Por encima de las normas, es la propia comunidad científica la que con su comportamiento ha de demostrar su rechazo a actuaciones negativas, porque una conducta impropia puede afectar el desarrollo científico por la desconfianza que genera.

Cuando la discusión sobre el alcance de los conocimientos científicos toma derroteros indeseados, se suele alegar que el científico no es responsable de la utilización de sus descubrimientos. Sobre esto habría que discutir, pero hay algo que muchas veces se olvida, y es que el afán de conocimiento y la curiosidad humanas no se pueden detener. Alcanzada una meta, ya habrá otra aguardando, y ello sin considerar qué uso se hará de ese nuevo conocimiento. Si a la vista de la repercusión social (sensacionalista) de un invento o un descubrimiento se hubiera detenido la investigación, hoy estaríamos padeciendo enfermedades que ya han sido combatidas o sometidos a incomodidades a las que de ninguna manera queremos volver. Se trata, pues, de una responsabilidad compartida.

La evaluación previa de la producción científica ("peer review")

El revisor ("referee", o "peer reviewer", o evaluador) es uno de los elementos más significativos y también más controvertidos en el proceso de publicación científica. Aunque el director de la revista tenga la última palabra, la aceptación de un artículo dependerá en gran parte del juicio del revisor. Al ser una figura normalmente anónima, suele suscitar más problemas éticos, especialmente desde la visión de los autores, que se encuentran en inferioridad de condiciones. Ellos —los autores— actúan a cara descubierta, cediendo para su revisión el resultado de mucho tiempo de trabajo. Aunque hay que esperar y confiar en la imparcialidad del revisor, a veces, aun actuando honestamente, éste puede hacer un juicio subjetivo del artículo; el idioma, el país de origen, la institución y el grupo en el que está encuadrado el autor, son factores que pueden influir en la evaluación. La revisión paritaria, ("peer review"), está actualmente en entredicho y desde diversos ámbitos se piden cambios profundos en el sistema. Además de la posible subjetividad, se alega que nadie evalúa los informes de los revisores y se objeta que es una figura reciente en la historia de la ciencia; que aparece a principios del siglo xx, cuando la investigación se especializa y los directores de las publicaciones buscan el consejo de expertos. Quizá perdure porque de momento no se ha encontrado otra manera mejor —o menos mala, algo que también se ha dicho de la democracia— de evaluar un trabajo científico. Que los revisores pueden equivocarse nadie lo duda y hay muchos ejemplos. Que la revisión paritaria suele mejorar los artículos que se publican, es también evidente. El tema merece discusión y se organizan congresos para hacerlo. El próximo será en Praga, en septiembre de 1997, dedicado al sistema de revisión paritaria en las revistas de biomedicina.

Dada la enorme capacidad de comunicación mundial de que disponemos hoy día, ¿por qué no poner los artículos en Internet, sin revisión alguna? Si los resultados son significativos, comprobables y repetibles, ya serán utilizados para

el avance del trabajo de otros científicos. La citación de un trabajo será la mejor garantía de su validez y calidad. ¿No será que las revistas que dependen de empresas editoras se niegan a ello porque perderían, además de su negocio, su capacidad de influir? ¿No será que los grupos dominantes en las áreas de donde se selecciona a la mayoría de los revisores no quieren abandonar su capacidad de control? Sinceramente, creemos que no, y que el sistema actual de revisión de momento debe ser mantenido.

El disfraz de ciencia

La última fase del proceso científico es la divulgación, cuya finalidad es poner al alcance de la población general el conocimiento científico. La divulgación, además de diarios, revistas y libros, utiliza el cine, televisión, exposiciones permanentes (museos) y temporales, etc. Recientemente, a estos medios se han añadido el CD-ROM e Internet. La divulgación científica puede ser un arma de doble filo, dado que no está sometida a revisión y que su destinatario no siempre es capaz de juzgar su calidad y fiabilidad.

Uno de los peligros que acechan a la divulgación es la presentación de pseudociencia como ciencia genuina. Serían las tinieblas que harían ver como real lo que no tiene una base sobre la que sustentarse. En Estados Unidos, donde los medios informativos tienen una gran influencia en la población, la difusión de las paraciencias alcanza grandes proporciones. Para ejercer algún control se creó el Committee for the Scientific Investigation of Claims of the Paranormal, CSICOP —apréciese el juego de palabras, ya que "cop" significa policía—, que fue fundado en 1976 por Paul Kurtz, filósofo de la Universidad de Buffalo. El CSICOP promueve la investigación crítica y razonada de los hechos paracientíficos y difunde los resultados. Cuenta con una red de personas para examinar los acontecimientos y las ciencias llamadas "alternativas" y publica la revista, *The Skeptical Inquirer*.

Carl Sagan, Isaac Asimov y Martin Gardner fueron algunos de los miembros fundadores del CSICOP. Entre los actuales se encuentran Richard Dawkins (zoólogo de Oxford, autor de *El gen egoista* y *El relojero ciego*), Bernard Dixon (microbiólogo y divulgador científico), Stephen Jay Gould (paleontólogo de Harvard y escritor de libros tan conocidos como *El pulgar del panda* o *La vida maravillosa*), John Maddox (director emérito de *Nature*), Gerard Piel (renovador de *Scientific American*) y John Rennie (actual director de dicha revista). Quienes se sienten perjudicados por los análisis del CSICOP dicen que es un comité hostil a cualquier idea innovadora, y lo acusan de ser la Inquisición de los tiempos modernos. Sagan admitió que a veces tienen razón, pero al CSICOP pueden dirigirse los medios de comunicación cuando desean oir la otra versión de la historia.

Hay que diferenciar entre pseudociencia y ciencia errónea. Una parte de nuestra ciencia actual es errónea con respecto a la del futuro. Los errores anteriores no son malos por sí mismos; alimentan la ciencia, que los va eliminando uno a uno. Decía Francis Bacon que "la verdad sale antes del error que de la duda". Al fin y al cabo, la historia de la ciencia está llena de falsas hipótesis y conclusiones erróneas, que el propio desarrollo científico ha ido desmontando. La pseudociencia es justamente lo contrario; parte de hipótesis que se dan como ciertas y, cuando se intenta demostrar su falacia surgen defensores que ven en la postura científica una conspiración.

Pero como afirmó Sagan, el escepticismo no vende. Un evolucionista duda; un creacionista "sabe". El público general prefiere que los artículos "científicos" le ofrezcan un mundo de esperanza y le abran las puertas a arcanos que creía

inaccesibles. La ciencia despierta admiración y sorpresa entre la población, pero lo mismo ocurre con la pseudociencia. Y ésta es aceptada en la misma proporción en que la ciencia es mal interpretada o no comprendida.

¡Hola, Dolly!

La fecha del 27 de febrero de 1997 será recordada en el futuro y pasará a los libros de texto. Señala la publicación en una revista científica (*Nature*) de los experimentos conducentes a la producción de un mamífero clónico (6). Aunque otros grupos tenían resultados similares, incluso con animales más cercanos a la especie humana, no se habían atrevido a publicarlos, o habían pasado desapercibidos.

¿Se hubiera detectado el artículo de no haber mediado un comunicado de prensa de la revista? El título que dio la prensa a la noticia —"La oveja clónica"— y el del artículo en *Nature*— "Descendencia viable procedente de células fetales y adultas de mamífero"— eran muy distintos. ¿Cuántos periodistas hubieran reparado en la importancia de la noticia viendo solamente el índice? Y aunque la imagen idílica de la portada, junto con el juego de palabras "A flock of clones", hubiera ayudado algo, ¿cuántos científicos?

En la sociedad, los ciudadanos disponen de unos derechos, logrados a veces a costa de sacrificios, propios o de otros que nos antecedieron. A menudo se olvida el precio que hubo que pagar por esos derechos; pero lo que es peligroso es que caigan en desuso. Si nos referimos a la libertad, posiblemente el bien más preciado del ser humano, no nos damos cuenta que es necesario ejercerla continuamente para que se mantenga viva. La libertad de expresión se puede debilitar de muchas formas; una de ellas, al rebajar los estándares educativos, despreciar la competencia intelectual y minimizar el debate de los conceptos y de las ideas. Una educación que destaque el valor de la libertad —de expresión, de elección, etc,— contribuye de la mejor forma a su mantenimiento. Cuando el individuo no se atreve a pensar por sí mismo ni a dudar de la autoridad, pasa a depender de quienes ejercen el poder. La forma de evitarlo y hacer que sean los gobernantes quienes trabajen para los ciudadanos, y no al revés, es estar capacitado para formar las propias opiniones. Los medios de comunicación son un arma poderosa en la formación de actitudes y opiniones. Por eso interesan tanto a los políticos.

Actualmente muchas personas no se atreven a expresar su opinión en materia de ciencia o de política científica, a pesar de estar en desacuerdo. ¿Cuál es la razón, si la libertad de expresión es un derecho constitucionalmente reconocido? Quizá el desánimo, la desesperanza en que los responsables de tomar las decisiones elijan las correctas. No se sabe si porque no aciertan con cuáles son o porque están sometidos a un mal entendido dominio de "lo popular". Y la consecuencia es que el reparto de los recursos económicos y humanos se establece según criterios supuestamente "objetivos" que eliminan la competencia de calidad cuando (muy a menudo) no se puede hacer frente a ella.

La investigación se exige que sea rentable, en el sentido amplio del término. En investigación la rentabilidad se mide: (i) por su contribución al avance del conocimiento, (ii) por las publicaciones que produce, consecuencia del punto anterior, (iii) por su aplicación práctica. Todo ello da lugar a un proceso de producción científica en el que intervienen distintos elementos.

En el último capítulo de su libro Sagan, reflexiona sobre política y sobre los políticos, y comenta la calidad humana de los líderes de los primeros tiempos de la historia de su país. Destaca en ellos la cultura y mente abierta. Conocedores de la naturaleza humana y de sus debili-

dades. Conocedores de su lengua, autores de sus propios discursos. Realistas y prácticos, a la vez que motivados. No les incomodaban los planes a largo plazo, aunque éstos superaran los períodos de su mandato. Eran autosuficientes y no buscaban grupos de presión para extender su influencia.

Con todas sus imperfecciones, la ciencia y los métodos que le son propios son excelentes modelos para mejorar los sistemas sociales, políticos y económicos. Y lo son: (i) Porque no son autocomplacientes y sí autocorrectivos. (ii) Porque de entre diferentes métodos para conseguir un resultado utilizan el más económico en todos los sentidos. (iii) Porque incorporan los cambios y mejoras que se producen aunque venga de otras áreas de la ciencia. (iv) Porque la aplicación de los resultados exige un período de prueba para controlar la eficacia y los efectos indeseados. (v) Porque no hay ninguna duda en desechar una idea o una hipótesis que se consideró adecuada en un tiempo hasta que nuevos conocimientos mostraron su inadecuación. Y muchas más. La ciencia, con su doble componente de curiosidad y escepticismo, junto a su capacidad de estimular la diversidad y el debate, constituye un elemento esencial para continuar ese delicado experimento que es la libertad en una sociedad industrial y altamente tecnológica.

¿Por qué creemos muchos en el valor de la ciencia y en su capacidad para extender la luz sobre muchos problemas? Porque es contraria al argumento de autoridad y es más eliminadora de barreras sociales que cualquier otro sistema, ya que una hipótesis o teoría de alguien desconocido puede desbancar otra anterior de alguien prestigioso. No es perfecta en su ejercicio, pero tiene más capacidad de autocorrección que otros sistemas. ¿De cuál de ellos, dirá uno de sus integrantes, algo como "la única pieza sólida de la verdad científica, es nuestra profunda ignorancia acerca de la naturaleza". La frase es de Lewis Thomas. De George Bernard Shaw (que no era científico), es la siguiente: "la ciencia solamente se vuelve peligrosa cuando se cree que ha alcanzado sus objetivos". Podríamos añadir que la ciencia es algo muy serio para dejarla en manos de gestores-administradores y de políticos, sobre todo en los casos en que carecen tanto de una mínima formación científica como del propio sentido de qué es la ciencia.

El coste de la educación es insignificante comparado con el coste de la ignorancia. Y la ignorancia sólo puede producir tinieblas y turbulencias que pueden llegar incluso a apagar la vela. Es una decisión que hay que tomar cada día con la propia actitud, decidir entre las tinieblas o la luz. Parece que tendría que ser una elección sencilla, pero algo dice que no siempre se toma la decisión más brillante.

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Revisión de libros
Book reviews

Geigy Scientific Tables, 8th ed.
Vol. 6. Bacteria, Fungi, Protozoa, Helminths.

Cornelius Lentner, Charlotte Lentner (ed.)
CIBA-GEIGY, Basel, Switzerland, 1992. 153 pp.
ISBN 3-905298-02-3

Las *Tablas Científicas de Geigy* constituyen una obra que ha resistido el paso del tiempo, gracias a la continua actualización de su contenido. Es precisamente esa puesta al día y la ampliación de los temas lo que aconsejó dividir en varios volúmenes el contenido, que hasta la séptima edición quedaba recogido en un único tomo (a veces, con algunos anexos monográficos, como los destinados a enfermedades infecciosas). La obra mantiene su objetivo de proporcionar a los investigadores y profesionales de las ciencias de la vida y de la salud un compendio preciso y detallado de datos científicos, debidamente referenciados. Los demás volúmenes de la serie, hasta un total de seis, incluyen unidades de medida, tablas estadísticas, composición del cuerpo y de la sangre, errores congénitos del metabolismo, farmacogenética, ecogenética y otros temas de interés en el desarrollo de la práctica médica y en el laboratorio.

El volumen 6 de esta 8ª edición, revisada y ampliada, recoge en formato de tablas, fácilmente consultables, los cerca de 500 organismos patógenos de la especie humana conocidos de importancia, que se incluyen en los siguientes grupos: bacterias, 250; hongos, 150; protistas, 40; y helmintos, 50. Las tablas indican, además del nombre científico del patógeno, las especies sinónimas y los nombres comunes, si los hubiera. Se incluyen las características más destacadas del organismo, su hábitat, huésped y mecanismo de transmisión, así como la enfermedad o enfermedades a la que están asociados. Las tablas se complementan muy adecuadamente con textos de especial relevancia, que destacan aspectos significativos de la microbiología clínica (tuberculosis, lepra, infeciones de *Escherichia coli*, entre otros). En el caso de los hongos, se acompañan unos dibujos explicativos de gran calidad. El índice al final del libro permite relacionar con facilidad una enfermedad infecciosa con su organismo causante, así como un patógeno con la enfermedad con la que está relacionado.

En este volumen se omiten los virus. Y no se prevé incluirlos en ninguno de los otros cinco volúmenes de la 8ª edición. Es una exclusión penosa, debido a la extrema importancia que tienen las infecciones víricas en todos los organismos. A pesar de ello, esta omisión no resta valor al libro como fuente de referencia rápida sobre las enfermedades infecciosas.

Jordi Mas-Castellà
Universidad de Barcelona

Tratado de Microbiología

Bernat D. Davis, Renato Dulbecco, Herman N. Eisen, Harold S. Ginsberg

MASSON, S.A., Barcelona, 1996. 1145 pp. Precio: 14.500 PTA. ISBN 84-458-0371-9

Si hubiera que hacer una selección de los principales libros de texto de microbiología, con todo merecimiento éste figuraría entre ellos. Reúne la principal condición que cabe exigir en un libro de estas características: tratamiento profundo de la materia y que los autores sean expertos en los temas tratados y que gran experiencia en la docencia.

Son muchos los que piensan que, desde su primera edición, este libro aportó una nueva concepción de lo que tenía que ser la enseñanza de la microbiología en las facultades de medicina. La principal enseñanza que puede extraerse de su lectura es hacer ver la importancia que tiene el conocimiento de los procesos básicos de la microbiología en la formación del médico. Como consecuencia, a partir de la publicación del "Davies" ningún texto enfocado a la simple descripción de casos y procesos de enfermedades podría considerarse adecuado.

Para dar cabida a todo el nuevo material que era necesario introducir desde la edición anterior, publicada en 1980, los autores han resumido el tema del metabolismo intermediario, reorganizando la sección sobre fisiología y genética bacteriana, y han optado por profundizar en los aspectos fisiológicos más que en la enorme cantidad de información sobre secuenciación del DNA y de proteínas o en las interacciones moleculares. Se mantiene la estructura de cuatro secciones: Fisiología y genética bacterianas, Inmunología, Bacterias patógenas y Virología, integrando en los tres últimos los aspectos biológicos y los clínicos. La sección de inmunología se ha redactado de nuevo y el capítulo sobre inmunidad y transplantes se ha ampliado, para incluir enfermedades autoinmuninatarias e inmunodeficiencias, en especial el sida. La virología se ha revisado extensamente, incorporando los datos más recientes sobre genomas víricos y sus funciones codificadoras, así como los mecanismos de patogeneidad de las enfermedades víricas. La parte de retrovirus, muy aumentada, incluye un extenso apartado sobre virus del sida. Se ha añadido un capítulo sobre hongos que, aunque breve, resultaba imprescindible en un texto de microbiología de estas características.

Con un enfoque didáctico, se aprecia el esfuerzo en exponer las pruebas que fundamentan las conclusiones, para evitar quedarse en la mera descripción de hechos. A este respecto, los autores siguen fieles a su idea original de presentar la ciencia como un hecho dinámico, en continuo crecimiento, y no como un simple cuerpo de conocimientos que debe ser memorizado sin profundización. Por ello, no falta la perspectiva histórica en la presentación del material, ni la naturaleza de los hechos que han llevado a cada una de las conclusiones más importantes. El "Davies" es, por tanto, un texto de consulta muy adecuado para estudiantes e investigadores de medicina, farmacia y biología, pero también de otras disciplinas relacionadas. La traducción al español de la cuarta edición aparece con un cierto retraso, seis años con respecto a la publicación de la edición inglesa. Es éste el principal problema de las traducciones, que habrá que pensar en corregir para evitar el desfase que supone, sobre todo en los textos de material científico.

Isabel Esteve
Universidad Autónoma de Barcelona

Manual of Environmental Microbiology

Christon J. Hurst (editor-in-chief)

American Society for Microbiology Press, Washington, D.C., 1997. 894 pp. Precio (miembros ASM): $ 85. ISBN 1-55581-087-X

"Si pudiese empezar de nuevo mi carrera, sería un ecólogo microbiano. Diez mil millones de bacterias viven en un gramo de suelo corriente, lo que podemos pellizcar con el pulgar y el índice". Quien así se expresa es Edward O. Wilson (en el párrafo final [p. 364] de su libro *Naturalist*, 1994 [Island Press, Washington, D.C.]), profesor de entomología (!) de Harvard, una de las grandes figuras del pensamiento biológico del siglo xx, y creador de los conceptos de sociobiología, biofilia y biodiversidad. Es difícil señalar la diferencia ecología microbiana y microbiología ambiental; quizás podría decirse que la primera es la ciencia básica, el substrato, de donde ha surgido la segunda. Ambas disciplinas son bastante recientes. El primer libro con el título de ecología microbiana, de Thomas D. Brock, se publicó en 1966. La microbiología ambiental empezó a desarrollarse en los años 70, utilizando las ideas y las técnicas tanto de la ecología microbiana como de la genética molecular.

A veces se tilda a los investigadores que trabajan en ciencia básica de ilusos, por esforzarse en la resolución de problemas que parecen no tener aplicación práctica. Brock ha dedicado dos décadas de su vida al estudio de los microorganismos que viven en las fuentes termales. Los que mejor conoce son los del parque nacional de Yellowstone, en Estados Unidos. Pero viajó por todo el mundo recogiendo muestras en ambientes de temperaturas elevadas, como solfataras, fuentes termales, géiseres (que deben su nombre a la ciudad de Geyser, en Islandia) y vertederos calentados por la fermentación de sus residuos. Brock quería determinar el límite superior de temperatura para la vida; un objetivo aparentemente alejado de cualquier aplicación práctica. Sin embargo, gracias a ese estudio, se cuenta hoy con una de las armas más potentes de la biología molecular: la reacción en cadena de la polimerasa (PCR). Brock consiguió aislar una bacteria (no arquea), *Thermus aquaticus*, que crece y se reproduce en agua hirviendo. Sus enzimas resisten las altas temperaturas del medio y su DNA-polimerasa, *Taq* polimerasa, se usa en la PCR. Luego se han utilizado otras enzimas similares, como la *Pfu* polimerasa, aislada de la arquea *Pyrococcus furiosus*, que tiene su óptimo a 100°C. Brock, que demostró no ser un iluso, sí que es un idealista. Depositó gratuitamente *T. aquaticus* en la Colección Americana de Cultivos Tipo (ATCC). Posteriormente, una empresa privada aisló un enzima, la *Taq* polimerasa, de dicho organismo y vendió la patente a una multinacional. En una carta a *Science*, Brock se lamentó de la actual comercialización de la ciencia (véase *Boletín SEM*, 16 [1993], p. 12). Este es un ejemplo de la aplicación de una ciencia básica; en este caso, la ecología microbiana. Y también de que, según Julian Davies, no hay ciencia básica o ciencia aplicada, sino ciencia bien hecha, o mal hecha.

"Manual" es, según el *Diccionario de la Lengua Española* (de la Real Academia), un "libro en que se compendia lo más sustancial de una materia". La definición del diccionario americano Webster amplía el significado. La primera acepción dice que es un "libro de pequeño tamaño que puede ser transportado o manejado fácilmente"; en otra pone que es un " libro que comprende, de forma concisa, los principios, normas e instrucciones necesarias para el dominio de un arte, ciencia o habilidad". Dejando de

lado lo de "pequeño tamaño", o lo de "forma concisa" (el libro mide 28,2 x 21,5 x 4,5 cm y pesa 2,325 kg), el *Manual* se ajusta tanto a la definición española como a la inglesa. A pesar de su extensión, expone en compendio lo más sustancial de la ecología microbiana. Y comprende los principios, normas e instrucciones necesarias para el dominio de esta ciencia, que es una área emergente y explosiva de la microbiología (véase la serie de artículos dedicados en un número reciente de *Science* [del 2.5.97]).

El *Manual* está distribuido en ocho secciones, a las que han contribuido cerca de ciento cincuenta especialistas. Aporta las nociones básicas para comprender el porqué de las técnicas descritas. La Sección I, básicamente descriptiva, es una introducción a la microbiología ambiental, al estudio de las comunidades microbianas y a las interacciones entre poblaciones microbianas y al conocimiento de la diversidad procariótica. La Sección II es un resumen de las técnicas utilizadas para medir y clasificar los microorganismos de la comunidad a estudiar: microscopia, tipos de cultivo (cultivo axénico, cultivo mixto, etc.), determinación de la biomasa, aplicación de la biología molecular a la determinación de la densidad, diversidad y filogenia, nuevas técnicas para el estudio de microambientes (biodescriptores, biosensores, microsondas). Dedica el capítulo final a los principios que aseguran la calidad de un proyecto de microbiología ambiental. Siguen dos secciones (III y IV) sobre la microbiología del agua. La primera trata de los aspectos relacionados con el agua y la salud pública en cuanto a la transmisión de enfermedades: el medio acuático como transportador de agentes infecciosos; detección de protistas parásitos; detección de virus en aguas ambientales, y en aguas y lodos residuales; indicadores de la calidad del agua de las playas; detección de microorganismos indicadores en aguas dulces y las destinadas al uso doméstico, así como su control; detección de bacterias y virus en moluscos destinados al consumo humano; estudio de la supervivencia de microorganismos en el agua y en el suelo; detección de bacterias patógenas en las aguas y lodos residuales; y evaluación de la eficiencia de los tratamientos de aguas residuales. La otra sección (Sección IV) está dedicada a la ecología del medio acuático: productividad primaria; productividad secundaria bacteriana; estructura de las comunidades (virus, bacterias, arqueas y protistas); métodos cuantitativos para el examen de organoosmotrofos en restos vegetales; fagotrofia en las cadenas alimenticias acuáticas microbianas; aplicaciones de isótopos para analizar los cambios en la materia orgánica béntica; ciclos del azufre, nitrógeno, fósforo, tolerancia y requerimientos de metales; colonización, adhesión y biofilmes; ambientes marinos poco corrientes, o sometidos a altas presiones.

La Sección V estudia el suelo, la rizosfera y la filosfera, y presenta las diferentes metodologías y técnicas específicas para aislar y cultivar microorganismos asociados al suelo y las plantas (bacterias, virus, hongos formadores de micorrizas; aislamiento y caracterización de bacterias y hongos endofíticos; aplicación de técnicas de biología molecular al análisis de la estructura de las comunidades microbianas del suelo; recuperación del DNA de las comunidades bacterianas; aplicación de la PCR a la amplificación de las secuencias genéticas de microorganismos; diferentes aspectos de la actividad y transformaciones químicas llevadas a cabo por microorganismos; aplicación de resistencia a antibióticos, marcadores cromogénicos o luminiscentes y de anticuerpos fosforescentes para el estudio de microorganismos asociados al suelo; empleo de genes descriptores para evaluar la expresión génica en bacterias asociadas a plantas y al suelo; producción de

antibióticos; cuantificación de la transferencia genética en el suelo y en la rizosfera; e interacciones microorganismos–insectos.

La Sección VI trata varios aspectos del estudio de los microorganismos que crecen en los vertederos y en las capas más profundas de la corteza terrestre: perforaciones y muestreo; estudio de la microbiología de los vertederos y de la descomposición anaeróbica; aspectos microbiológicos del acuífero; métodos para el estudio in situ y ex situ del transporte microbiano en profundidad; microbiología del petróleo; y localización y protección de pozos de agua potable. La Sección VII está dedicada a los microorganismos que son transportados por el aire, de interés principalmente en agricultura y medicina. Se tratan el muestro y análisis; transporte; producción de toxinas; algunas enfermedades humanas con agentes transportados por el aire (bacterias [*Legionella*; tuberculosis] y virus) ; aerobiología de patógenos vegetales. La Sección VIII presenta aspectos diferentes de la biotransformación y la biodegradación, que serán muy útiles para quienes trabajen en biorremediación: determinación de la biodegradabilidad o la biodisponibilidad de contaminantes; técnicas para estimular la biodegradación; métodos para la biodegradación de contaminantes específicos y para la extracción de metales; mecanismos de corrosión por microorganismos; escalado de los procesos en biorremediación.

Al ser un libro en el que han participado muchos autores, la exposición de los diferentes temas es desigual; pero la falta de uniformidad no resta valor a su contenido. Es una obra imprescindible en los departamentos que imparten microbiología ambiental, así como en los laboratorios dedicados a realizar controles de calidad y auditorías ambientales.

Ricard Guerrero
Universidad de Barcelona

In the company of mushrooms

A Biologist Tale

Elio Schaechter

Harvard University Press. Cambridge, Massachusetts. London, England, 1997. 280 pp. Precio: $ 24.95. ISBN: 0-674-44554-6

La micofilia, actitud cultural de determinados pueblos, en los que los hongos y las setas juegan un papel importante en sus tradiciones, su cultura, su gastronomía, su ciencia, e incluso en su religión, no es patrimonio exclusivo de rusos, franceses, italianos, vascos y catalanes. En la cultura anglosajona es posible también el desarrollo de una actitud de micofilia. Buena prueba de ello es el excelente libro que comentamos. Se trata de una obra bien escrita, documentada y rigurosa, con información útil para quien desee adquirir o ampliar conocimientos sobre micología. Nada en relación con esta ciencia queda fuera de sus páginas. Como ejemplos, sus referencias a la paleonto-micología (hongos fósiles del ámbar cretácico), o sus comentarios sobre antropología y etnología en relación al hombre hallado en un glaciar en el centro de Europa hace pocos años. Su texto es también divulgador y su lectura resulta fácil incluso para profanos en este mundo subyugante de las setas. Lo adorna un fino humor, amable y agradable. Pero sobre todo, traduce algo más profundo y significativo que los amplios conocimientos de su autor sobre hongos, setas y micología. Expone su actitud vital, su amistad, su respeto, su admiración, su simpatía hacia ellos. No es ninguna casualidad que el autor de este pequeño libro sea también autor o editor de obras que han marcado hitos en distintos campos de la microbiología; mencionemos, entre otros, *Mechanisms of Microbial Disease*, *Physiology of Bacterial Cell*, o *Escherichia coli*

and Salmonella tiphymurium, Cellular and Molecular Biology. Moselio —o Elio, como firma el libro de hongos, y como todos los amigos le conocen— Schaechter ha sido durante veinticinco años jefe —y, al mismo tiempo, investigador activísimo— del departamento de biología molecular y microbiologíam de la Tufts University, en Boston. El autor hace en *In the Company of Mushrooms* una incursión en otro campo de la microbiología que podría sorprender a muchos de sus colegas. Es un relato de su historia de amor por las setas, como lo describe Laurence A. Marschall en la elogiosa crítica que le dedica en de *The Sciences*, revista de la Academia de Ciencias de Nueva York.

Este bonito y entretenido libro expresa y lleva hasta el lector toda una filosofía y una actitud a entender, ver, apreciar y disfrutar con los más variados aspectos en relación a las setas. Y ello lo observamos tanto en sus comentarios sobre la nomenclatura de los hongos y setas y en sus descripciones de los diversos grupos de macromicetos, como en aquellos párrafos en los que con excelente humor y lenguaje ameno nos habla de la "caza" de setas, y nos la pone en su justo lugar. Creo que nunca antes he leído una descripción tan completa del conjunto de matices y aspectos lúdico-culturales que se encierran tras la afición de acudir a la naturaleza a recolectar setas. Schaechter sabe, y sabe explicarlo muy bien, que esa afición nos lleva al bosque y al monte para algo más que conseguir un complemento culinario para nuestra mesa. El encontrar un espécimen raro, que supone un reto para nuestra habilidad como taxonomistas; el encontrar un hermoso ejemplar que nos proporcionará una luminosa y coloreada fotografía para nuestras charlas en el futuro; el descubrir una especie tóxica o muy cerca de ella otra muy similar, comestible, y admirarnos así de la dualidad belleza–peligro que encierra la micofagia. Y aun más: el disfrutar del aire libre y del contacto con la naturaleza.

Muchos aspectos destacaría de este libro, pero el que más me agrada resaltar es, precisamente, esa amable, clara y sencilla forma de describir en todos sus matices la vivencia del "amateur", del que tiene por "hobby" a las setas, o alguno de los muchos caminos que su estudio nos abre: científicos, culturales, humanísticos, antropológicos, religiosos, artísticos, geográficos, históricos, botánicos, químicos, ecológicos, etc. A quienes hemos compartido con Schaechter la aventura de salir al bosque un día de otoño en busca de tan preciado tesoro, no nos sorprende su destreza y maestría en la descripción de esa afición.Una bibliografía —nada convencional, por cierto— permitirá buscar nuevas fuentes de información. Así mismo, una relación de asociaciones y clubes micológicos, al final del libro, será útil para el que desee desarrollar y compartir su afición.

Sin duda alguna, este es un libro del que todos podemos aprender algo. Quien se inicie en el "hobby" micológico encontrará consejos sobre cómo, cuándo y dónde recolectar setas. Quien se interese por la historia de la micología, hallará datos de interés en relación a los micólogos del pasado. Y, por último, a quien le interesen los aspectos descriptivos, taxonómicos, bioquímicos o biológicos sobre las setas, los hallará igualmente. Por lo que hace al aspecto formal, no podemos dejar de resaltar lo cuidado de la edición. La sobrecubierta, el papel, los numerosos dibujos y las bellas fotografías, hacen de él un libro excelente. Un libro que no dudamos en recomendar, no sólo a quienes sienten interés por la micología, sino también a cualquier persona que desee una lectura amena sobre un tema relacionado con la naturaleza.

Josep Piqueras
Hospital General Universitario Vall d'Hebron
y *Asociación Española de Micología*

Microbial Diversity in Time and Space

Rita R. Colwell, Usio Simidu, Kouichi Ohwada (ed.)

Plenum Press, New York, 1996. 172 pp.
ISBN 0-306-45194-8

El libro recoge las ponencias presentadas en el Simposio internacional *Microbial Diversity in Time and Space* (Tokio, octubre 1994). Organizado por la Sociedad Japonesa de Ecología Microbiana, contó con la participación de destacados microbiólogos como Rita R. Colwell, Erko Stackebrandt, Yehuda Cohen, Usio Simudu, entre otros.

El objetivo del simposio era destacar la contribución de los microorganismos en los ciclos globales de los elementos como soporte básico a la biodiversidad en el planeta. Para un microbiólogo, cualquier iniciativa encaminada a poner de manifiesto el papel de los microorganismos en el funcionamiento del planeta y en el mantenimiento de la biodiversidad, es motivo de satisfacción. En este sentido, el libro trata aspectos de singular importancia como, por ejemplo, la diversidad de los virus, los hongos, las algas, la simbiosis en insectos y plantas, la diversidad microbiana en los ciclos de los elementos, etc., todo ello a cargo de especialistas reconocidos.

En el siglo pasado, los primeros microbiólogos taxónomos siguieron el sistema que habían iniciado sus colegas botánicos (probablemente, muchos de esos microbiólogos eran también botánicos) y durante 80 años las descripciones de especies se basaban únicamente en rasgos fenotípicos. Si bien entonces esto supuso una contribución ante la falta de otros criterios de clasificación, pronto fueron perdiendo utilidad e incluso introduciendo confusión. En 1970 habían sido descritas erróneamente unas 45.000 especies de bacterias. Por ello, el 1 de enero de 1980 puede considerarse una fecha destacada para la microbiología moderna en su intento de resolver problemas de sistemática, cuando V. B. D. Skerman et al. publicaron la *Aproved list of bacterial names* en el número de enero del *International Journal of Systematic Bacteriology*. A partir de su publicación, cualquier especie no incluida dejaba de considerarse válida a efectos de nomenclatura. En un sólo día se redujo en número de especies bacterianas de 45.000 a 2500.

Desde entonces, los avances tecnológicos han permitido desarrollar métodos basados en la hibridación del DNA y en la quimiotaxonomía. Todo ello ha conducido a un enfoque polifásico —término utilizado por R. R. Colwell por primera vez en 1970—. En la actualidad, el número de especies bacterianas descritas se sitúa en un margen de 2000 a 4000, mientras que el número total estimado de especies existentes ronda los 3.000.000. En el caso de los virus, el número de especies podría ser del mismo orden.

Los casos particulares donde se relaciona la diversidad bacteriana con los ciclos de los elementos constituyen quizá una de las aportaciones más novedosas de este libro. Por ejemplo, el estudio de la diversidad genética de bacterias del suelo en relación con el ciclo del nitrógeno, como también la coevolución entre plásmidos en rizobios y especies de plantas leguminosas. La obra presenta, en definitiva, un conjunto de artículos que reúnen calidad, interés y novedad, y que subrayan la necesidad de ahondar en el estudio de la diversidad microbiana como eje básico del funcionamiento de los procesos biogeoquímicos, tanto a pequeña escala como a escala global.

Jordi Mas-Castellà
Universitat de Barcelona

The World of Science and Technology

A Theme-Based, Study-Skills Approach

Frances Luttikhuizen

The University of Michigan Press, Michigan, 1995. 222 pp. ISBN 0-472-08269-8

An Introduction to English for Academic Purpose

A Theme-Based, Study-Skills Approach

Frances Luttikhuizen

Publicacions Universitat de Barcelona, Barcelona, 1994. 156 pp. ISBN 84-475-0770-X

El primero de los dos libros que comentamos es un claro ejemplo del avance de la metodología aplicada a la enseñanza de las lenguas. Basado en un sistema de aprendizaje que incide en los aspectos significativos, la obra introduce al estudiante de inglés en el amplio universo de la ciencia, y lo hace de forma interdisciplinaria.

Dividida en diez unidades; cada una de ellas incluye diversas lecturas seguidas de actividades que abarcan desde los clásicos comentarios, ejercicios léxico-gramaticales, las modernas "cloze activities" hasta una enorme variedad de ejercicios analíticos, de interpretación e iniciación a la investigación, con estilo dialogante y poco rígido pero sin eludir el rigor lingüístico. Tal como manifiesta, poéticamente, la autora, "El elemento unificador de la selección de las lecturas es el relato de la búsqueda del hombre para descubrir lo desconocido" o , también, " la lucha del hombre para comprender, dominar y explotar los recursos de la Tierra".

El libro evoca conceptos humanistas y se aprecia la dialéctica Hombre-Naturaleza, los peligros y las buenaventuras de esta relación épica y profunda. Sus numerosos gráficos y dibujos complementarios lo hacen visualmente atractivo. No hay que olvidar la revolución estética sufrida por las artes gráficas, en general, y los libros de texto, en particular, a consecuencia de la evolución de los programas informáticos y su influencia en el lenguaje iconográfico. Es, en definitiva, algo más que un simple manual de lengua. Su contenido, histórico y científico, contribuye de forma amena, casi lúdica, a proporcionar y mantener la cultura general científica del lector, a la vez que le prepara para comunicarse en inglés a un nivel medio–avanzado. Aunque se dirija fundamentalmente a estudiantes de ciencias, preuniversitarios y universitarios, también podrá resultar de utilidad a los licenciados que deseen profundizar en sus conocimientos de inglés.

A un nivel más elemental, como corresponde a una introducción, pero igualmente útil y, sobre todo, realizado con el mismo rigor, cabe referirse al segundo libro, *An Introduction to English for Academic Purpose*, donde se ofrece al estudiante los instrumentos y técnicas que ayudan en el aprendizaje y correcta utilización de una lengua extranjera.

Recordemos, por último, el papel relevante de la lengua inglesa, sobre todo en las últimas décadas, al servir de vínculo de unión entre los pueblos para la transmisión del saber tecnológico y científico, en un mundo en el que, según el escritor y Premio Nobel de Literatura, Gabriel García Márquez, "los idiomas se dispersan sueltos de madrina, se mezclan y confunden, disparados hacia el destino ineluctable de un lenguaje global".

Asunción Peral Socías
I. B. Torras i Bages, Barcelona

Science International
A history of the International Council of Scientific Unions

Frank Greenaway

Cambridge University Press, Cambridge. 1996. 280 pp. ISBN 0-521-58015-3

Como leemos en la sobrecubierta, nos hallamos ante la primera historia de una organización mundial de científicos (International Council of Scientific Unions, ICSU) cuyas raíces se remontan a la International Association of Academies (IAA, 1899–1919) y pasan por el International Research Council (IRC 1913–1931), que la precedieron. Sin embargo, el libro es bastante más que la historia de ICSU. Los avatares políticos, el progreso social y científico, los cambios en las comunicaciones y la geografía política a lo largo del siglo XX se reflejan en esta obra; son varias historias entremezcladas en un único relato.

Y está bien que se haya escrito esta historia. El público ajeno al mundo de la ciencia, o incluso el investigador que no forma parte de ninguna organización científica, puede llegar a sentirse abrumado por el baile de siglas que dan nombre a asociaciones y supraasociaciones y puede tener la impresión de que tantas entidades no son más que una excusa para que una elite de científicos viaje y se reúna de vez en cuando. La lectura de *Science International* es muy posible que cambie esa visión, al menos por lo que a ICSU se refiere. La función de dicha entidad como defensora de la libertad de conducta del científico queda reflejada en el capítulo 8; es un tema de sumo interés que podría cubrir por sí solo un libro entero. ICSU luchó por la abolición de la discriminación política que en muchos casos impedía a los investigadores, no sólo emigrar hacia otros países para desarrollar su actividad, sino simplemente su participación en reuniones internacionales. En 1963 se creó un comité (SCFSC, Standing Committee on the Free Circulation of Scientists) que convirtió a ICSU en un grupo de presión ante gobiernos totalitarios o ante aquéllos que olvidaban que la ciencia es ajena a la política. Los archivos de ICSU, que son confidenciales, guardan la historia de muchas injusticias. Aunque, como hemos indicado, la ciencia sea —o deba ser— ajena a la política, no puede cruzarse de brazos ante actitudes que niegan los más elementales derechos de la persona. Así, ICSU, se vio forzada a ampliar los propósitos expresados en sus estatutos iniciales, que consistían básicamente en coordinar las organizaciones nacionales y las asociaciones internacionales ya existentes que se adhiriesen a esta nueva organización; dirigir la actividad científica internacional en campos que no estuviesen ya cubiertos por otras asociaciones; y establecer, por medio de las entidades nacionales adheridas, relaciones con los países representados en ICSU para promover en ellos la investigación científica.

Los cuatro primeros capítulos de *Science International* describen los orígenes, la fundación y el desarrollo inicial de ICSU. Los capítulos 5, 6 y 7 nos cuentan la actuación de ICSU durante la II Guerra Mundial, el despertar de 1945 y su desarrollo en la época de postguerra. El ya mencionado capítulo 8 (The free conduct in science), trata uno de los temas más arduos a los que se han de enfrentar los científicos: la libertad para desarrollar su profesión. Los capítulos 9, 10 y 11 están dedicados a la actividad en el seno de ICSU, que refleja el desarrollo que la ciencia ha seguido en el exterior. Los grupos que se han ido constituyendo en las últimas décadas responden a la aparición de nuevas áreas del conocimiento científico. El autor ve en ICSU una maquinaria viva (capítulo 9: Living machinery: officers and staff) accionada por los diferentes grupos que la integran.

Siguen cuatro capítulos dedicados a temas que parecen dispares, pero que reflejan varios aspectos de la situación actual de la investigación: la expansión de la ciencia (el paso de ciencia "regional" a ciencia "global"), la información científica, los proyectos mundiales, relacionados principalmente con problemas ambientales, y la relación entre ICSU y UNESCO. Aunque ICSU es anterior a UNESCO, sin la existencia de esta entidad y de otras organizaciones al amparo de la ONU, ICSU sería muy distinta de lo que es y representa en la actualidad. ICSU canaliza una parte del presupuesto de UNESCO hacia proyectos de todo el mundo y actúa de consejera científica de dicha entidad. Por su parte, UNESCO proporciona el trasfondo intelectual y político para las actividades de ICSU, y le sirve de enlace con el ambiente político de la ONU. También tienen programas conjuntos.

El capítulo 16 (Membership) describe el tipo de entidades que forman parte de ICSU, que son básicamente asociaciones científicas de carácter nacional, de países adheridos a ICSU; organizaciones internacionales que agrupan a diferentes entidades de una misma especialidad; y miembros asociados, que suelen ser entidades con finalidades semejantes a las de ICSU, pero de acción restringida a países con características comunes. Entre estas últimas la Academia de Ciencias del Tercer Mundo (Third World Academy of Sciences, TWAS), fundada en 1983 y con sede en Trieste (Italia); la Academia de Ciencias de América Latina, creada en 1982; o la Federation of Asian Scientific Academies and Societies, que data de 1984. En los últimos años el número de miembros nacionales ha aumentado considerablemente, especialmente tras la caída de la URSS. Hay que destacar que cuando ICSU se refiere a "miembros nacionales" no lo hace en términos geopolíticos. Se describen algunas de las entidades de carácter gremial e internacional integradas en ICSU: IUPAP (International Union of Pure and Applied Physics), creada en 1922; IUBS (International Union of Biological Sciences), en 1923, entra en ICSU en 1925; IUMS (International Union of Microbiological Societies), creada en 1927 como International Society of Microbiology; se integró en ICSU en 1982; IUNS (International Union of Nutritional Sciences) miembro de ICSU desde 1968). IBRO (International Brain Research Organization; una de las más recientes que han entrado en ICSU, en 1993).

Los tres últimos capítulos (Ringberg [1985] to Visegrad [1990]: self-examination, The road to Rio and beyond, y ICSU at the end of a century) muestran el compromiso de la ciencia, y por ende, de ICSU, con el mundo actual para buscar soluciones a los problemas que afronta la humanidad en el umbral del siglo xx. Esto significa una participación cada vez mayor en programas conjuntos con entidades políticas internacionales.

El libro incluye unos útiles apéndices con los acrónimos de más de 150 asociaciones internacionales y una serie de tablas, figuras y estadísticas, que permiten comprender la estructura de ICSU, su funcionamiento y su propio desarrollo histórico. *Science International* es una obra que, además de ilustrar, hace pensar. ¿Cómo será el futuro de estas asociaciones en un mundo que, por una parte parece tender a la uniformización —la aldea global—, pero, por otra, ve acrecentar las diferencias entre los países más ricos y los más pobres? ¿Cómo evolucionará la relación ciencia–sociedad, teniendo en cuenta que el desarrollo de esta última depende de la primera más que en ningún otro momento de la historia? Estas son algunas de las incógnitas que el libro plantea.

Mercè Piqueras
Redacción de Microbiología SEM

Instructions to authors

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

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

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

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

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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.

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Continued from p. 124

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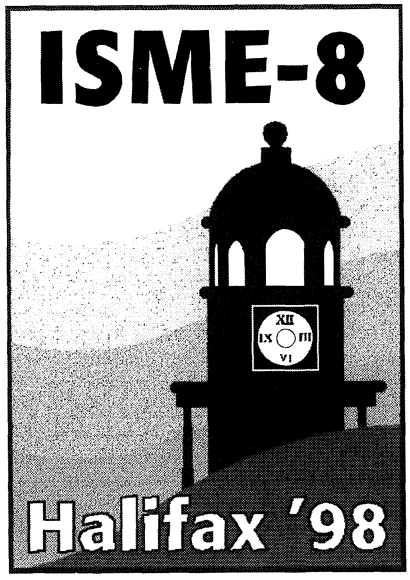

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