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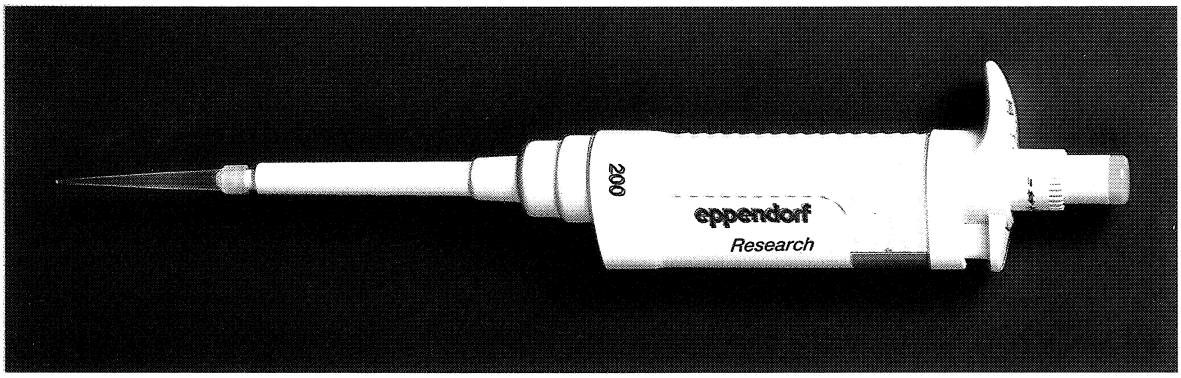

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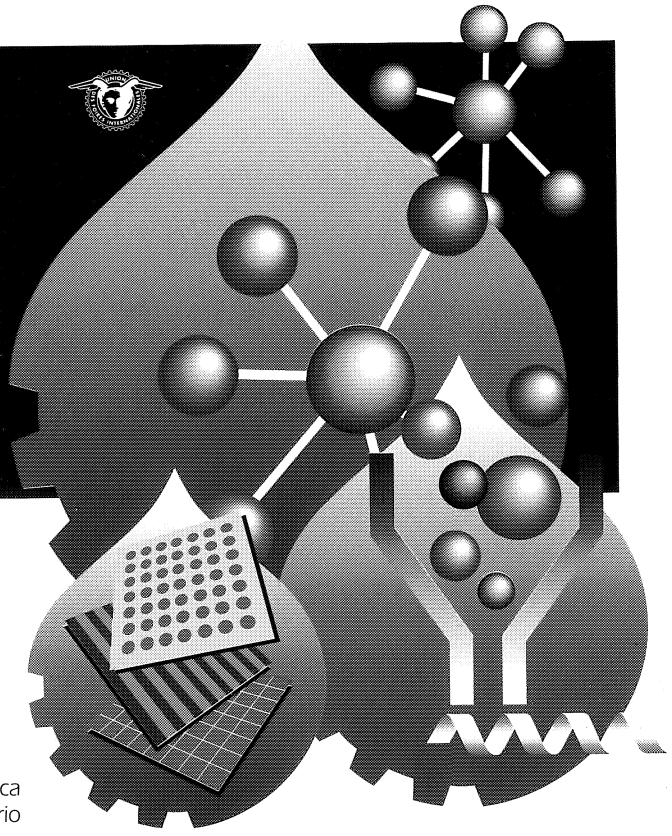

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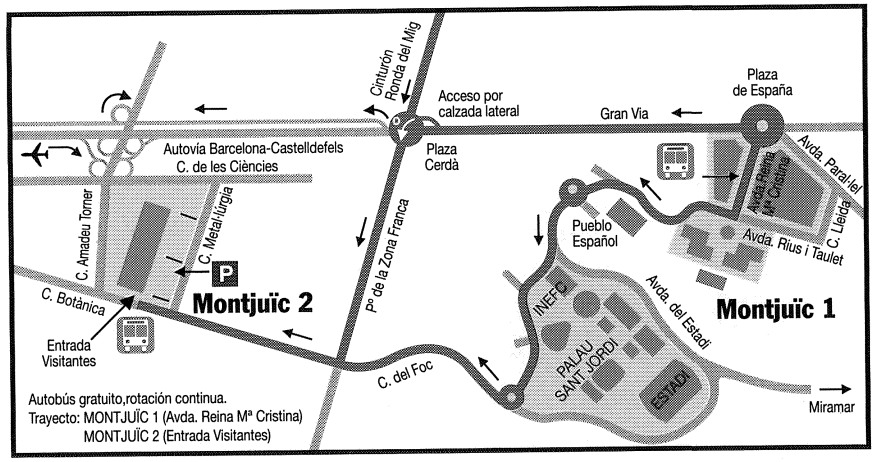

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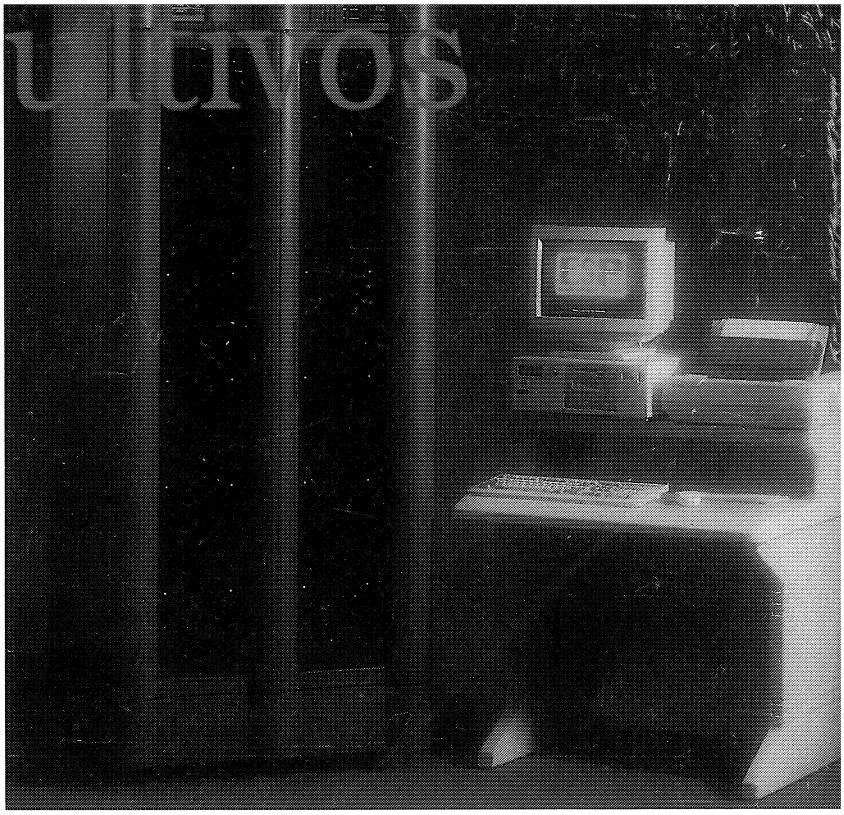

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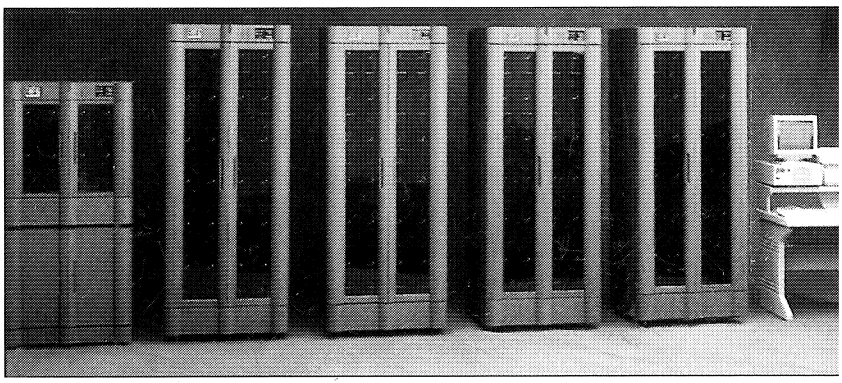

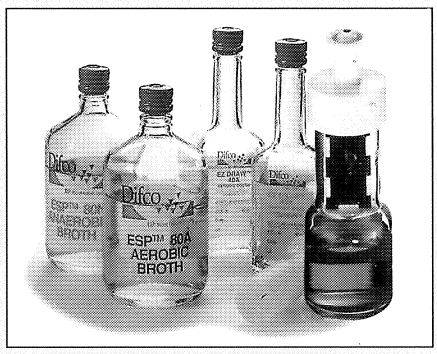

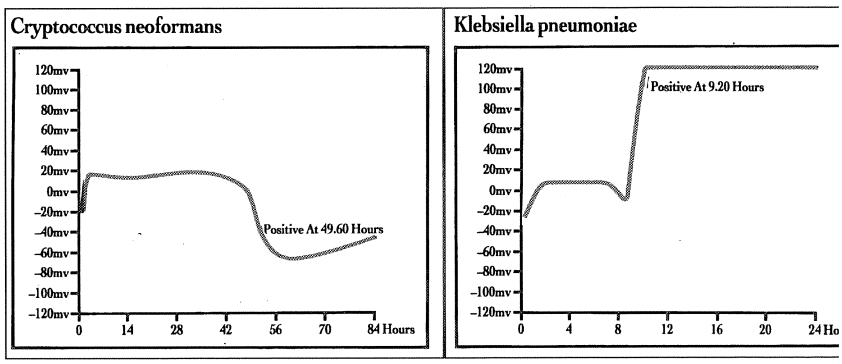

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MICROBIOLOGÍA SEM **12** (1996), 343–346

Editorial*

Instituciones de apoyo a la microbiología en Cuba

A principios de la década de1980, un grupo de científicos de Estados Unidos y Cuba crearon el NACSEX (Programa de Intercambio Científico Norteamericano–Cubano), cuyo objetivo, explícito en el propio nombre, era establecer mecanismos de intercambio científico e información sobre el desarrollo de la ciencia, especialmente biomédica, entre ambos países. El programa, de carácter no gubernamental por parte de Estados Unidos, permitió que algunos de sus científicos conocieran el estado de la microbiología en Cuba.

En 1981, con motivo de un programa sobre política de investigación pública, el Dr. Ernesto Bravo, de la Facultad de Medicina de La Habana, visitó la Boston University. Poco tiempo después empezábamos a organizar juntos el programa NACSEX. El Dr. Bravo, que había emigrado desde Argentina a Cuba, ha desempeñado un papel muy destacado en el establecimiento de programas de investigación biomédica y atención sanitaria, y también de política científica, en Cuba. En el verano de 1982, un grupo de científicos de Massachusetts del área de biomedicina organizaron, junto con el Dr. Bravo, visitas periódicas a Cuba.

En 1983, varios investigadores y escritores científicos de Estados Unidos pasaron una semana en Cuba visitando la Universidad de La Habana y otros centros de investigación, invitados por la Academia de Ciencias. Como presidente de la rama estadounidense de NACSEX, tuve oportunidad de observar el desarrollo de las ciencias biomédicas y biológicas (incluida la microbiología); también pude seguir el desarrollo de la atención sanitaria, y apreciar el quehacer de una comunidad científica que se abría a las modernas investigaciones en biotecnología. En 1984, en otro viaje a la isla, se impartió un curso teórico-práctico sobre biología molecular, al que asistieron investigadores

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* Prosiguiendo con el análisis de la situación científica en América Latina, en este número dedicamos el editorial al desarrollo de la microbiología en Cuba en los últimos años. Para ello hemos contado con la inestimable colaboración del Prof. Harlyn O. Halvorson (University of Massachusetts, en Dartmouth), conocedor de primera mano del tema.

jóvenes y estudiantes de diferentes centros. Ese mismo año, un grupo de microbiólogos de Estados Unidos y Europa organizaron un curso sobre ecología microbiana y evolución de la vida, con trabajo de campo incluido, que dio lugar a una publicación conjunta entre los investigadores participantes.

El año1985 nuevamente dos grupos del NACSEX, entre ellos escritores científicos, asistieron al Segundo Seminario Internacional sobre Biotecnología e Interferón. Después, el Dr. Silva Rodríguez, único cubano que consiguió visado de Estados Unidos, pasó tres meses en la University of Massachusetts, en Amherst, aprendiendo técnicas de secuenciación de ácidos nucleicos con el Dr. Robert Zimmerman.

Con alguna breve interrupción por problemas administrativos, las visitas a Cuba promovidas por NACSEX prosiguieron hasta junio de 1992. Durante el período de actividad de la organización, aproximadamente ochenta investigadores y escritores científicos impartieron conferencias, cursos breves, seminarios y clases prácticas sobre biología molecular, oncología, microbiología, biotecnología, botánica, ecología y otras materias. Varios escritores científicos publicaron diez artículos sobre Cuba en revistas de Estados Unidos e Inglaterra. En materia de ciencia, durante la década pasada, la única organización que ha ofrecido una vía de intercambio entre Estados Unidos y Cuba ha sido NACSEX.

Instituciones científicas de Cuba que realizan investigaciones en microbiología

En 1994 y en el curso de uno de mis viaje a Cuba, visité detenidamente diversas instituciones científicas. De la detallada memoria que realicé posteriormente, he extractado la información que sigue sobre los centros existentes en las diversas áreas y la actividad que se lleva a cabo en ellos. Sin duda, son muchas las dificultades, pero también es cierto que existe una comunidad científica en la que cada vez son más los jóvenes que se preparan y participan en su desarrollo, del cual se saben protagonistas. El futuro de la ciencia en Cuba depende de ellos y del apoyo que la comunidad científica internacional le preste, apoyo que, afortunadamente, se realiza desde bastantes países.

La Academia de Ciencias de Cuba es el principal organismo financiador de la mayoría de centros de investigación. Otros son la Facultad de Medicina de La Habana y el Ministerio de Salud. La Dra. Rosa E. Simeón Negrín, virólogа, es Presidenta de la Academia, que cuenta con un especialista en cooperación internacional científico-técnica. Las instituciones científicas se organizan en cinco Polos, que representan las regiones del país.

El Centro Nacional de Investigación Científica (CNIC), creado en 1965, constituye el pilar de la investigación básica. Sus investigadores han nutrido muchos de los institutos de más reciente creación. Se organiza en diez Divisiones, que incluyen, entre otros, Productos Naturales, Química Farmacéutica (síntesis química de drogas, transformaciones biológicas, antibióticos, fermentaciones), Toxicología, Servicios Analíticos, Neurociencia y Biología (Biología Molecular, Genética), Enfermedades Infecciosas (vacunas contra el cólera, vacunas BCG) y Botánica. En todos los departamentos se realiza ciencia básica. El CNIC publica unos 100 artículos anuales y envía alrededor de 100 investigadores fuera del país para realizar estancias de diferente duración en Francia, Inglaterra, Suiza, España, México, Brasil y Chile. El estudio y control de una epidemia vírica (virus RNA) a gran escala, con síntomas neurológicos y que afectó a un número de entre 10.000

y 20.000 personas, fue llevada a cabo por investigadores del Centro de Neurociencias. Sólo se tiene noticia de una epidemia similar en Japón. La Organización Mundial de la Salud (OMS), y un equipo de 15 científicos del CDC (Centers for Disease Control, EE. UU.), participaron en la investigación. La epidemia fue detenida con suplementos vitamínicos. Los cubanos montaron 40 laboratorios con 140 personas (80 de la plantilla de investigadores) y diseñaron los instrumentos para el seguimiento de la población y poder controlar la epidemia. Este centro, que cuenta con un laboratorio de biología molecular, participa en el mapado del genoma humano y en el estudio de las bases moleculares de la esquizofrenia.

El Centro de Ingeniería Genética y Biotecnología (CIGB) se inauguró en 1986, bajo la dirección del Dr. Manuel Limonta. Incorporó departamentos del CNIC y otras instituciones, entre ellos Inmunología, Biotecnología y Farmacología Industrial, Biotecnología Animal y Vegetal, División Analítica, División Clínica y la recientemente creada División de Química Física. Su actual director se ha formado en el Instituto de Proteínas de Osaka, en Japón. Se dispone de buen equipamiento para secuenciar péptidos mediante espectroscopía de masas, microscopía electrónica y de barrido, separación de péptidos en geles y cromatografía de afinidad, modelado de proteínas y cinética enzimática. Se desarrollan componentes de equipos de diagnóstico —anticuerpos monoclonales, proteínas recombinantes y péptidos sintéticos— para la validación de sangre y productos sanguíneos. Sus productos clave incluyen interferones recombinantes y naturales, factor de crecimiento epidérmico recombinante (rEGF), estreptoquinasa recombinante (para cuyo desarrollo Cuba obtuvo la primera patente), y vacuna recombinante de la hepatitis B. Tuve la oportunidad de visitar la División de Biotecnología Animal y Vegetal, donde se trabaja en los mecanismos de control de garrapatas de ganado, se estudia el uso de organismos transgénicos para controlar la expresión de la glándula mamaria y la embriogénesis temprana en animales pequeños, y se investiga en la manipulación del crecimiento en organismos acuáticos.

En 1994, la Academia de Ciencias inauguró el Instituto de Oceanografía, que está bajo la dirección del Dr. Mariano Bellota. Distribuidos en dos centros, tiene una plantilla de 90 trabajadores entre técnicos, doctores, estudiantes de doctorado y demás personal. Dispone de los departamentos de Farmacia, Electrofisiología, y Aislamiento y Purificación. Se estudian 74 especies de organismos marinos, a la búsqueda de toxinas (especialmente neurotoxinas de canales de K y Na) en ratones y cangrejos, compuestos antivirales y proteasas. Los estudios se realizan en colaboración con el Instituto de Medicina Tropical, y con la Facultad de Química para las determinaciones estructurales. Otros proyectos incluyen la obtención de antihepáticos, pigmentos, compuestos antitumorales, emulsionantes de bacterias y acuicultura (ostricultura y piscicultura).

En 1991 se creó el Instituto de Vacunas Finley —nombre del científico cubano descubridor del agente causante de la fiebre amarilla—. Incluye cuatro líneas de producción (tres bacterianas y una vírica) y las áreas de actuación son: *a*) centro de referencia de control de calidad sobre la hepatitis B; *b*) diagnóstico especial de *Neisseria* para comprobar la eficacia de su vacuna en países del tercer mundo; *c*) vacunas del cólera, meningococo múltiple, BCG, DPT, antirrábica, antitifoidea oral y sida. Se están produciendo vacunas para el tétanos, difteria, sarampión, BCG recombinante y leptospira celular completa.

El Centro Nacional de Bioproductos comenzó a funcionar en 1992. Situado a unos 60 quilómetros de La Habana, ocupa 11,6 hectáreas. Bajo la dirección del Dr. Silva Rodríguez, el centro trabaja sobre

a) fabricación de vacunas, medios de cultivo y equipos de diagnóstico a escala industrial; *b*) desarrollo de procesos biotecnológicos a gran escala; y *c*) formación y perfeccionamiento de investigadores.

El Instituto de Medicina Tropical tiene una larga historia. Fundado en 1937 por el Dr. Flori, un virólogo del Departamento de Parasitología de la Facultad de Medicina, participó en la década de los cuarenta en programas de colaboración con América Latina y Estados Unidos a través de la Cornell Medical School. El hospital trata enfermedades infecciosas, parasíticas y sida. Los casos positivos de HIV detectados en Cuba han sido 1.017, de ellos, 250 con sida, la mitad de los cuales han fallecido. El centro cuenta con cuatro plantas para microbiología y tres para parasitología. Se imparten enseñanzas en niveles universitario y de postgrado. Con ayuda de Unesco se imparten cursos cortos del programa "Pan American Health". El Centro recibe financiación del gobierno cubano, OMS, gobierno francés y fundaciones interesadas en las enfermedades tropicales. Se han diseñado cuatro equipos: *a*) antígenos toxoplásmicos para sida, *b)* ELISA fosfolipáticas de humanos y ganado, *c*) detección de proteasas en amebas, toxoplasma, y *d*) detección del dengue.

Los estudios sobre anticuerpos monoclonales empezaron en Cuba en 1982, en el Instituto Nacional de Oncología y Radiobiología (INOR). El trabajo con anticuerpos anti-linfocitos T (CD6) llevó a ensayos clínicos de anticuerpos t3 (anti CD3), usados en inmunosupresión en transplantes de órganos. Estos datos se obtuvieron en 1989 y al año siguiente se decidió la construcción de un edificio que albergaría el Centro de Inmunología Molecular (CIMAB); el edificio fue finalizado en 1994. La investigación se centra sobre nuevos anticuerpos monoclonales, purificación de antígenos y análisis. Disponen de instalaciones para la preparación de medios, control de calidad, estabulario, biblioteca y unidades de cultivos a gran escala, todo ello con un excelente equipamiento suizo. El diseño de ingeniería se realizó en Cuba y es muy impresionante. Además, se están preparando equipos de diagnóstico, incluyendo ELISA, para detección en suero o excrementos. Cabe destacar que todos los centros visitados cumplen las normas OMS y FDA (Food and Drug Administration, EE. UU.) en materia de control y seguridad a todos los niveles.

Todo lo expuesto, observado en los diversos viajes a la isla de Cuba por el autor, da idea del interés en el desarrollo de la ciencia y en la formación profesional de sus investigadores que muestran los cubanos. Sin duda, es algo que, más allá de consideraciones políticas, merece atención, interés y respeto por parte de toda la comunidad científica.

Harlyn O. Halvorson*
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Traducido por Alfredo Mayor y Carmen Chica
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Harlyn O. Halvorson es fundador y director del Police Center for Marine Biosciences and Technology, de la University of Massachussetts, en Dartmouth. Es experto en temas de microbiología marina y ha presidido diversas organizaciones científicas, entre ellas la American Society for Microbiology (ASM) y la American Association for the Advancement of Science (AAAS). Persona de gran experiencia, ha prestado su apoyo y colaboración al desarrollo científico de diversos países de América Latina, entre ellos Cuba.

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MICROBIOLOGÍA SEM **12** (1996), 347–358 **MINIREVIEW**

Are bacterial biofilms constrained to Darwin's concept of evolution through natural selection?

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Summary

Numerous antimicrobial agents have been developed which act at the molecular, cellular, and organismal levels. However, few have been developed which act at the community-level. This results largely from the failure of Darwinian selection theory to envision communities as units of proliferation and evolution. It is thus difficult to conceive of microbial communities as causative agents and to develop antimicrobials which are effective against them. Consequently, we find it necessary to consider a more comprehensive biological paradigm which envisions biofilm communities and other microbial associations (e.g. mixed infections, food spoilage, tooth decay) as units of existence, activity, ecology, proliferation, survival, and evolution. These communities exist in the same sense that organisms exist as units of ecological activity. This is a simpler, more comprehensive, and more unifying theory of ecology. It is simpler in that it no longer requires convoluted explanations of altruistic behavior in terms of individual selection. It is more comprehensive by not constraining evolution to the selection of any single level of biological organization (genes, races, lineages, or groups). It unifies in that it bridges the boundaries between microbial ecology, evolutionary ecology and ecosystem ecology. The basis for this theory lies in recognizing that life consists of various forms of information (order) which evolve not only through genetic recombination and mutation, but also through the recombination of organisms within communities (as well as other mechanisms, some of which are considered beyond the realm of biology). It also involves setting aside the concept of evolution through selection and competition, in favor of evolution through proliferation and association.

Key words: Darwinian selection theory, proliferation theory, community theory, Gaia, autopoiesis

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Resumen

Hasta el presente, se han desarrollado muchos agentes antimicrobianos que actúan a los niveles molecular, celular y del organismo. Sin embargo, se han producido muy pocos que actúen a nivel de comunidad. Esto es consecuencia, en gran parte, de la incapacidad de la teoría de la selección natural darviniana para considerar las comunidades como unidades de proliferación y evolución. Por ello, es difícil imaginar las comunidades microbianas como entidades causativas y desarrollar agentes antimicrobianos que sean efectivos contra ellas. En consecuencia, se hace necesario ampliar el paradigma microbiológico, que considera las comunidades de los biofilmes y otras asociaciones microbianas (p. ej. infecciones mixtas, degradación de alimentos, caries) como unidades de existencia, actividad, proliferación, supervivencia y evolución. Tales comunidades existen en el mismo sentido en que los organismos son unidades de actividad ecológica. Esta teoría ecológica es más simple, más comprehensiva y más unificadora. Es más simple porque ya no necesita explicaciones rebuscadas de un comportamiento altruista de los individuos. Es más exhaustiva porque no limita la evolución a la selección de un único nivel de organización biológica (genes, razas, linajes o grupos). Y más unificadora, porque difumina las fronteras existentes entre la ecología microbiana, la ecología evolutiva y la ecología de ecosistemas. La base de esta teoría reside en reconocer que la vida comprende varias maneras de información (orden), que evolucionan no sólo a través de la recombinación genética y de la mutación, sino también a través de la recombinación de los organismos dentro de las comunidades (así como de otros mecanismos, algunos de los cuales se considera actualmente que están más allá de los límites de la biología). La nueva teoría se aparta del concepto de evolución a través de la selección y competencia y escoge la idea de que la evolución se produce mediante la proliferación y la asociación.

Introduction

Bacteria proliferate and evolve as consortia, bioaggregates, biofilms and other biological networks in which the synergisms between associated organisms determine the success or failure of the entire association (12, 13, 14, 15, 17, 18, 19, 20, 21) as opposed to the success of solitary individuals. Some of these have evolved to become eukaryotic cells, each containing several prokaryotic lineages (1, 39, 40, 41, 42, 43, 44). This new perception of life and evolution raises the question of whether bacteria are unique in transcending Darwinian selection theory (22, 31), or whether eukaryotes might also be in the formative stages of evolving and proliferating not only as individuals but also as networks of organisms (13, 14). This includes a wide range of associations from lichens and *Ophrydium versatile* communities (25) to the possibility of establishing ecosystems on Mars (45, 46).

Community-level bacterial processes include biodegradation, gingivitis, calculus formation, tooth decay, denitrification, methanogenesis, biofouling, food spoilage, natural fermentations, biologically mediated corrosion, wastewater treatment, composting, sulfate reduction, and other processes. In these situations, a solitary species is not optimal and a consortium or community is required (14). If these organisms pro-

liferate more effectively in association, then it would be logical to study them as intact associations in the laboratory. However, for the past century microbiologists have been guided primarily by Koch's postulates and the germ theory (32, 33). This requires that causative relationships be determined exclusively through the study of pure cultures and precludes the possibility of culturing communities and consortia under controlled laboratory conditions. This concept has served the needs of microbiologists for more than a century and was most succinctly stated by Brefeld in 1881 (3): "Work with impure cultures yields nothing but nonsense".

However, if a bacterial community functions as a causative agent, it is also the community which succeeds or fails and survives or perishes as a consequence of the network of interactions among the organisms within it. Thus devising antimicrobial systems which are effective against pure cultures (at the cellular level) is no guarantee that they will be equally effective against biofilm communities (at the community level) (13, 15, 20, 21). In this situation one member of the community may be protected by the rest through exopolymer production or other synergisms, and this leads to false positive results in the testing of antimicrobial agents.

Under these circumstances an organism is susceptible to an antimicrobial agent when tested in isolation. However, the same organism is resistant when protected by other members of its normal community.

A false negative result can occur if the agent being tested is functional at the community level, and acts to inhibit the mechanisms of interaction between synergistic organisms (blocking chemoreceptors, inhibiting motility, etc.), as opposed to being able to inhibit the growth of organisms cultivated in isolation. Consequently, when establishing cause and effect relationships and testing antimicrobial agents, it is often necessary to use communities as units of laboratory study if community-level mechanisms are involved.

Many technical barriers to the study of microbial communities have been removed due to advances in microscopy, fluorescent molecular probes, and genetic engineering. Community culture methods have been developed (10,12, 50, 51, 52) and criteria have been established to determine when communities have been successfully cultivated as opposed to the cultivation of mixed cultures and enrichments (15). Using scanning confocal laser microscopy (SCLM) and fluorescent molecular probes; the growth, biochemistry, genetics, metabolism, and behavior of microorganisms can often be studied nondestructively, without uncoupling an organism from its community and microenvironment (7, 8, 9, 14, 20). Communities are cultured by providing a well-defined steady-state microenvironment. This allows individual organisms to organize into communities that are ultimately defined by their environment (12, 57). Emigration, immigration, and succession replace streaking and aseptic technique when subculturing these associations (along with their predators and parasites). Thus community culture has become a necessary step in studying communities as functional systems (12, 15), just as pure culture (organismal culture) is used to understand organisms as functional systems.

Cultivation of bacterial communities

Community cultures are unlike enrichments, or pure cultures. They are self-organizing networks, with member populations that are adapted to coaggregate, survive, and proliferate as a unit. They are the consequence of specific be-

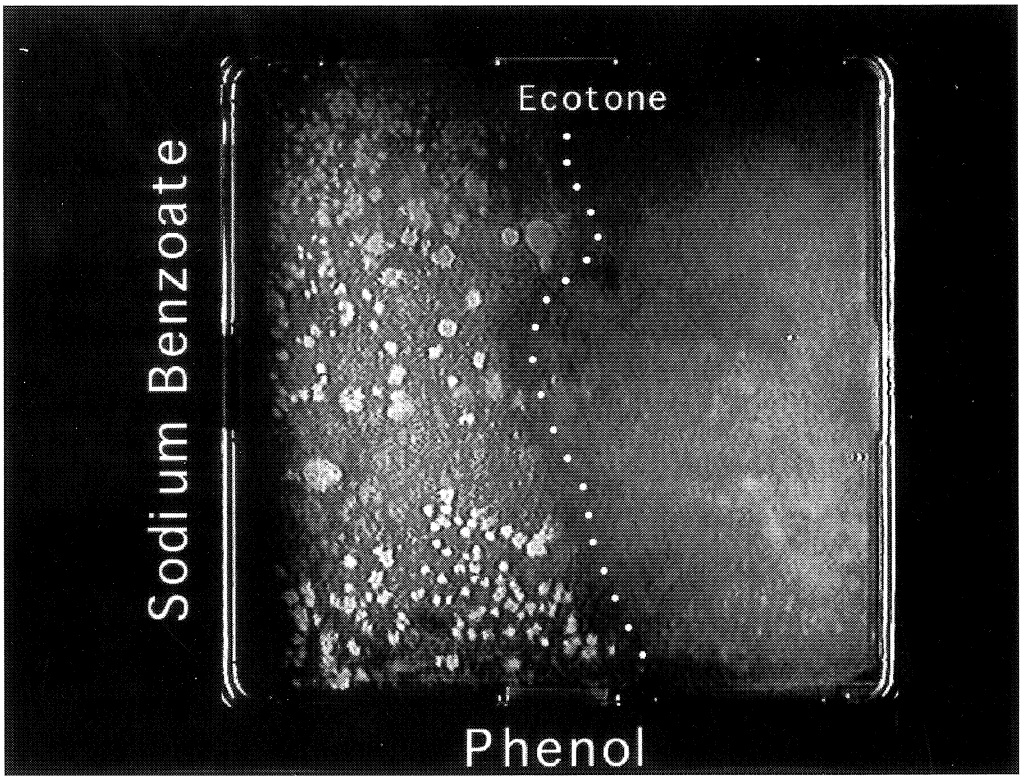

FIG. 1. A benzoic acid versus phenol gradient showing two bacterial biofilm communities and the ecotone between them (white dots).

havioral mechanisms (23, 34, 35, 37, 38) involving chemotaxis, motility, chemoreceptors, exopolymer production, phagocytosis, etc. These community cultures are products of their environment. Thus they are defined by defining the environment, rather than by using aseptic technique to isolate one organism from others. The controlled environment allows development of an autopoietic network of individuals that optimizes conversion of the abiotic to the biotic (14). Confirming that a community culture has been successfully obtained requires that criteria of communality, synergy, autopoiesis, and homeostasis have been satisfied (15). Isolation of communities under defined laboratory conditions also requires that community inputs and outputs be maintained to prevent the depletion of substrates and the accumulation of toxic endproducts or intermediates (11).

Fulfilling the criterion of communality requires that the culture be grown along an environmental gradient, as shown in Fig. 1, to determine whether an ecotone (a sharp inflection in community composition) occurs at some point along the length of the gradient. Ecotones confirm the existence of communities, and define their range as well as the boundaries between them (5, 6). The presence of an ecotone indicates that the community culture contains an interactive network of interdependent organisms. If each of the organisms in the culture were completely independent, then each would respond to the gradient individually at a unique location along the gradient, rather than responding in

unison at the ecotone. However, if two or more of the organisms represent an interactive network, then they respond together at a single location. Thus the existence of an ecotone confirms that the community is not simply a mixture of unrelated organisms, but an interactive network that must go through a reorganizational transition to a new set of interrelationships while responding to changing environmental conditions. Wolfaardt et al. (58, 59, 60, 61) have shown that in some cases degradative communities contain several consortia each of which might go through a distinct ecological shift at a unique location along a spatial or temporal environmental gradient.

Meeting the criterion of synergy requires that community members function more effectively in association than individually. This can be confirmed by comparing the performance of organisms grown alone and in association. Synergy can take many forms. These include; an increase in the collective cell yield for the association as opposed to its isolated members, a decrease in the half-saturation constant, an increase the rate of proliferation, an increase in habitat range, etc. Fulfilling the criterion of autopoiesis requires that the culture be defined by its physical environment rather than aseptic technique. This also requires that it be capable of forming spontaneously under septic conditions.

Fulfilling the criterion of homeostasis requires that the community be equally resilient when challenged by pure cultures which are foreign to the association or when challenged by changes in the ambient physical environment.

Enrichment theory versus community theory

Enrichment culture is used primarily as a method of isolating bacteria with specific physiological capabilities (28). Enrichment culture provides the specialized conditions often required to isolate organisms with a specific type of physiology or behavior. For example, the lack of fixed nitrogen in an enrichment medium leads to the proliferation of organisms which are capable of nitrogen fixation. Once these organisms have become numerically dominant, they can then be isolated on streak plates, pour plates, or in dilution tubes. Each of these isolation techniques relies primarily upon dilution to obtain an effective separation. The primary objective of enrichment culture is not to preserve or obtain diversity, but to decrease it as much as possible by favoring the organism which is to be isolated. As stated by Beijerinck in 1901 (2):

"Because of our very imperfect understanding of the environmental requirements of the majority of microbes, it is impossible in most enrichment culture experiments to go further than to bring about a relative increase in the numbers of a desired form without leading to a complete disappearance of the other species present. Often this partial enrichment only occurs at a particular stage of the experiment, whereas earlier and later other forms predominate. Because of this, enrichment culture experiments can be called "perfect" or "imperfect". In a perfect experiment a single species is isolated in all its varieties."

It is thus often assumed that the product of evolution in a defined environment should be a pure culture. However, it is clear from numerous continuous culture experiments that microorganisms tend to diversify when placed in a constant environment over a prolonged period (4, 16, 24, 26, 27, 48, 49, 55). Decreases in diversity occur only as a transient phase during the early stages of colonization when rapidly growing organisms have the opportunity to colonize before a community network has yet become organized and established. Consequently, the ob-

jective of enrichment culture is usually to reduce diversity within a culture, while the objective of community culture is to sustain or increase diversity.

Proliferation theory

Proliferation theory (13, 14, 15) postulates that biological organization arises through the association of evolving systems, as well as through the mutation and genetic recombination of these systems. Higher levels of order thus gradually emerge from lower levels as shown in Fig. 2. Evolving systems at lower levels of the hierarchy associate and result in the formation of more complex systems. Systems which evolve at higher levels of organization thus originate through the adaptation of individual systems as they proliferate, as well as through the association of evolving systems from lower levels of organization. As stated previously (14):

"The proliferation hypothesis involves the assumption that organisms proliferate more effectively by adapting through genetic mutation and genetic recombination. However, it also assumes that self-replicating molecules sometimes proliferate more effectively if they associate and then propagate as macromolecules (through covalent and ionic bonding), that self-replicating macromolecules sometimes proliferate more effectively if they associate and then propagate as prokaryotic cells (through formation of cell membranes and walls), that prokaryotic cells sometimes proliferate more effectively if they associate and then propagate as eukaryotic organisms (through endosymbioses and attachment), that both prokaryotic and eukaryotic organisms sometimes proliferate more effectively if they associate and then propagate as communities (through behavioral adaptations), and that communities sometimes proliferate more effectively if they then associate to form ecosystems and the biosphere as a whole".

There is little possibility of a single ordered subsystem evolving in isolation from the rest through a process of gradual adaptation as currently envisioned by contemporary selection theorists (31). The distribution and abundance of life in its totality, is a form of order which links all of life together as a single, evolving, hierarchical system irrespective of the relative importance of the various subordinate levels of organization which occur within. However, simple evolving systems must originate first before they can then associate to form more complicated systems which can then also evolve simultaneously along with their subordinate levels of organization (14). This implies that there must be a chronology involved, and that the simplest forms of life are more likely to have had a longer period to associate and then evolve at higher levels of organization (the community and ecosystem level). Thus it is possible that plants and animals may not have developed obvious community-level mechanisms of survival and reproduction. They may still be in the formative stages of developing these mechanisms. It is not that these mechanisms do not exist or are inactive, it is simply that they have not yet had time to be fully expressed. Consequently, the evidence for such systems of organization may not yet be as easy to discern for plant and animal communities as they are for microbial communities.

Community theory is thus a special case of proliferation theory and communities are only one of many ordered systems capable of proliferating by creating localized decreases in

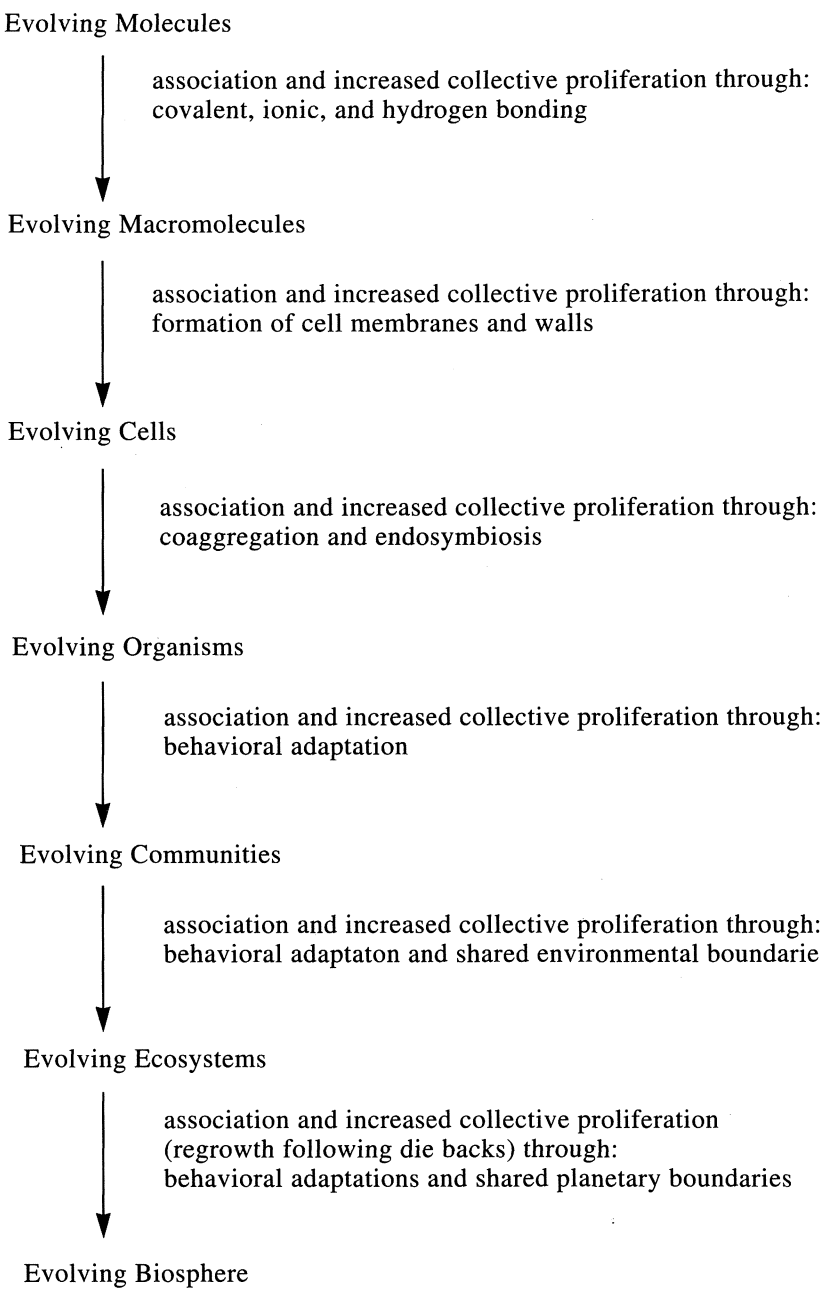

FIG. 2. Life as an evolving hierarchy of nested information systems. The evolution of information in these systems proceeds not only through the evolution of isolated systems (individuals) but also through the association of evolving systems to form composite systems which then evolve as independent units, which may themselves associate yet again to form another level in the hierarchy. Evolution is not restricted to any one level of this hierarchy and the evolution of the subcomponents within this hierarchy also constitutes evolution of the hierarchy of life as a whole. This is due to the common planetary boundary shared by all life forms. The importance of a common boundary in evolution (the membrane of a cell, the cortex of a lichen, the basin of a lake, etc.) is that spatial positioning of systems within defined boundaries constitutes a form of information and order which evolves in the same sense that the genetic code evolves. Boundaries also tend to link the collective success or failure of the subsystems enclosed within, but this is not their only role in the evolutionary process.

entropy at the expensive of their physical environment. Bacteria have had 3.5 billion years to develop community-level associations as compared to 0.4 billion for vertebrate animals. Consequently, microbial systems are more amenable to the formulation of testable community-level hypotheses than are plant and animal communities.

Proliferation theory is shown in Fig. 3 where it has been illustrated as a Forrester diagram. Isolated systems (units A, B, and C) evolve by undergoing genetic mutation or recombination and increasing their probability of proliferation as individual systems (units A', B', and C'). They may alternatively undergo mutation or recombination resulting in association with one or more other systems to form a composite system (unit ABC) which itself is then capable of undergoing a mutation or recombination event which may occur within one of the ABC subunits, but that affects the reproductive success of the composite as a whole rather than affecting only the subunit carrying the gene.

Natural selection as a theory of bacterial ecology and evolution

The driving force in ecology is the evolution of information systems that optimize conversion of the abiotic to the biotic. This is a question of

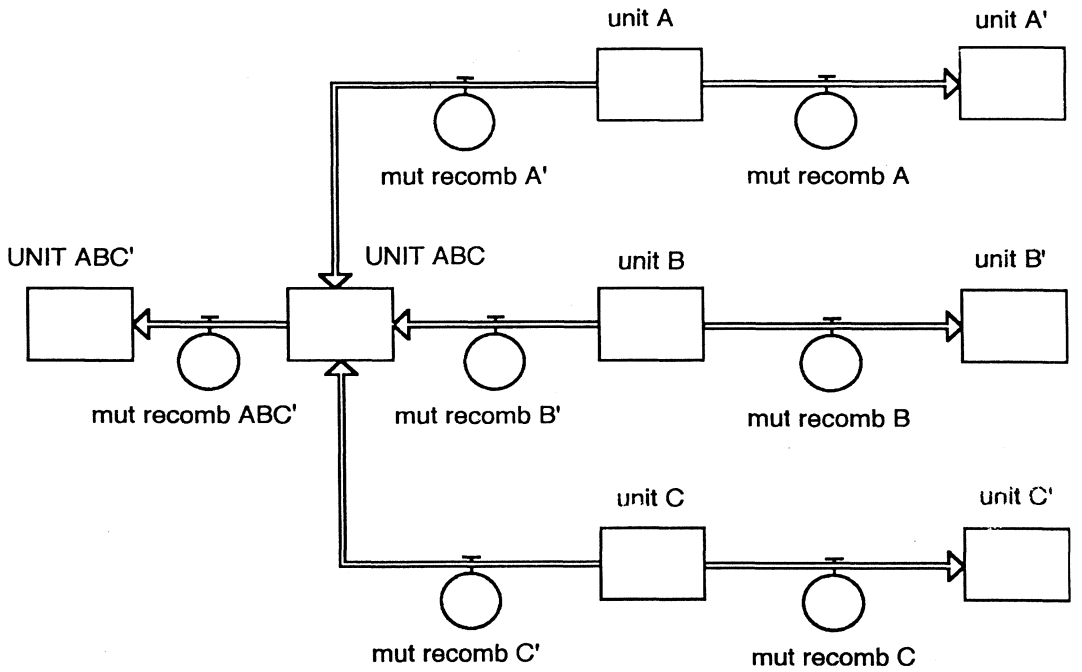

FIG. 3. A Forrester diagram of proliferation theory. Each of the boxes represents a self-replicating, evolving unit (clay minerals, genes, plasmids, cells, communities, ecosystems etc.) which is capable of proliferating. Each unit may evolve by undergoing a genetic recombination or mutation event that improves the system's probability of proliferation. Each unit may also evolve by undergoing a genetic change that results in the association of two or more systems to form a composite which then increases the composite's probability of successful proliferation.

the proliferation of life as a whole, as well as the occurrence and proliferation of specific individuals. These information systems include reproductive strategies (evolving concepts) that arise simultaneously at all the levels of biological organization. Consequently, life must be conceived as an evolving hierarchy of nested systems. It is not plausible to consider the evolution of one level of organization while denying the evolution of the other elements.

There is no question that Darwin's theory of natural selection can be used to explain the diversity of life at the organismal level (species). However, proliferation theory is simpler and more comprehensive. It explains not only the diversity of species but also the diversity of plasmids, genes, communities, ecosystems, human thought and language, as well as digital and other forms of evolving information. Thus according to the law of frugality (Ockham's razor), proliferation theory should be used preferentially if bacterial communities and other systems of organization are to be adequately studied and understood.

Conclusions

Most evolutionary theorists base their understanding of ecology on the assumption that natural selection results in an optimal strategy for the reproductive success of individual organisms and that biological complexity at other levels of organization (genes, plasmids, communities, ecosystems, and the biosphere) is a coincidental by product of this individual selection process (22, 31). This assumption has been completely rejected by some (14) and in part by others (29, 30, 40, 43, 44, 56). For many, the species concept itself is inadequate to understand and formulate hypotheses concerning the origin and nature of life (43, 47, 53, 54).

Confocal laser microscopy, fluorescent molecular probes and digital image analysis (8, 12, 36) provide the opportunity to focus on alternative hypotheses which are readily amenable to direct experimental testing using cultured microbial communities (10, 12, 15). Thus it is no longer necessary to rely primarily on descriptive studies and historical data (the fossil record, genetic sequences, and studies of natural communities) to resolve theoretical questions relating to ecology and evolution. The repercussions of these advances in knowledge will be profound. Darwin's theory of evolution through natural selection has become an integral part of western culture and language, as well as its political, economic, social, and educational institutions.

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Recombinant *Acremonium chrysogenum* strains for the industrial production of cephalosporin†

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Summary

Conventional strain improvement programs based on random mutagenesis and rational screening have meant valuable results to the antibiotic producing companies. The development of recombinant DNA techniques and their applications to the industrially-used cephalosporin-producing fungus *Acremonium chrysogenum* has provided a new tool, complementary to classical mutation, promoting the design of alternative biosynthetic pathways making it possible to obtain new antibiotics and to improve cephalosporin production. Yield increases have been achieved by increasing the dosage of the biosynthetic genes *cefEF* (deacetoxycephalosporin C expandase/hydroxylase) and *cefG* (deacetylcephalosporin C acetyltransferase) or enhancing the oxygen uptake by expressing a bacterial oxygen-binding heme protein (*Vitreoscilla* hemoglobin). New biosynthetic capacities such as the production of 7-aminocephalosporanic acid (7-ACA) or penicillin G have been achieved through the expression of the foreign genes *dao* (D-amino acid oxidase) coupled with cephalosporin acylase or *penDE*(acyl-CoA:6-APA acyltransferase) respectively. Confined manipulation of the above-mentioned recombinant strains must be performed according to standing rules.

Key words: *Acremonium chrysogenum,* 7-aminocephalosporanic acid (7-ACA), penicillin, strain improvement, hemoglobin

† Dedicated to the memory of Elvira Gil.

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Resumen

Los programas de mejora de cepas basados tradicionalmente en mutación al azar y selección racional de mutantes han proporcionado valiosos resultados a las compañías productoras de antibióticos. El desarrollo de las técnicas de DNA recombinante y su aplicación en *Acremonium chrysogenum*, hongo utilizado industrialmente para la producción de cefalosporina, ha permitido disponer de una nueva herramienta, complementaria de la mutación clásica, que ha hecho posible diseñar rutas biosintéticas alternativas, con la consiguiente síntesis de nuevos antibióticos y el incremento de la capacidad de producción de cefalosporina. Una gran mejora en la productividad se ha conseguido mediante el aumento de la dosis génica de los genes biosintéticos *cefEF* (desacetoxicefalosporina C expandasa/hidroxilasa) y *cefG* (desacetilcefalosporina C acetiltransferasa), o aumentando la disponibilidad de oxígeno gracias a la expresión de una hemoglobina bacteriana procedente de *Vitreoscilla*. La capacidad de biosintetizar ácido 7-aminocefalosporánico (7-ACA) o penicilina G se ha conseguido mediante la expresión heteróloga del gen *dao* (D-aminoácido oxidasa) acoplado con cefalosporina acilasa o del gen *penDE* (acil-CoA:6-APA aciltransferasa) respectivamente. La utilización confinada de las cepas recombinantes mencionadas anteriormente debe realizarse de acuerdo con la normativa vigente.

Introduction

Antibiotics, as secondary metabolites, are produced through multistep biosynthetic pathways, starting from intermediates of primary metabolism to specific moieties. The component moieties of secondary metabolites, activated as adenylated, phosphorylated or CoA derivatives, are finally linked to form the final products. Biosynthetic steps are catalysed by specific enzymes for each antibiotic (38).

Strain improvement by random mutation and screening has proven to be a rewarded process over the past decades. This essential part of the traditional programs of the improvement of antibiotic production, has allowed to block undesired enzymatic activities, remove negative regulations, increase gene dosage, etc. Strains to produce new antibiotic can be selected to cover specific purposes: to increase antibiotic production, to use unexpensive raw materials, to modify cell morphology, to control temperature and oxygen needs, etc. A classical example of this strategy is the production of penicillin by the fungus *Penicillium chrysogenum*: penicillin yield has been increased more than two orders of magnitude from the 1950s to present time.

The use of recombinant DNA techniques, available from the 70s, is expanding the opportunities for human profit of living resources. The application of genetics to industrial production processes focuses in obtaining new products and in a more efficient manufacturing of those already established. Reports on the cloning of antibiotic biosynthetic genes from industrially exploited fungi were first published in the mid-1980s, and the first use of recombinant DNA to improve an industrial antibiotic production in 1989 (52).

This article offers an overview of the applications of recombinant DNA technology to the improvement of *Acremonium chrysogenum* strains used in the industrial production of cephalosporin.

Cephalosporin biosynthesis

Cephalosporins are chemically characterized by a cephem nucleus in which a β-lactam ring is fused to a dihydrothiazine ring. Their biosynthetic pathway (Fig. 1) has been subject of detailed study over the last 20 years, but many aspects of the reactions carried out by the different biosynthetic enzymes remain still obscure. β-lactam antibiotics come from the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthesized from the amino acids L-α-aminoadipic acid, L-cysteine and L-valine by the enzymatic activity ACV synthetase (ACVS). Pioneer studies proved the presence of ACVS activity in crude cell-free extracts of *A. chrysogenum* (3), and afterwards this enzyme was purified and characterized from *A. chrysogenum* (2), *Aspergillus nidulans* (56) and *Streptomyces clavuligerus* (31). ACVS catalyzes the ATP-dependent activation of each L-amino acid precursor, the binding of the activated amino acids as thioesters, the epimerization of L-valine to D-valine and finally the two sequential transpeptidation reactions giving ACV (56).

ACV is cyclized by the isopenicillin N synthase (IPNS) to form isopenicillin N (IPN), an intermediate that has an L-α-aminoadipyl side chain attached to the 6-aminopenicillanic (6-APA) nucleus and shows weak antibiotic activity. 6-APA is structured by a β-lactam fused to a thiazolidine ring. This reaction was first observed in cell-free extracts of *P. chrysogenum* (16); later on, the IPNS activity was described and the enzyme purified from *A. chrysogenum* (42), *P. chrysogenum* (44), *S. clavuligerus* (29), *Nocardia lactamdurans* (7), etc. Because its broad substrate specificity, IPNS has been used for the synthesis of new penicillins from ACV analogs (58).

IPN is transformed to penicillin N by the isopenicillin N epimerase (IPNE), which isomerizes the L-α-aminoadipyl side chain to the D configuration. This enzyme was first described in cell-free preparations of *A. chrysogenum* (32), and highly labile activity was observed (27). However, the corresponding enzymes from *S. clavuligerus* (28) and *N. lactamdurans* (34) resulted more stable.

In *A. chrysogenum*, penicillin N is transformed into deacetylcephalosporin C (DAC) by the bifunctional enzyme deacetoxycephalosporin C synthase/hydroxylase (so-called expandase/hydroxylase) (49). This enzyme first converts the five-membered thiazolidine ring of the penicillin N into the six-membered dihydrothiazine ring of cephalosporins, to form deacetoxycephalosporin C (DAOC), and then hydroxylates DAOC rendering DAC. Both enzymatic activities are located in a single protein in *A. chrysogenum* (15), while in actinomycetes there are two separate enzymes (10, 30).

Acetylation of DAC to form cephalosporin C is the final reaction in cephalosporin producing fungi (17). Cephalosporin C can be degraded to DAC by acetylhydrolase activity present in *A. chrysogenum* (23).

Cephalosporin biosynthetic genes

As proposed by Ingolia and Queener (24), the common genes to the penicillin and cephalosporin biosynthetic pathways are named *pcb*, the genes that are only involved in penicillin biosynthesis are named *pen*, whereas those only involved in cephalosporin biosynthesis are named *cef*.

The *pcbAB* gene encoding ACVS has been characterized from several filamentous fungi, including *P. chrysogenum* (14, 55), *A. chrysogenum* (20) and *Aspergillus nidulans* (37). The transcription direction of the above mentioned *pcbAB* genes is opposite to that of the *pcbC* gene. In contrast, studies achieved in *S. clavuligerus*,

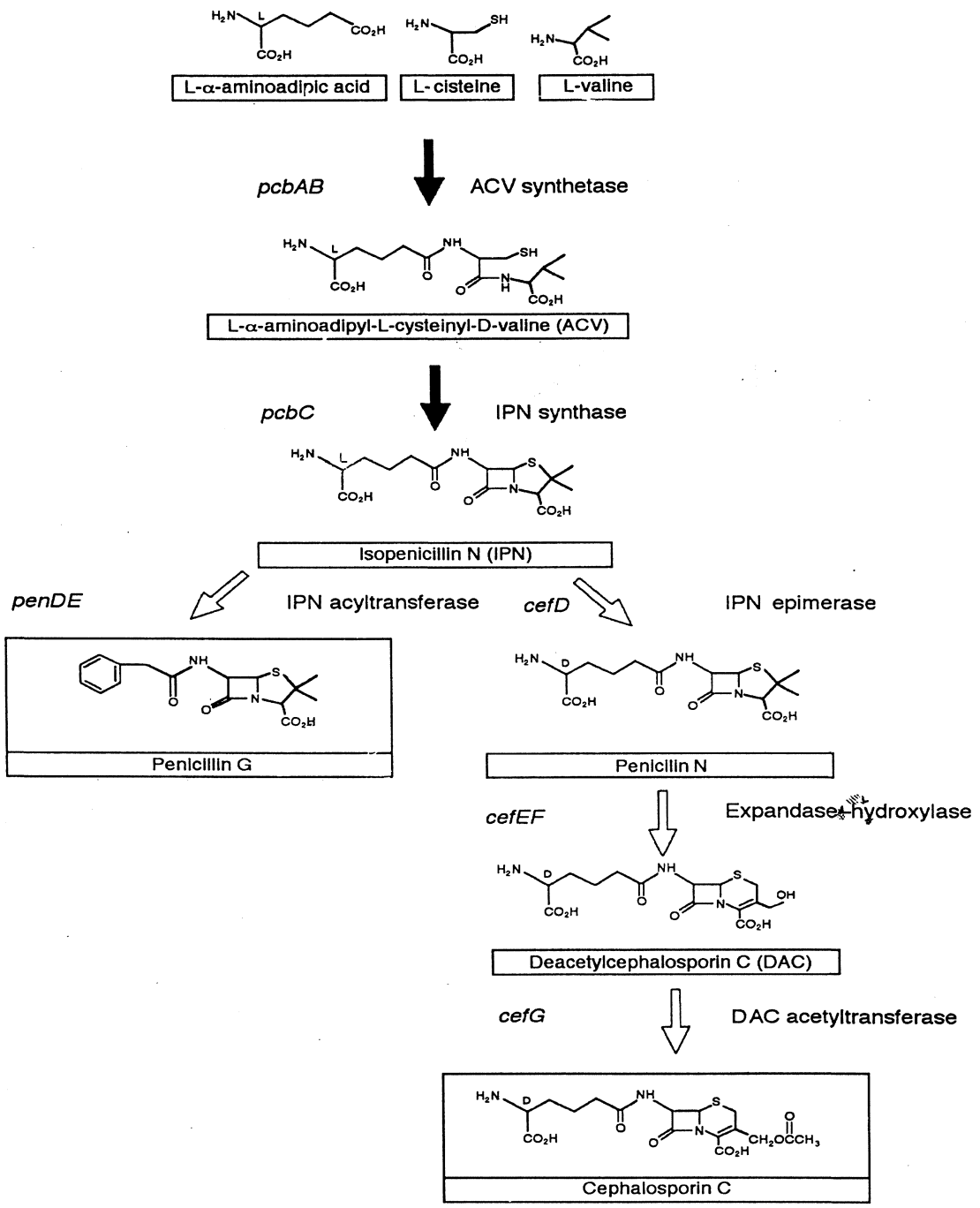

FIG. 1. Cephalosporin C and penicillin G biosynthetic pathways. Dark arrows indicate common biosynthetic steps and open arrows show specific steps. *pcbAB* and *pcbC* genes, encoding ACVS and IPNS respectively, are common to both pathways. *cefD* gene encoding IPNE has been identified in *Streptomyces* spp. but not yet in *A. chrysogenum*. *cefEF* gene encodes an enzyme with both DAOC synthase (expandase) and DAC synthase (hydroxylase) activities in *A. chrysogenum*. Prokaryotic species have *cefE* and *cefF* individual genes, coding for separate enzymes. *penDE* codes for AAT in *Penicillium chrysogenum* and *cefG* for DAC acetyltransferase in *A. chrysogenum*.

N. lactamdurans, *Flavobacterium* sp. and *Lysobacter lactamgenus* YK90 indicate that, in bacteria, the direction of transcription of the *pcbAB* and *pcbC* genes is the same (38). ACVS contains three domains with conserved amino acid sequences that are homologous to enzymes involved in ATP-mediated amino acid activation. The existence of three amino acid activating domains in the ACV synthetases is consistent with the organization in tyrocidine synthetase II and III, which activate 3 and 6 amino acids respectively during tyrocidine biosynthesis.

IPNS activity is present in a polypeptide encoded by the *pcbC* gene. This gene was first cloned from *A. chrysogenum* and expressed in *E. coli,* where it produced an enzyme with the same kinetics and substrate specificity of the fungal enzyme (46). *The pcbC* genes of several other microorganisms which produce β-lactam antibiotics have been cloned and characterized: *P. chrysogenum* (4, 6), *A. nidulans* (43, 57), *Streptomyces clavuligerus* (35), *Streptomyces lipmanii* (50, 57), *Streptomyces griseus* (18), *Streptomyces jumonjinensis* (50), *Flavobacterium* sp. (50), *N. lactamdurans* (8), etc. The similarity found between the IPNS from actinomycetes and fungi suggests a close evolutive relationship. It has been proposed that the genes encoding enzymes involved in β-lactam biosynthesis evolved first in *Streptomyces,* or other bacteria, being later transferred to filamentous fungi (6, 43, 57). All known IPNS enzymes, except that of *N. lactamdurans,* contain two conserved cysteine residues (8). Mutated *A. chrysogenum* IPNS lacking the cysteine-106 showed a 5% specific activity as compared to the enzyme derived from the native gene and the K_M value increased five-fold. However, the mutation in the cysteine-225 produced only a small decrease of enzyme activity (47). This second cysteine corresponds to the cysteine absent in the *N. lactamdurans* enzyme. The recently described crystal structure of IPNS will allow in-depth investigations on its catalytic mechanisms (45).

Amino terminal sequence of the purified IPNE was used to clone the *cefD* gene from *S. clavuligerus*. The gene was located immediately upstream of the *cefE* gene, and both are expressed from the same transcript (33). The *cefD* genes from *S. lipmanii* (54) and *N. lactamdurans* (9) have also been characterized, and show broad identity between them. Nevertheless, the putative *cefD* gene of *A. chrysogenum* has not been identified yet. The *cefEF* gene encodes DAOC synthase/hydroxylase in *A. chrysogenum*, a 36,460 Da bifunctional protein that includes both expandase and hydroxylase enzymatic activities (48). In *S. clavuligerus* and *N. lactamdurans cefE* and *cefF* are individual genes coding for separate enzymes (38). Comparison of the *cefE* and *cefEF* genes showed close identity, which supports the hypothesis that the *A. chrysogenum cefEF* gene has been originated by fusion of separate primordial genes arised in *Streptomyces*. In *S. clavuligerus,* genes *pcbAB*, *pcbC*, *cefE* and *cefF* are physically linked in the genome, whereas in *A. chrysogenum,* the genes encoding the first two enzymes of the cephalosporin biosynthetic pathway (*pcbAB* and *pcbC*) are clustered in chromosome VI, and the genes coding for the enzymes that catalyze the three final steps of the pathway (*cefEF* and *cefG*) are clustered in chromosome II (53).

cefG gene encoding DAC acetyltransferase is linked to the *cefEF* gene in *A. chrysogenum*, and is expressed in opposite orientation (21). The expression level of the *cefG* gene in *A. chrysogenum* is clearly smaller than that of the *cefEF* gene (38, 39).

Cephalosporin yield increase in *A. chrysogenum*

The first recombinant DNA improvement of a high producing industrial strain was described

by Eli Lilly researchers on *A. chrysogenum* (52). The approach involved: (i) identification of significant accumulation of an intermediate (penicillin N) by the highly productive strain, (ii) isolation of the enzyme (expandase/hydroxylase) that acts on that intermediate, (iii) cloning of the corresponding gene (*cefEF*), (iv) introduction of a stably maintained copy of the gene into the genome of the high producing strain, and (v) screening of a small number of transformants for improved conversion of the accumulated intermediate and cephalosporin overproduction.

During industrial scale cultivation, *A. chrysogenum* strain 394-4 was observed to repetitively accumulate a substantial quantity of penicillin N in the broth. Data from HPLC analysis of 33 fed-batch stirred cultivations were collected, finding a ratio of penicillin N to IPN of about 5 to 1, and a molar ratio of penicillin N to cephalosporin C of 0.3. These results suggested that expandase/hydroxylase activity encoded in *cefEF* gene should be the limiting step in cephalosporin C synthesis in the strain 394-4. To test this hypothesis, plasmid pPS56 was constructed for the introduction of one or more extra copies of *cefEF* gene in strain 394-4. The plasmid included the hygromycin phosphotransferase gene, as a dominant selection marker conferring hygromycin B resistance, and the *cefEF* gene in a 7 kb *Bam*HI genomic DNA fragment. Using previously described conditions (51), pPS56 was used to generate hygromycin B resistant transformants of *A. chrysogenum*. After verification of hygromycin B resistance, 30 transformants were selected for laboratory scale testing of cephalosporin C and penicillin N production. Five of these transformants ranged between 20 and 39% of cephalosporin C yield improvement in shaked flasks. In every transformant with cephalosporin C improved titer, the amount of penicillin N in the medium was reduced in comparison with the untransformed recipient strain.

The highest producing transformant, strain LU4-79-6, was selected for expandase/hydroxylase enzymatic level analysis, showing a near two-fold increase in the specific activity of this enzyme. Southern analysis of this transformant strain showed the presence of a single heterologous integration event. Stability of the transforming DNA was observed after laboratory scale and pilot plant cultivations without selective pressure as could be expected for chromosomal integration. The 15% increase of cephalosporin C production at pilot scale was accompanied by an equivalent reduction of the penicillin N accumulated with regard to the untransformed recipient strain. Eli Lilly scientists remarked that this 15% improvement was found by screening the first 8 isolates produced by genetic transformation of the highly developed strain, whereas no improved strains were found among tens of thousands of nitrosoguanidine- and UV-treated derivatives of the same production strain.

Nevertheless, posterior cloning of the *cefG* gene (21) showed that it was linked to the *cefEF* gene and included in the 7 kb *Bam*HI fragment used in the above mentioned transformations. This point raised the possibility that the effects observed in the transformants were probably due to the enhanced expression of the *cefG* gene, as its expression has been reported to be limiting in *A. chrysogenum* (39).

Industrial scale cultivation of the strain LU4-79-6 was examined and approved by the National Institutes of Health following recommendation of its Recombinant DNA Advisory Committee.

Penicillin G production by *A. chrysogenum*

P. chrysogenum and other penicillin-producing fungi exchange the α-aminoadipyl side chain of IPN for aromatic acids, such as phenylacetic or

phenoxyacetic acids, by means of the isopenicillin N acyltransferase activity (AAT) (1), encoded by the *penDE*gene (5), following the biosynthesis of penicillin G or V respectively (see Fig. 1) (36). *A. chrysogenum* and other cephalosporin producers lack AAT activity, and no DNA sequence homologous to the *penDE* gene of *P. chrysogenum* was found in their genome. Filamentous fungi produce either penicillins or cephalosporins; however, none of the known β-lactam producers is able to synthesize both types of antibiotics.

The heterologous expression in *A. chrysogenum* of the *penDE* gene of *P. chrysogenum* caused simultaneous production of benzylpenicillin (penicillin G) and cephalosporin C in phenylacetate fed cultivation of the recombinant strains (19). Northern analysis of the transformants revealed a single 1.15 kb band indistinguishable in size from the original transcript of *P. chrysogenum*. This result suggested that the gene should be expressed in *A. chrysogenum* from its own promoter, since the transcription signals and expression motifs appear to be conserved in filamentous fungi. Furthermore, the three introns of the *P. chrysogenum penDE* gene were properly processed by *A. chrysogenum*. The 40 kDa pre-AAT encoded by the *penDE* gene is post-translationally processed in *P. chrysogenum* giving rise to two (11.5 and 28.5 kDa) polypeptides. It is not established if this processing also occurs in *A. chrysogenum*.

The branching of the biosynthetic pathway to penicillin G and cephalosporin C at the IPN step resulted in a reduced level of cephalosporin C accumulated, but approximately the same total production of β-lactam. The relative amounts of each antibiotic were determined by the specific activities and substrate affinities of the two competing enzymes IPNE and AAT. *A. chrysogenum* strains accumulating penicillin N may favor penicillin production in this fungus, since penicillin N seems to be in balance with IPN. Inactivation of the IPNE or expandase/hydroxylase activities in AAT expressing strains of *A. chrysogenum* might result in penicillin overproduction. Another possible industrial application of these transformants could be the adipyl-cephalosporin production (11), which would simplify the enzymatic production of 7-aminocephalosporanic acid (7-ACA).

7-aminocephalosporanic acid production by *A. chrysogenum*

7-aminocephalosporanic acid (7-ACA) has a great industrial interest for the preparation of clinically important semisynthetic cephalosporins, and has traditionally been produced by complex chemical methods which imply the use of organic solvents. The development of an alternate enzymatic procedure, performed in water, is a major goal of the pharmaceutical companies. A two-step enzymatic procedure (Fig. 2) has been proposed (25), which involves the conversion of cephalosporin C to 7-β-(5-carboxy-5-oxopentanamido)-cephalosporanic acid (keto-AD-7-ACA) by the activity D-amino acid oxidase (DAO). The keto-AD-7-ACA reacts nonenzymatically with the hydrogen peroxide coproduced in the above reaction to give 7-β-(4-carboxybutanamido)-cephalosporanic acid, also named glutaryl-7-ACA (GL-7-ACA). GL-7-ACA, keto-AD-7-ACA and cephalosporin C are hydrolyzed at different rates to 7-ACA by cephalosporin acylase activity (12, 40).

In a posterior report Isogai and coworkers from Fujisawa Pharmaceutical Company (26) described the construction in *A. chrysogenum* of a 7-ACA biosynthetic gene cluster, consisting of the genes encoding the enzymatic activities DAO from *Fusarium solani* M-0718 and cephalosporin acylase from *Pseudomonas diminuta* V22. Both genes were

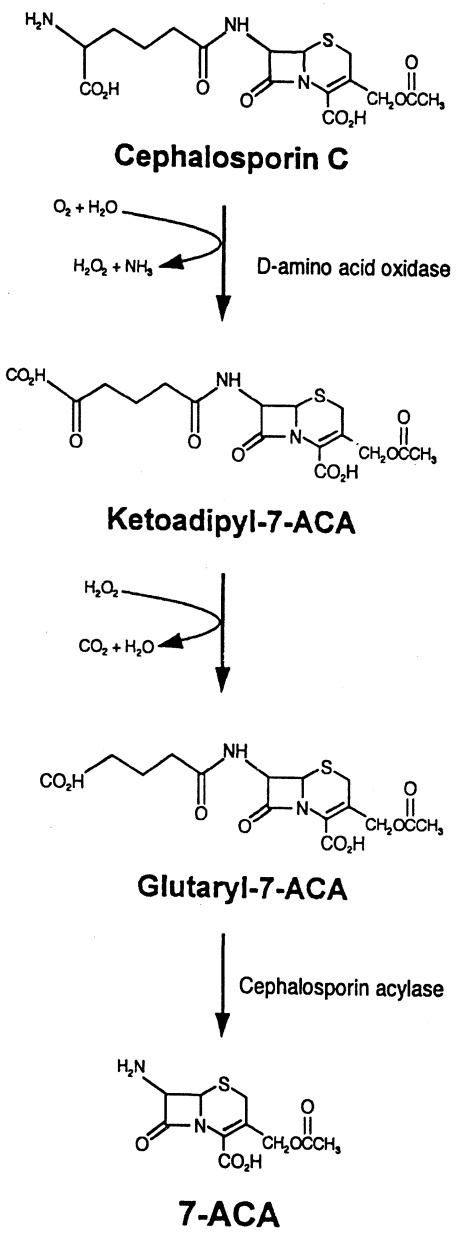

FIG. 2. Enzymatic synthesis of 7-ACA from cephalosporin C using the two-step enzymatic procedure involving D-amino acid oxidase (DAO) and cephalosporin acylase (GL-7-ACA acylase). Ketoadipyl-7-ACA reacts nonenzymatically with the hydrogen peroxide coproduced in the preceding reaction, giving glutaryl-7-ACA.

cloned under transcriptional control sequences of *A. chrysogenum* alkaline protease gene together with the hygromycin B resistance marker, and the resulting plasmid pHDV11 was introduced in the high cephalosporin-producing strain *A. chrysogenum* BC2116. Transformant strains expressing this 7-ACA biosynthetic pathway produced about 150 mg/ml of 7-ACA and no GL-7-ACA, but two side-products were detected: 7-aminodeacetylcephalosporanic acid (7-ADACA) and 7-aminodeacetoxycephalosporanic (7-ADCA). 7-ADACA and 7-ADCA have probably been synthesized from DAC and DAOC respectively, two intermediate compounds in the biosynthetic pathway of cephalosporin C.

Although the cephalosporin C to 7-ACA conversion yield of pHDV11 transformants resulted commercially insignificant (1.7%), and there was a 25% reduction in the cephalosporin C titer, this represents the first report of 7-ACA microbial production and proves the feasibility of introducing new biosynthetic capabilities into industrial microorganisms by combining fungal and bacterial genes. Yield decrease could be related with the use of the β-lactamase gene as bacterial selection marker in pHDV11, as low level gratuitous expression of a cloned β-lactamase gene in *Saccharomyces cerevisiae* transformants has been observed (52).

Improvement of oxygen availability and cephalosporin production in *A. chrysogenum*

Cephalosporin C biosynthesis is regulated by environmental factors including nutrients or oxygen availability. *A. chrysogenum* cultivation under low-oxygen conditions severely reduces the overall rate of cephalosporin C synthesis, possibly affecting the biosynthetic oxidation reactions of the pathway: ACV cyclization, thiazolidine ring expansion and DAOC hydroxylation, leading to the accumulation of penicillin N in stirred tank reactor (22). The development of technologies which improve the aerobic metab-

olism of the fungus should have a positive effect on cephalosporin C production.

Intracellular expression of a heme-binding protein (hemoglobin) from the gliding bacterium *Vitreoscilla*, a member of the *Beggiatoa* group, in several industrial antibiotic-producing microorganisms, resulted in improvements probably originated by the increment of the oxygen flux to the respiratory apparatus. Expression of this hemoglobin in *A. chrysogenum* under the control of the TR1 promoter of *Trichoderma reseii* originated recombinant strains that produced significantly higher yields of cephalosporin C than the untransformed parental strain (13). Using standard cultivation programs, two selected transformants produced 4.0 g/l cephalosporin C, significant productivity increase over the untransformed strains (1–2 g/l). Reduced aeration conditions resulted in this transformants in 5-fold higher cephalosporin C levels than the parental strain (3.0 g/l vs. 0.6 g/l).

Northern analysis of the overproducing transformants revealed a poor level of hemoglobin transcript when compared with highly expressed genes as *cefEF*. Their expression under the control of stronger transcriptional promoters as well as the replacement of unfrequent fungal codons might increase its efficacy.

Future prospects

DNA fragments including regulatory and/or structural genes that affect the biosynthesis of β-lactam antibiotics constitute a suitable source of information that can be applied to strain improvement and to the design of new antibiotics. Expression studies of cis-regulating promoter sequences of cephalosporin biosynthesis genes will provide additional information about possible bottlenecks in the pathway.

In this way, Menne and coworkers (41) performed studies with the bidirectional *pcbAB-pcbC* promoter region from *A. chrysogenum*, using reporter gene fusions, concluding that the *pcbC* promoter is at least five times stronger than *pcbAB* gene promoter. These results address a new direction in cephalosporin yield improvement by the increase of *pcbAB* gene expression.

Genetically manipulated organisms (GMOs) so obtained must be wisely handled in order to prevent adverse effects to both human health and the environment. The European Union directives 90/219/CEE (confined manipulation) and 90/220/CEE (field release) regulate the GMOs utilization. In Spain, the law 15/1994 (BOE 4 June 1994) rules the confined manipulation, field release and commercialisation of GMOs in order to prevent human health and environmental risks. The potential of genetic engineering is great and it must be safely exploited for the benefit of both humans and the environment.

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Genetic constitution of industrial yeast

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Summary

Saccharomyces cerevisiae industrial yeast strains are highly heterogeneous. These industrial strains, including bakers', wine, brewing and distillers', have been compared with respect to their DNA content, number and size of chromosomes, homologies between their genes and those of laboratory strains, and restriction fragment lengths of their mitDNA. A high variability, and the presence of multigenic families, were observed in some industrial yeast groups. The occurrence or the lack of chromosomal polymorphism, as well as the presence of multiple copies of some genes, could be related to a selective process occurring under specific industrial conditions. This polymorphism is generated by reorganization events, that take place mainly during meiosis and are mediated by repetitive Y' and Ty elements. These elements give rise to ectopic and asymmetric recombination and to gene conversion. The polymorphism displayed by the mitDNA could also result from specific industrial conditions. However, in enological strains the selective process is masked by the mutagenic effect that ethanol exerts on this DNA.

Key words: *Saccharomyces cerevisiae*, electrophoretic karyotype, chromosomal polymorphism, mitDNA polymorphism, industrial yeast

Resumen

Las cepas industriales de *Saccharomyces cerevisiae* son muy heterogéneas. Cuando estas cepas de utilización en la industria alimentaria (producción de pan, vino, cerveza y destilería) se comparan, en cuanto al contenido en DNA, al número y tamaño de los cromosomas, a las homologías de genes con las cepas de laboratorio y a la longitud de los fragmentos de restricción del DNA mitocondrial, se observa una gran variabilidad, así como la presencia de familias multigénicas en sólo algunos

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grupos industriales de levaduras. La presencia o ausencia de polimorfismo cromosómico, y la presencia de copias múltiples de algunos genes, podrían estar relacionadas con un proceso de selección en condiciones industriales específicas. El polimorfismo se genera por reorganizaciones producidas fundamentalmente durante la meiosis y mediadas por elementos repetitivos Y' y Ty, que originan recombinaciones asimétricas y ectópicas y fenómenos de conversión génica. El polimorfismo del DNA mitocondrial también podría responder a condiciones industriales concretas, aunque en cepas enológicas el proceso selectivo se ve enmascarado por el efecto mutagénico que el etanol ejerce específicamente en este DNA.

Introduction

Yeast are unicellular fungi which usually grow and divide asexually by budding. Although defined as unicellular, yeast can in fact be a phase of the life cycle of a filamentous fungus (5). Taxonomically, they are grouped under different genera of ascomycetes, basidiomycetes and deuteromycetes. This diversity indicates that "yeast" is a morphological structure which has been favorably selected and which has recurrently appeared throughout evolution. In fact, there are yeast with sexual and asexual cycles, very distinct metabolisms and even with chromosome numbers that range from 3 to more than 20 (ref. 5). Even within a given species such as *Saccharomyces cerevisiae*, great variations among different strains are observed with regards to their metabolic and molecular characteristics, e.g., chromosome number. Some of these characteristics could reflect the artificial selection exerted by mankind on specific industrial yeast groups through history.

Characteristics of industrial yeast

Traditional applications of industrial yeast include the formation of important products, the result of specific fermentation carried out by strains of *S. cerevisiae*. These strains share common features such as efficient sugar utilization, high ethanol tolerance and production, aroma and flavor formation, high yield and fermentation rate, and genetic stability (8, 38). In addition, each industrial group possesses other specific properties, such as the capacity to utilize trisaccharides, dextrins and starch (distillers' yeast), flocculating capacity (brewers' yeast), low H_2S production (wine yeast), high glycolytic potential, capacity to ferment maltose and resistance to storage, freezing and desiccation (bakers' yeast), etc. (6, 8, 38). In spite of this diversity, all the industrial processes could be divided according to two main goals. Maximal biomass/substrate yield ($Y_{x/s}$) (2), which is the first goal, (i.e., bakers' yeast biomass formation), require very specific conditions. The second goal is the optimization of the product/substrate yield ($Y_{p/s}$) (2) (i.e., wine production). In this review, we will discuss the mechanisms responsible for the heterogeneity found in the different groups of industrial yeast strains. We will also try to relate this heterogeneity to different selective mechanisms, mainly those that aim at either maximal $Y_{x/s}$ or $Y_{p/s}$, according to specific industrial conditions

With regards to the first goal, let us take, as an example, bakers' yeast which are cultivated in molasses, where the main carbon source is sucrose (34). This is a fermentable sugar hydrolyzed by the enzyme invertase into glucose and fructose (38). However, the production of bakers' yeast biomass takes place by a scale up

process under strong aerobic conditions and limited addition of substrate (38). By maintaining low substrate concentrations, yeast metabolism is oxidative and biomass production is maximal (glucose at low concentrations does not exert catabolic repression on respiration) (38). Conditions therefore favor good functional mitochondria, even if the carbon source is fermentable.

In the second case, maximal ethanol formation is desirable. Therefore, the substrate is in excess, and ethanol production is only limited by the inhibitory effect of this compound on the fermenting yeast (6, 10). Within a certain strain, ethanol tolerance is an inheritable characteristic genetically controlled (19). For this reason, genetic improvement of wine yeast can be achieved through the selection of those strains more ethanol tolerant and with the highest $Y_{p/s}$ values (8, 38). In addition to this efficiency in ethanol formation, in some cases, such as those of the elaboration of Sherry wine, yeast (flor strains) are also responsible for aging and are subjected to extreme conditions. Ethanol concentration is over 16% and there is no fermentable sugar, so that the metabolism is exclusively oxidative (9). These conditions also favor the selection of strains with good functional mitochondria.

Identification of industrial yeast

Traditionally, industrial yeast were characterized according to morphological and metabolic criteria such as capacities to ferment and/or assimilate certain nitrogen and carbon sources, variables easy to measure (5). These methods allowed to differentiate genera and species, although it was not possible to identify a strain unequivocally. Interfertility followed the metabolic criteria, with the inconvenience of the lack of sexual cycles in most industrial yeast (5). Recently, other variables are used, such as GC content, percentage of hybridization DNA–DNA, polymorphism in the length of the restriction fragments after incubating purified DNA with restrictases (RFLP), chromosome electrophoresis, polymorphism after hybridization with specific probes, etc. (38). These new procedures allow unequivocally to identify a specific industrial strain, even in the absence of morphological or biochemical indicators (industrial strains are polyploids, lacking those markers which allow to radidly identified a haploid strain) (38).

Electrophoretic karyotypes, which allow the separation of individual chromosomes (12) (Fig. 1), are particularly useful because most industrial strains have their own characteristic pattern (24). In combination with other techniques, they allow to determine the number of copies of a specific chromosome present in the strain, the variations in the size of homologous chromosomes, as well as the presence of aneuploidies, translocations, deletions, and so on (40).

The techniques to determine genomic constitution are complemented with the analysis of restriction fragments (RFLP) shown by mitochondrial DNA (mitDNA) (Fig. 2) (12). The RFLP of mitDNA has been used successfully as the best technique of individual characterization in multipopulational processes such as wine elaboration, due to its efficiency, accessibility and availability (34). This identification and control has allowed researchers to establish the role that a selected strain plays in the aroma, flavor, and organoleptic characteristics of wine as compared to the natural microbiota during the vinification process (34).

Variability of the industrial yeast nuclear genomes

DNA content. Some authors (1, 26, 33) have reported great variations in the DNA content and

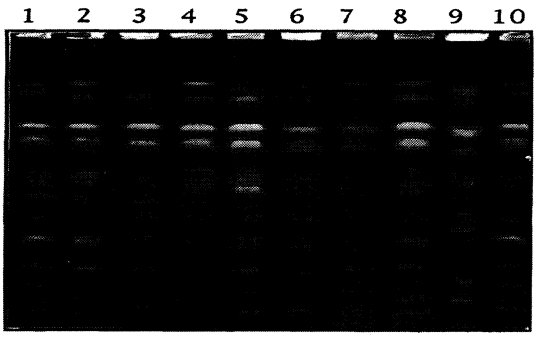

FIG. 1. Electrophoretic profiles of laboratory and industrial yeast. Lanes 1 and 10: laboratory diploid strain DS81. Lanes 2–8, bakers' yeast: DADI (lane 2), VS (lane 3), CT (lane 4), SB2 (lane 5), SB11 (lane 6), V1 (lane 7), and V2 (lane 8). Lane 9, wine strain: IFI256. (See ref.12.)

the chromosome number of different industrial yeast, and have suggested that these variations may respond to specific industrial environments. However, when comparing simultaneously different industrial yeast groups, i.e., bakers', distillers', brewers' and wine strains, similar variations in DNA content have been found among yeast from the same group (i.e., bakers') and those from different groups (i.e., bakers' vs. brewers') (13, and A. C. Codón, unpublished). This indicates that, at least, the DNA content does not reflect specific adaptation to industrial environments.

Electrophoretic karyotype. The polymorphism found in the electrophoretic karyotype patterns of the chromosomes from yeast strains belonging to different industrial groups, is very high (30). Whereas a standard haploid laboratory strain of *S. cerevisiae* (or its isogenic diploid) displays 15 bands corresponding to 16 chromosomes (ref. 15) (Fig. 1), great variations have been found in industrial yeast, both in the number and size of the chromosomes, resulting in variations both in the number and position of the chromosomal bands (Fig. 1) (12, 13). These differences in the number of chromosomal bands is mostly the result of homologous chromosomes of different size. Polymorphism is so high that nearly each industrial yeast strain can be identified unequivocally by its specific chromosomal pattern (Table 1 and Fig. 1) (12). These differences in size and number of chromosomal bands have been displayed by yeast strains from either the same (i.e., bakers' yeast) (Fig. 1) (12) or different industrial groups (10). This indicates that global changes in the number and size of the chromosomes do not reflect selection to specific environments either.

Chromosomal polymorphism has been described in all cases to be higher in industrial yeast than in laboratory strains (30). Furthermore, whereas the literature concerning chromosomal polymorphism of brewers', bakers' or distillers' yeast is very scarce (13), that of wine yeast is more abundant and in all cases, a fairly high chromosomal polymorphism among these strains has been reported (39, 40). Exceptionally, when flor yeast have been analyzed, an almost unique standard chromosomal pattern has been found (27) (more than 85% of the strains analyzed displayed the standard pattern). Flor yeast are gathered in four races of *S. cerevisiae*, and all the four showed this standard pattern (27). It was indicated above that the conditions under which flor yeast grow and survive are very extreme, with ethanol concentrations over 16% and a total absence of fermentable carbon source. It is therefore expected that these selective conditions have propitiated a unique chromosomal pattern, the same for all the flor yeast, and different from those of other industrial or laboratory yeast (27). Alternatively, it has been suggested that, in the absence of recombination, a population becomes monomorphic (36). Some authors supported this suggestion by describing sexual isolation of wine yeast which stopped them from mixing their taxonomical features (37). In a similar way, flor yeast do not sporulate under

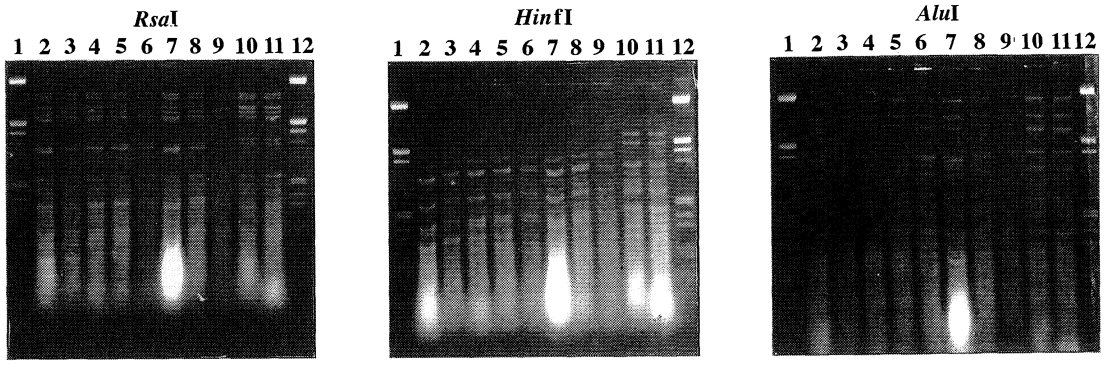

FIG. 2. Mitochondrial DNA restriction analysis with three restriction endonucleases, *Rsa*I, *Hin*fI and *Alu*I. Lanes 1 and 12: size markers, in kb, which correspond to a mixture of lambda phage DNA fragments, obtained with *Hin*dIII and *Eco*RI. Lanes 2–8, bakers' yeast: DADI (lane 2), VS (lane 3), CT (lane 4), SB2 (lane 5), SB11 (lane 6), V1(lane 7), and V2 (lane 8). Lane 9: laboratory diploid strain DS81. Lanes 10 and 11: wine strains ACA21 (lane10) and ACA22 (lane 11). (See ref.12.)

any tested condition (27); thus recombination processes are very restricted, and this would explain the single chromosomal pattern shown by these strains.

Chromosomal location of specific genes. To identify the different chromosomes specifically, they are usually hybridized, once they have been separated by pulse-field electrophoresis, with probes corresponding to genes which are known to be present once in the genome (11, 30). The chromosome of the industrial yeast, bakers', brewers', distillers' and wine strains, have hybridized in all cases with probes from laboratory strain genes, thus indicating that the DNA homology with these genes is very high (13, 30). Chromosome location is also the same in both laboratory and industrial yeast. Hybridization with several bands, near to each other, seemed to confirm the existence of several homologous chromosomes of different size (13). Exceptionally, the fact that multiple bands hybridized with the gene probe has clearly indicated translocation to other chromosomes (i.e., *CUP* gene) (11, 12, 30).

When comparing brewers' and wine yeast with laboratory strains, other authors (24) have found both interchromosomal (translocations) and intrachromosomal (deletions, duplications, inversions) reorganization, as well as a pair of homologous chromosomes, one of them having high homology with the corresponding chromosome of the laboratory strain, whereas the other homologous had none (23). This homology has been such that, after hybridizing brewers' and laboratory strains (33), one chromosome was able to complement auxotrophic markers of its homeologous (homologous chromosomes from different species), but there was no recombination between them (33).

If, as suggested (1, 38), polymorphism of industrial yeast chromosomes reflects selection under specific environmental conditions, in bakers' yeast, the procedure for biomass production would favor any chromosomal reorganization which resulted in an increase in the growth rate. In fact, if there is a limited addition of the substrate (molasses), amplification of the *SUC* gene which codes for invertase would result in a more efficient utilization of the sucrose (38). When hybridization of the *SUC* gene probe and chromosomal bands of industrial strains was carried out (11, 26), in bakers' strains, *SUC* gene

seemed to be amplified and translocated to several chromosomes, as judged by the many bands which hybridize with the probe (11, 31) (Table 1). This phenomenon occurring in just bakers' and distillers' yeast could therefore be interpreted as an adaptive mechanism in specific industrial environments. In fact, some wine strains (which in their natural environments ferment glucose and other fermentable sugars but not sucrose) have a single band (12) (or even no detectable band able to hybridize with the *SUC* probe, these yeast being totally unable to metabolize sucrose) (7, 18, 28).

The reasons in favor of amplification of the *SUC* gene to be a true adaptive mechanism are the following: (i) When measuring invertase of numerous bakers' yeast strains as an average, this activity is 11-fold or above that of laboratory strains. Distillers' yeast also have about 11-fold the activity of the laboratory strains. Wine yeast have an increase of only 2-fold (A. C. Codón, unpublished). (ii) When grown in molasses, laboratory strains reduced their growth rate by 80%, as compared to that in laboratory media; wine yeast did so by 45% (18) or 30% (28). Bakers' yeast, however, grew at a higher growth rate in molasses than in laboratory media (12). (iii) Other authors found that, during growth in molasses with *S. cerevisiae* strains, biomass production increased by adding fungal invertase to the culture medium (32), indicating that the capacity to hydrolyze sucrose is the limiting step for growth and productivity.

Chromosomal reorganization as a source of variability

Chromosomal reorganization is a natural phenomenon in the life cycle of many organisms, and, in some cases, it is part of their development pattern (3). As an example, we may consider the de novo telomere formation in *S. cerevisiae*, the reposition of the surface antigen genes in *Trypanosoma*, the mating type switch in fungi, the fragmentation of the chromosomes occurring in the somatic nucleus of ciliate or, in higher eukaryotes, chromosome reorganization of the immunoglobuline genes (3, 22, 25, 26). Besides, there are other non-programmed genome reorganizations which include deletions, translocations etc., of amazing consequences, such as dramatic changes in the viability of the meiotic products of a strain heterozygous for one of these reorganization. Apart from changes in chromosome structure (size),

TABLE 1. Characteristics of some industrial yeast groups and the environment were they are ususally isolated

Origin of the strains	CP	mitDNA	TY	SPO	MI	INV	SUC	ETH
Laboratory yeast (control)	+	NR	+++	++++	+/–	+	+	+/–
Bakers' yeast	++++	+/–	++++	+	++++	++++	++++	+/–
Brewers' yeast	+	+	++	+/–	NR	++	+++	++
Distillers' yeast	+++	NR	++	+/–	NR	+++	+++	+++
Wine yeast	++	+++	+	++	++	+/–	+/–	+++
Flor yeast	+/–	++++	++	+/–	+/–	+/–	+/–	++++

Columns: Variability of the chromosomal electrophoretic pattern (chromosomal polymorfism) (CP) and of the restriction fragment length polymorphism of the mitochondrial DNA (mitDNA); frequency of Ty and Y' repeat elements (TY); frequency of sporulation and meiosis (SPO); variation of the chromosomal patterns at meiosis (meiotic instability) (MI); copy number of *SUC* genes coding for invertase (INV); sucrose (SUC) and ethanol (ETH) concentration in their environment.

Symbols: NR, not reported; ++++, very high; +++, high; ++, medium; +, low, +/–, very low or absent.

there are also changes in chromosome number as a consequence of lack of pairing and recombination of homeologous chromosomes (with low homology), and non-disjunction at meiosis, which results in an unequal distribution of the chromosomes in the progeny, low viability and aneuploidies in the surviving products (15, 22).

In *S. cerevisiae*, genomic reorganization takes place *via* recombination, and this phenomenon occurs between homologous sequences. Therefore, the substrate for recombinations leading to genome reorganization are repeat sequences which, in yeast, are very scarce: telomeric and subtelomeric Y' and X regions, Ty transposable elements and, to a lesser extent, rRNA and tRNA genes (3, 22). The recombination frequency between Ty elements present in the same (allelic or asymmetrical) or different (ectopic) chromosomes (Fig. 3) in diploid cells has been estimated to be 1% in meiosis and 10^{-7} in mitosis (26), whereas the transposition frequency was of 10^{-4} to 10^{-3} per generation, in equilibrium with excisions to maintain a stable copy number (26). Those Ty elements of the same family (i.e., the Ty1 family) are highly homologous. The reason is that they become homozygous due to gene conversion mechanisms (22), which take place in addition to those described of transposition and recombination. In fact, it seems that most "recombination" events occurring between Ty elements result in gene conversion.

The best characterized subtelomeric regions are the X and Y' regions, which possess replicating *ARS* origins (Fig. 3) and which appear repeated 4 to 5 times at the end of the chromosomes. Because of these tandem repetitions, there are reports of genome reorganization at meiosis, as a consequence of telomeric interactions, and of mitotic recombinations between Y' regions giving to duplications, deletions and insertions (Fig. 3) (3). The fact that some gene families such as *SUC*, *MAL*, *MEL*, etc., are located at the telomeric regions, made believe that it had favored genome reorganization of these genes, so that they appear in a variable copy number according to the strain (39). These gene families are examples of non-lethal genomic reorganization leading to chromosome polymorphism (39).

As indicated for the Ty elements, when the Y' sequences were analyzed, they displayed a degree of homology superior to that expected for independent evolution, and the reason has also been attributed to allelic, asymmetrical or ectopic recombination events which have resulted in gene conversion (23). In addition, the Y' elements possess replication origins, so that they can exist as circular autoreplicative elements, which can insert in any chromosomes which possess any Y' elements (Fig. 3) (22). When there is a different copy number of the Y' elements in two homologous chromosomes, recombination between them resulted in unequal distribution of the Y' elements and therefore in changes in the size of the chromosomes involved in the event (Fig. 4). The estimated frequencies of recombination events are of 2×10^{-6} per generation in mitosis, and 2% in meiosis (22, 24, 25, 26).

Molecular bases for the chromosomal polymorphism found in industrial yeast

Both Ty transposable elements and Y' subtelomeric regions have been described then to contribute to chromosome reorganization and therefore to introduce variability in chromosome size and number (Figs. 3 and 4) (3, 14, 23, 25, 26, 29). Thus, whereas hybridization with gene probes represented once in the genome allows to identify a specific chromosome, the use of probes of Ty transposable elements (3, 41) as well as subtelomeric Y' regions, which have multiple location in the yeast genome (23, 25),

could render information about the degree of polymorphism of industrial yeast.

Ty1 and Ty2 are highly represented in most industrial yeast, but these transposable elements are more frequent in bakers' than in the remaining yeast groups (11). Different intensities of the hybridization signals have been suggested to result from amplification (whether in tandem or not) of these elements in some chromosomes (11). Furthermore, copy number of Ty elements progressively diminished in laboratory yeast respect to bakers' strains, and in brewers', distillers' and wine yeast respect to laboratory strains, flor yeast being the group with the lowest Ty copy number (Table 1) (11).

With regards to the subtelomeric Y' regions (30), great differences among industrial yeast groups respect to the number of chromosomal bands able to hybridize with these Y' sequences have been described (11), but again, they seemed to be much less abundant in wine yeast (mostly flor yeast) than in the other industrial groups (33). Bakers' strains have shown once more to be the group whith the maximal hybridization, in relation to both the number of chromosomal bands able to hybridize and the intensity of hybridization (Table 1) (11).

Consequences of meiosis on the polymorphism of industrial yeast

Hybridization of specific gene probes with parental industrial yeast (bakers' strains) and their meiotic products (complete tetrads) have sometimes indicated the correct distribution of homologous chromosomes of different size in the corresponding four meiotic products (13). However, the appearance of new bands absent in the parental, the total absence of chromosomal bands of the parental in any of the meiotic products as well as bands which, being in the parental, segregate in a totally abnormal manner have frequently been seen (A. C. Codón, unpublished). These changes have been attributed to the many copies of Ty and Y' elements found in bakers' strains (11). Reorganization at meiosis has been observed both in all the tetrads of the same bakers' strains, and in all the bakers' yeast analyzed; these reorganizations differ according to the gene probe used and the specific tetrad analyzed (13). The fact that these reorganizations seem to be limited to the meiotic period is particularly worth noticing, as judged for the repetitive pattern found in the electrophoretic karyotype of these yeast when maintained for succes-

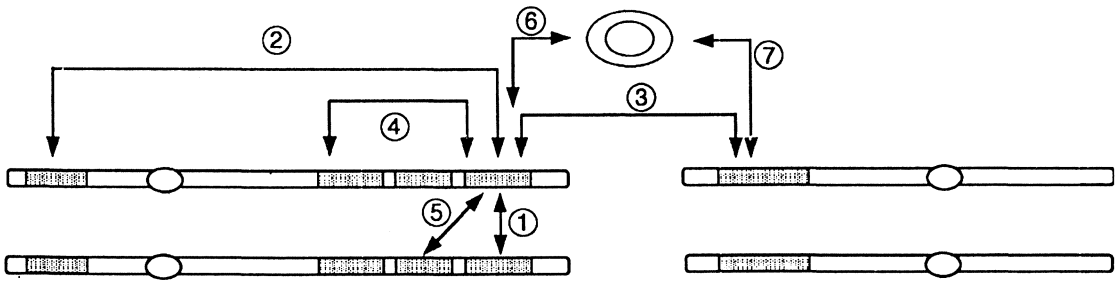

FIG. 3. Chromosomal reorganizations caused by Ty and Y' repeat elements. 1: Allelic recombination, 2: intrachromosomal recombination, 3: ectopic recombination, 4: recombination between tandem repeats, 5: asymmetrical recombination, 6: excision, 7: insertion.

sive mitotic generations (12). If there seems to be such a high frequency of reorganization at meiosis, undergoing meiosis should have major consequences for these industrial yeast. For instance, the following:

Increase or appearance of aneuploidy. Most industrial yeast analyzed have shown to be highly polyploid and/or aneuploid (13, 27). To start with, this aneuploidy could account for the lack of complete tetrad formation (5 to 10%) (11) and viability of the meiotic products (0 to 10%) (11) of those strains able to undergo meiosis and sporulation. Tetrad analysis of genetic markers such as auxotrophies, petite phenotype, ability to sporulate and/or conjugate and so on, indicates a very significant uneven distribution of chromosome number among the meiotic products (13). The presence of several bands of different size in only some of the meiotic products confirm the inheritance of unequal chromosome copy number.

Increase of appearance of differences in chromosome size. The presence of so many Ty and Y' repeat elements in bakers' yeast (13) introduce polymorphism in their chromosomal pattern. But this polymorphism of chromosome number and size highly increases if the strain undergoes meiosis. Comparisons have been made only with complete tetrads (i.e. those that allow the four meiotic products to survive), which represents a small fraction of the total meiotic processes. Incomplete tetrads probably showed even more dramatic reorganization than those where all the four meiotic products are viable.

How do the cells tolerate genome reorganization

Limitation of large reorganizations. Whereas Ty and Y' elements in laboratory strains have undergone many reorganizations which could have resulted in translocations, deletions, insertions and so on, of chromosomal fragments, most genes have not undergone such changes, so that they are usually localized at specific positions in the chromosomes. The frequency of gene conversion due to recombination between Ty elements located at different chromosome position, is only 2×10^{-6}. This suggests that there

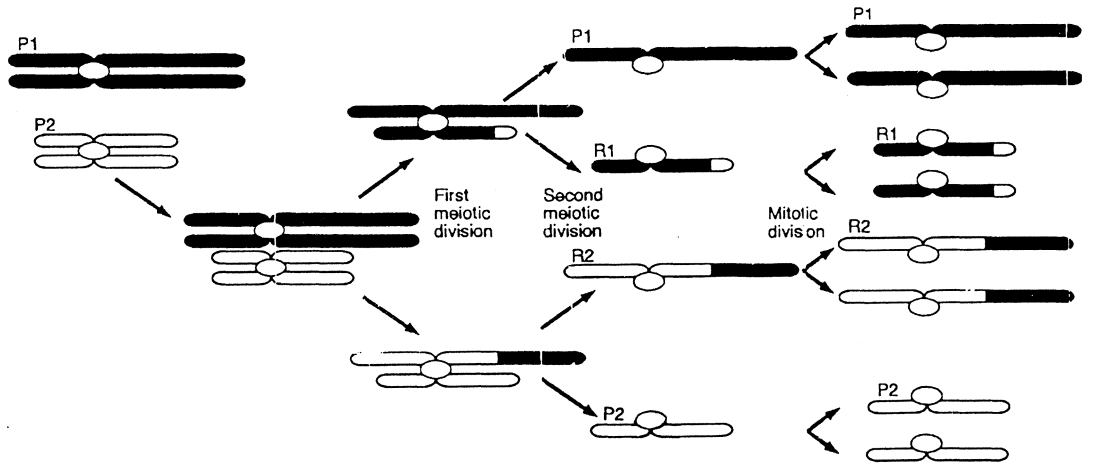

FIG. 4. Recombination occuring between two homologous chromosomes of different size (P1 and P2) results in four meiotic products, two of the same size as the two parentals (P1 and P2) and two whose sizes differ from those of the parentals (R1 and R2).

are some mechanisms which repress ectopic exchanges. The reason for this could be that most of the resulting recombinants are not viable. However, there is at least one gene (*EDR1*, presently *TOP3*) which is known to repress meiotic recombination events in chromosomal regions which are near a δ element (23). On the contrary, allelic recombination between Ty elements is neither repressed at meiosis nor at mitosis (23).

With regards to industrial yeast, the mitotic stability shown by the repetitive pattern of the electrophoretic karyotypes could be explained by a similar process, so that ectopic recombination between repeat sequences could also be repressed (23, 36). In addition and in contrast to laboratory strains, meioses are very scarce in most industrial yeast (brewers', bakers') and are practically absent in flor yeast (27). However, if meiosis takes place, the reorganizations are numerous, as if those mechanisms repressing ectopic recombination in laboratory yeast were not present, or did not function, in industrial yeast (17).

Reorganization of non-essential sequences. Polymorphism in size and number of yeast chromosomes is a common feature (so that these variations within populations belonging to the same species is the rule rather than the exception), from which the concept of "genome plasticity" has been developed. This concept refers to spontaneous reorganization happening at a very high frequencies without the phenomenon seriously affecting strain fitness, as if the possibilities of adaptation of the strains to specific environments were very high (1).

In addition, when analyzing the telomeric regions of the chromosomes, a variable number of repeat regions have been found in most cases. This indicates that, either there are selective advantages in having these repeat sequences or, rather, there are common mechanisms which lead to their accumulation and maintenance in the chromosome (26). It has also been demonstrated that, in some cases, repeat sequences are advantageous for the organisms which have them. In *est1* yeast mutants the senescence caused by telomeric shortening is avoided by a massive proliferation of Y' subtelomeric elements which takes place at the extremes of the chromosomes (26). Besides, repeat subtelomeric regions protect adjacent unique sequences from heterochromatinization and therefore, gene inactivation (26).

Favorable selection of reorganized chromosome regions. Reorganization mediated by Ty and Y' elements, giving rise to deletions, inversion, duplications and translocations are not reciprocal and the result is a heterozygous cell. Under certain circumstances, these reorganizations are favored and maintained because heterozygosis, that is, the presence of more than one allele in the reorganized region, allows a better fitness to specific environments (14, 29). Alternatively, reorganization can be unfavorable with regards to the wild type, or even lethal under the same environmental conditions, but can also allow to colonize new environments, not available to the wild type (29).

Attenuation of the negative effects caused by chromosomal reorganization

Besides the fact that reorganization can be advantageous, yeast can palliate those situations under which the effects of the reorganization are negative or unfavorable:

Apomixia. Apomictic yeast are those which have undergone mutations which impede them to complete either the first or the second meiotic division (16). As a consequence, the resulting asci form diads instead of tetrads, and the two meiotic products have the same DNA content as the parental. This mechanism sensibly reduces the loss of viability of the meiotic products of those numerous industrial yeast strains whose DNA content vary between 1 and 2n (7, 19).

Homothallism. Most *S. cerevisiae* yeast strains isolated from wild or industrial environments are homothallic (22). A meiotic product from a homothallic strain undergoes a mating-type switch when it has divided once. This change allows the cell to mate with cells from the same population and therefore to double its DNA content, and becomes homozygous for all the genes, except the mating type locus. Homozygosis attenuates the possible deleterous effect which could cause a meiotic reorganization when it is present in heterozygosis, for instance a translocation or a change in chromosome number, which would stop recombination and the correct chromosome separation. Homothallism of an aneuploid strain guarantees that, after meiosis, the products will have an even number of extra chromosomes, decreasing enormously the frequency of non-disjunction of the chromosomes and of viability. In wine yeast, as a consequence of their homothallism (19), the increase in viability for some strains has been from 60% in the first generation to 80 to 100% in the second generation.

Variability of the mitochondrial genome of industrial yeast

Variability in the length of the restriction fragments generated from the mitDNA has open the way to identify unequivocally specific strains from some industrial yeast groups, such as wine yeast (35). However, bakers' strains displayed exactly the same RFLP pattern of their mitDNA (Fig. 2) (12). Whereas the interaction within the same cell of gene products from nuclear and mitochondrial genomes would predict these genomes to coevolve (35), comparison between mitochondrial and nuclear genomes in bakers' strains suggests that the mitDNA is very stable whereas the nuclear DNA is highly unstable.

With regards to the wine yeast, these strains display both a high tolerance to ethanol and temperature as well as to the mutagenic effect that ethanol exerts on mitDNA as compared to other yeast groups (20, 21). The fact that the transference of mitochondria from wine to laboratory strains confer to the laboratory recipient yeast a considerable increase in tolerance to these variables, indicates that the mitochondrial is the main responsible for this tolerance rather than the nuclear genome (20, 21).

In spite of the fact that mitochondria from wine yeast have undergone a selective process of adaptation to ethanol, and in consequence are particularly tolerant to this compound, they are permanently exposed to its mutagenic effects which are specific for the mitDNA but do not affect the nuclear genome (20, 21). In conclusion, the differences of the industrial conditions could account for the differences found between the mitDNA of bakers' yeast (strongly selected and invariable because their mitDNA is never in the presence of ethanol) and the mitDNA of wine yeast (also strongly selected but always in the presence of high concentrations of ethanol) (see Table 1). The mutagenic effect of the ethanol is always masking the selective process.

The paradox of the genomes of industrial yeast

When flor yeast were analyzed, their nuclear genomes displayed a unique chromosomal pattern whereas the RFLP of the mitDNA was highly variable (27). However, bakers' yeast showed enormous variations of their nuclear DNA patterns whereas the RFLP of their mitDNA was identical (see Table 1 and Figs. 1 and 2) (12).

A possible explanation non-related to the sensitivity of the method of analysis is the fol-

lowing: an optimal karyotype has been selected in flor yeast. The conditions are so extreme that most strains analyzed showed identical DNA chromosomal patterns (27). These conditions have also allowed the selection of an optimal mitDNA. However, the presence of high ethanol concentrations (over 16%) are permanently introducing variations in the mitDNA, so that the patterns of the RFLP are highly variable (27). The level of spontaneous petite mutants (unable to respire) increases 10-fold when a strain is maintained in 24% ethanol (4). With regards to the nuclear genome, flor yeast display an almost unique chromosomal pattern which probably is the best selected under the extreme conditions which support these yeast (27). Thus, both mitDNA and nuclear genomes are subjected to strong selective conditions. But in contrast to the mitDNA (4), ethanol does not affect nuclear DNA and therefore variations of this DNA are minimal.

Other industrial yeast such as bakers' strains also undergo selective conditions leading to the best mitochondrial pattern, which is maintained because ethanol is absent. Limited addition of molasses leads to an exclusive respiratory metabolism, where good functional mitochondria and maximal $Y_{x/s}$ yield (which drops dramatically in petite mutants) are selected (38). Therefore, although petite mutants could survive under these conditions, the drop of their growth rate and yield would eliminate these mutants in few generations (2, 28). Data supporting the strong selection exerted on the mitDNA of the bakers's yeast are the following: (i) the frequency of spontaneous petite mutants in bakers' strains is about 0.1% (12). This frequency is extremely low, when compared with the usual frequencies found in other yeast groups (1–5%) (20). (ii) The RFLP of the mitDNA is identical for bakers' strains isolated from places geographically very distant, but sharing similar environmental conditions.

Polymorphism of the nuclear DNA of bakers' yeast is, however, very high (11) and could indicate either that the conditions for this nuclear DNA are not so selective (absence of ethanol), as they are for other industrial yeast (27), or the possible existence of several nuclear patterns, compatible with similar degrees of adaptation (fitness). Another explanation is that bakers' strains are able to undergo meiosis, sporulate and produce viable meiotic products, although with very low frequency (11, 12, 13). From data given above, it seems that whereas mitoses are very stable, the presence of Ty and Y' elements originate many reorganization at meiosis (13). The possibility of undergoing meiosis only once is enough for a bakers' strain to completely and extensively alter its chromosomal pattern. Polymorphism could therefore be the result of the meioses which these bakers' strains might have undergone, followed by the selective process which the resultant meiotic products underwent, in order to support the specific conditions of industrial bakers' strains. These phenomena of reorganization of the nuclear genome are almost absent in flor and other industrial yeast, unable to conjugate or undergo meiosis and sporulation.

Conclusions

Variability shown by industrial yeast, with regards to the number and size of the chromosomes, seems to be generated at meiosis, and to be the result of asymmetrical recombinations between homologous chromosomes of different size or ectopic recombinations between non-homologous chromosomes. These recombinations seem to be mediated by Ty and Y' repeat elements. Polymorphism seems therefore to depend on both the number of Ty and Y' elements present in a strain and its capacity to undergo meiosis.

Variability generated at meiosis is not exempt from further selection. This can be demonstrated by the presence of *SUC* genes, which appear in multiple copies only in those industrial yeast cultivated in molasses where high levels of invertase are required; by contrast, there is just one or none *SUC* genes in other industrial yeast groups.

Mitochondria undergo a strong selection in the industrial yeast groups. These yeast need a very high respiratory capacity either to exclusively respire the carbon source and give maximal $Y_{x/s}$ yield, to grow at maximal growth rate, to survive in high concentrations of ethanol in the absence of fermentable carbon source and others. This selection is not always obvious. The mutagenic effect of the ethanol in the DNA increases with increasing ethanol concentrations. As a result, mutagenicity masks the selective procedure, and variability of the RFLP of the mitDNA depends on the ethanol concentration.

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Detection of enterohaemorrhagic *Escherichia coli* O157:H7 in minced beef using immunomagnetic separation

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Summary

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 has been recently recognized as a human pathogen associated with haemorrhagic colitis and haemolytic uraemic syndrome. Most outbreaks of haemorrhagic colitis resulted from the consumption of undercooked minced beef or raw milk. Dairy cattle have been identified as a reservoir of EHEC O157:H7. In this study *E. coli* O157 specific antibody, coated on magnetic beads, was used to concentrate and release EHEC O157:H7 from meat samples. A survey of retail fresh minced beef and hamburger samples using this procedure revealed that 3 (5%) of 58 beef samples were positive for EHEC O157:H7. Two of the strains produced both VT1 and VT2 verotoxins, and one produced only VT2. Immunomagnetic separation is a sensitive and simple technique for the isolation of *E. coli* O157 from food, and could be useful for a further elucidation of the epidemiology of this organism. The relatively high prevalence of EHEC O157:H7 in beef samples may constitute a risk for public health. Thus, a suitable epidemiologic control and effective methods of prevention should be applied.

Key words: *Escherichia coli*, enterohaemorrhagic strains, serotype O157:H7, verotoxins, food microbiology

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Resumen

Escherichia coli enterohemorrágica (ECEH) del serotipo O157:H7 representa un nuevo patógeno humano asociado con la colitis hemorrágica y el síndrome urémico hemolítico. La mayoría de los brotes epidémicos de colitis hemorrágica se han asociado con el consumo de carne de vacuno poco cocinada o de leche no pasteurizada, estando claramente identificado el ganado bovino como el principal reservorio de ECEH O157:H7. En el presente estudio se ha empleado la separación inmunomagnética para concentrar y recuperar ECEH de muestras de carne picada y hamburguesas de vacuno. Se ha logrado aislar ECEH O157:H7 en 3 (5%) de las 58 muestras de carne investigadas. Dos de las cepas resultaron ser productoras de las verotoxinas VT1 y VT2, y una tercera de solamente VT2. Los resultados indican que la separación inmunomagnética es una técnica sencilla y sensible que puede ser útil en los laboratorios de diagnóstico rutinario y en los estudios epidemiológicos. La relativamente alta frecuencia de aislamiento de ECEH O157:H7 en carne de vacuno puede suponer un grave riesgo para la salud pública, por lo que se deben extremar las medidas preventivas de vigilancia y realizar un control epidemiológico adecuado.

Introduction

Verotoxin (VT)-producing enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is now recognised as a new enteropathogen that causes haemorrhagic colitis (HC) and the haemolytic-uraemic syndrome (HUS) in humans (7, 8, 9, 21, 23, 24, 25, 29, 38).

In the USA, Canada, and UK, EHEC O157:H7 has been reported as the major cause of bloody diarrhoea (15% to 41%), and as the second or third bacterial pathogen more frequently isolated from stool specimens, after *Salmonella* and *Campylobacter* (21, 38). Lately, an exponential rise has been observed in *E. coli* O157:H7 infections. In Scotland, for example, only 10 cases identified from 1984 to 1986 were followed by 202 in 1991 (38). The highest rates of infection by EHEC O157:H7 have been observed in Canada, where most testing laboratories perform routinely the detection of this pathogen; 1342 cases were identified in 1987, which means 5.2 cases per 100,000 inhabitants (21). In Scotland, with rates of infection slightly lower (4.0 cases per 100,000 inhabitants in 1991), 17 outbreaks of HC were reported between 1989 and 1992. In the USA, the number of cases of diarrhoea from *E. coli* O157:H7 is estimated at 10,000–20,000 cases per year, with 16 outbreaks reported during 1993 and other 11 reported during the first 6 months of 1994. The most serious outbreak happened in 1985 in an elderly people's home in Ontario (Canada); 55 of 169 residents and 18 of 137 employees were affected. Nineteen (35%) of the affected residents died as a result of the infection associated with eating minced beef sandwiches (16). Most outbreaks have been associated with eating hamburgers; that is why HC is known as "hamburger-illness" in Anglo-Saxon countries (1, 24, 36, 37).

Cattle appear to be a major reservoir of EHEC O157:H7. This microorganism is found as part of the normal intestinal microbiota of 1% of animals (4, 8, 11, 12, 14, 17, 42). During slaughtering and especially during skinning and eviscerating, *E. coli* strains present in the intestines of cattle inevitably contact the carcasses.

By using high sensitive immunologic and genetic techniques, some studies showed that contaminated food products associated with out-

breaks may contain ten O157:H7 bacteria per gramme only. These bacteria have the ability to survive in acidic conditions (pH 2.5 to 3.0), to grow at very low temperatures (7°C), and to remain viable for months in frozen meat at –20°C. However, they can be easily eliminated by heating (68.3°C). Hamburgers and minced meat are specially dangerous, for microorganisms remain viable if affected meat is not thoroughly cooked (3, 24, 27, 32, 39).

Unlike other *E. coli* strains, EHEC O157:H7 do not ferment sorbitol and are β-glucuronidase negative. These differences make it easy to identify O157:H7 strains in food products and clinic samples, though some sorbitol positive or H^- strains have been detected.

We found EHEC O157:H7 or H^- in 4 (0.8%) out of 510 calves from some farms in Spain (4, 8, 11, 12, 13). So, we decided to complete this epidemiologic study with the sampling of beef meat which was aimed to human consumption.

Materials and methods

Sample collection. A total of 58 samples (33 mince beef and 25 beefburgers) were collected from 20 local markets in Lugo city during May and July 1995. All meat samples were packaged individually in sterile plastic bags, refrigerated for transportation to the laboratory and processed within 2 h of collection. Results were compared with those obtained in a previous sampling, from 1994.

Immunomagnetic separation of *Escherichia coli* O157. A 25 g portion of each meat sample was homogenized in a stomacher with 225 ml of buffered peptone water supplemented with vancomycin (8 mg/l), cefixime (0.05 mg/l) and cefsulodin (10 mg/l) (Fig. 1). After mixing, broths were incubated at 37°C for 6 h, and 1 ml of broth was added to 20 μl of magnetic beads coated with an antibody against O157 antigen (Dynabeads anti-*E. coli* O157; Dynal, Oxoid, Hampshire, UK) in a 1.5 ml microcentrifuge tube for detection of *E. coli* O157 by immunomagnetic separation (IMS). The IMS was performed as previously described by Chapman et al. (17). The beads were suspended evenly in the broth culture by vortex mixing and were placed in a rotating mixer so that they were mixed by inversion every 2–3 s for 30 min at room temperature. Tubes were placed in a magnetic separator rack (MPC-10; Dynal, Oxoid) and the magnets were placed in position and left for 5 min. The culture supernate was removed by aspiration with a Pasteur pipette, the magnetic slide was removed from the rack, the beads were washed by resuspension in 1 ml of PBS, pH 7.2, with Tween 20 0.002% v/v (PBST) and the magnetic slide was replaced for 3 min. The beads were washed in PBST in this way a further two times, the magnetic slide was replaced for 3 min, the supernate was removed, and the beads were resuspended in 50 μl of PBS. The beads were inoculated on to sorbitol MacConkey (SMAC) and cefixime (0.05 mg/l), tellurite (2.5 mg/l), sorbitol MacConkey (CT-SMAC) media. After incubation overnight at 37°C, eight sorbitol non-fermenting colonies and two sorbitol positive colonies were selected.

Characterization of isolates. *(i) Biotyping.* Identification was based on biochemical tests, including fermentation of glucose (+) and lactose (+ or –), H_2S production (–), citrate (–), urease (–) and indole (+). O157 strains were confirmed as *E. coli* using the API 20E system (BioMérieux, Marcy L'Etoile, France). β-Glucuronidase activity was established on SMAC with MUG (Biolife, Milano, Italy).

(ii) Serotyping. Presence of O157 and H7 antigens was determined by the method described by Guinée et al. (22) and modified by us (6) with antisera obtained from the Dutch National Insti-

tute of Public Health and Environmental Protection (Bilthoven, the Netherlands). The antisera were adsorbed with the corresponding cross-reacting antigens to remove the unspecific agglutinins.

(iii) Production of verotoxins and detection on cells. One loopful of each *E. coli* strain was inoculated into a 50 ml Erlenmeyer flask containing 5 ml of tryptone soya broth with mitomycin C (5) and incubated for 20 h at 37°C in an orbital shaker (200 rpm). Detection of verotoxins was performed on Vero and HeLa cells as described in previous papers (5, 11). Briefly, Vero and HeLa cell assays were performed on cell monolayers grown nearly to confluence in plates with 24 wells. At the time of assay, the growth was changed (0.5 ml per well) and 75 µl of undiluted filtrate of culture treated with mitomycin C was added. Vero and HeLa cells were incubated at 37°C in 5% CO_2 atmosphere, and morphological changes in cells were observed under a phase contrast inverted microscope after 24 and 48 h of incubation.

(iv) PCR for amplification of VT1 and VT2 genes. DNA to be amplified was released from whole organisms by boiling. The pairs selected to amplify VT1 and VT2 gene segments (5'–3'):

VT1: CAGTTAATGTGGTGGCGAAG and CTGCTAATAGTTCTGCGCATC; and

VT2: CTTCGGTATCCTATTCCCGG and GGATGCATCTCTGGTCATTG,

have been used as previously described (31). The VT1 primers amplify a 894-bp fragment (nucleotides 215 to 1109 of the VT1 gene) and the VT2 primers amplify a 478-bp fragment (nucleotides 288 to 766 of the VT2 gene). Oligonucleotide primers were synthesized by using a Gene Assembler Special (Pharmacia, LKB Biotechnology, Inc., Uppsala, Sweden) according to the protocol provided by the manufacturer. Amplification of bacterial DNA was performed with 50 µl volumes containing 10 µl of the prepared sample supernatant; the oligonucleotide primers (150 ng for VT1 primers, and 90 ng for VT2 primers); 0.2 mM (each) dATP, dGTP, dCTP, and dTTP; 10 mM Tris HCl (pH 8.8); 1.5 mM $MgCl_2$; 50 mM KCl; and 1 U of DynaZyme DNA polymerase (Finnzymes OY, Finland). The reaction mixtures were overlaid with an equal volume of mineral oil. The PCR was performed with a thermal cycler (model Gene ATAQ Controller; Pharmacia, LKB Biotechnology, Inc.) at 94°C for 2 min for 1 cycle, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The amplified product was visualized by standard submarine gel electrophoresis of 10 µl of the final reaction mixture on a 2% agarose gel. Amplified DNA fragments of specific sizes were located by UV fluorescence after staining with ethidium bromide. Molecular size markers (*Hae*I-II digest of ϕX174 DNA) were included in each gel. The above described procedure(s) is schematized in Fig. 1.

Most probable number determination and detection of verotoxigenic *Escherichia coli* by conventional methods. Most probable number (MPN) determination was performed in BRILA broth with MUG (Fluorocult BRILA-Bouillon, Merck, Darmstadt, Germany) by inoculation of 1 ml of each decimal dilution (1/10, $1/10^2$, $1/10^3$) into three BRILA-MUG tubes. Decimal dilutions were made in peptone water. After incubation (37°C/48 h) each tube was examined for gas (lactose fermentation), fluorescence (β-glucuronidase action) and indole production. Only the tubes with these three characteristics were considered positive for the presence of *E. coli* (33).

Detection of verotoxigenic *E. coli* (VTEC) by conventional methods included: (i) resuscitation in peptone water, (ii) selective enrichment in MacConkey broth, (iii) selective plating on Lactose MacConkey agar and on SMAC with tellurite. A total of 25 colonies with typical

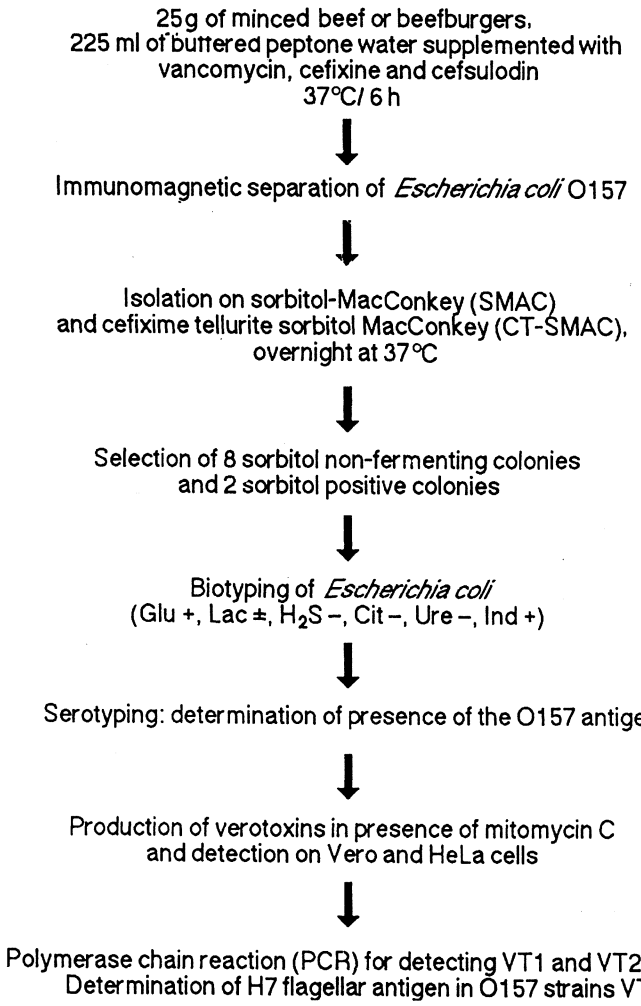

FIG. 1. Methodology for the detection of ECEH O157 in food.

morphology of *E. coli* were selected per sample, including lactose-positive, lactose-negative, sorbitol-positive, and sorbitol-negative colonies. After identification, *E. coli* colonies were investigated for verotoxin production on Vero and HeLa cells.

Results

E. coli colonies were isolated from 100% (33/33) of the minced meat samples and from 96% (24/25) of the hamburgers. More than 10^2 *E. coli* per gramme were detected in 45% (15/33) and in 20% (5/25) of the samples, respectively. VT-positive EHEC O157:H7 were detected in three minced meat samples, two of which gave 480 *E. coli* colonies per gramme and one gave more than 2,400 *E. coli* colonies per gramme (Table 1). The three positive samples (with EHEC O157:H7) came from the same supermarket. The first sample (CP22) was obtained on May 25, 1995, the second (CP44) on June 21, and the third (CP47) was obtained on

TABLE 1. Frequency of EHEC O157:H7 in relation to the most probable number (MPN) of *Escherichia coli* per gramme of meat

MPN *E. coli*	No. of samples	
	Total	EHEC O157:H7
$< 10^2$	38	0
$> 10^2 < 10^3$	12	2 (17%)
$> 10^3$	8	1 (13%)

June 27. A total of 11 minced meat samples and 5 hamburgers were investigated from this supermarket. The last month of the study, we obtained samples only from this supermarket: 10 minced meat samples (two of which were positive for EHEC O157:H7) and five hamburgers.

The IMS appeared to be decisive for the detection of EHEC O157:H7 strains, which would have not been detected if we had applied conventional methods (Table 2). From one of the positive samples, 111 *E. coli* colonies obtained from conventional methods were assayed for toxins without detecting EHEC O157:H7 strains (Table 2).

The three O157 strains isolated showed the features of EHEC: O157:H7 serotype, sorbitol-negative and β-glucuronidase-negative. Toxigenicity determined in Vero and HeLa cells and confirmed by PCR showed that one strain had the VT2 gene (strains CP22) and two strains had both VT1 and VT2 genes (strains CP44 and CP47). The titles obtained for the O157 antigen were: 1/2560 (strain CP47), 1/5120 (strain CP22) and 1/10,240 (strain CP44).

Discussion

Two samplings were performed during 1994 and 1995. During 1994 we investigated the presence of EHEC O157:H7 in 72 fresh beef samples (54 hamburgers and 18 minced meat samples). The resuscitation of the *E. coli* was realized in tryptone soy broth, the selective enrichment in MacConkey broth, and the isolation on SMAC with tellurite. Though a special selective medium for the isolation of sorbitol-negative EHEC O157:H7 strains was used, we found no evidence of O157:H7 strains in the 72 samples examined (Table 3) (10). IMS was included in the study of 1995. This technique allows to recover EHEC strains at concentrations as low as one bacterium per gramme and mixed with other *E. coli* strains or bacterial species (17, 30). Furthermore, the most adequate conditions for the recovery of EHEC O157 were applied: (i) enrichment in buffered peptone water supplemented with vancomycin, cefixime and cefsulodin, and (ii) isolation on CT-SMAC (17). This procedure let us to detect sorbitol and

TABLE 2. Detection of EHEC O157:H7 by conventional methods and by immunomagnetic separation

Positive samples for EHEC O157:H7	No. of EHEC O157:H7 colonies / No. of total *E. coli* colonies investigated	
	Conventional methods	Immunomagnetic separation
CP22	0/21	8/10 (1%)[a]
CP44	0/111	1/10 (25%)[a]
CP47	0/20	1/10 (60%)[a]

[a] Percentage of sorbitol non-fermenting colonies grown on CT-SMAC after immunomagnetic separation.

TABLE 3. EHEC O157 in beef: comparison of results obtained in different countries

Location (country)	Year	No. with EHEC O157 / No. total of samples	Method used for detection of EHEC O157	Reference
Manitoba (Canada)	1988	0/71 (0%)	ELISA-O157[a]	37
	1989	4/164 (2%)	ELISA-O157[a]	37
Ontario (Canada)	1988	0/225 (0%)	Verocytotoxin neutralization assays[a]	35
Calgary (Canada)	1985	5/17 (29%)	HGMF-immunoblot[a]	18
Wisconsin (USA)	1985–86	1/147 (0.7%)	HGMF-immunoblot[a]	18
Wisconsin (USA)	1990–91	3/107 (3%)	ELISA-O157[a]	32
Bangkok (Thailand)	1989	0/93 (0%)	DNA Hybridization-VT1 and VT2 genes (5 colonies)	40
London (UK)	1992	0/310 (0%)	DNA Hybridization-VT1 and VT2 genes[a]	41
Madrid (Spain)	1993	2/75 (3%)[b]	Serotyping (O157)	2
Lugo (Spain)	1994	0/72 (0%)	Vero and HeLa cells (20 colonies)	10
	1995	3/58 (5%)	Immunomagnetic separation (10 col.)	This work

[a] Cultures with up to 1000 colonies were tested for the presence of *Escherichia coli* O157 or verotoxin-producing *E. coli*.
[b] In this study the production of verotoxins was not investigated.
ELISA-O157, Enzyme-linked immunosorbent assay with an antibody specific for *E. coli* O157 antigen; HGMF-immunoblot, Hydrophobic grid membrane filter-immunoblot procedure using antiserum to *E. coli* O157:H7 culture filtrate.

β-glucuronidase negative EHEC O157:H7 strains in 3 (5%) of the 58 samples investigated in 1995 (see Table 1). This high percentage of detection has been improved only by Doyle and Schoeni (18) in Calgary (Alberta, Canada), where the rates of EHEC O157:H7 infection are the highest found in the world. Nevertheless, it must be considered that our three EHEC O157:H7 samples came from the same supermarket, though they were obtained within a month. It seems to be two different types of O157:H7 strains, one $VT2^+$ and two $VT1^+$ $VT2^+$. However, they might come from the same clone, for VT1 and VT2 genes can be easily lost. Specific epidemiologic markers should be studied to identify the strains. Benezet et al. (2) investigated the presence of *E. coli* O157:H7 in 125 fresh beef and pork samples, and also in 20 boiled ham samples. Most of those samples came from factories in Madrid. *E. coli* O157:H7 strains were isolated from 2 (3%) of the 75 beef samples, and from 1 (2%) of the 50 fresh pork samples. Nevertheless, it cannot be assured that these strains are VT^+, as they were not probed for verocytotoxicity. It must be pointed out that IMS was conclusive for the detection of EHEC O157. Padhey and Doyle (32) found EHEC O157:H7 in 3 (3%) of 107 beef minced meat samples by immunoassay, using a policlonal antiserum specific for the O157 antigen. They also determined the MPN of EHEC O157:H7 for the three samples, which ranged from 0.4 to 1.5 bacteria/g. Our study confirms these results, as the concentration of EHEC O157:H7 in one sample (CP44) was so low that we could not recover it after testing 111 colonies by conventional methods (see Table 2). Our results show that IMS may be a useful technique for routine detection of EHEC O157:H7 from outbreaks in microbiological laboratories. The reactives for IMS (Oxoid), as well as the O157

and H7 antisera (Difco) are available. Sorbitol and β-glucuronidase negative O157:H7 strains may be considered presumptively as verotoxigenic, but it is advisable to send them to a reference laboratory to confirm the production of verotoxins.

Considering that EHEC O157:H7 strains have been isolated from beef meat with a relative frequency, and that they have been found in the intestinal content of several cows and calves from farms in Spain, outbreaks in humans could be expected to happen frequently too. Marne et al. (26), Gaztelerrutia et al. (20), Elcuaz et al. (19), Canut et al. (15), and Navarro et al. (28) studied the presence of sorbitol-negative EHEC O157:H7 strains in 17,843 stool specimens, which were recovered from only 9 cases. This means a rate of incidence of 0.05% (7, 34). We have recovered them from three (0.6%) of the 514 stool specimens obtained from patients of the Complejo Hospitalario Xeral-Calde (Lugo) during 1995, from January to September (data not published).

The low incidence of human infection in relation to the potential reservoir can be explained by the fact that bovine strains isolated in Spain might lack pathogenic factors for human infection, or because meat processing and cooking habits inactivate them. It is also possible that contact with this bacterium for many generations has made the population immune against it. However, adequate control measures and an epidemiologic screening should be considered, since this problem broke out suddenly in Canada, the USA, and UK in the early eighties. So, consumers need to be informed about hazards from undercooked beef meat. The most effective measure is to cook meat adequately until an internal minimum temperature of 68.3°C. To reduce the incidence of pathogenic microorganisms in meat products, a series of Hazard Analysis and Critical Control Point (HACCP) programmes should be developed and implemented in farms, slaughter facilities, and postlaughter meat processing operations, as well as at further points along the system from the farm to food consumption. Economic analyses show that the public health benefits expected from the reduced number and severity of foodborne diseases resulting from the use of irradiation are greater than the costs associated with the implementation of the irradiation process. Detection of animal carriers has also been suggested to reduce their occurrence in farms (27).

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Evaluation of an enzyme immunoassay for verotoxin detection in *Escherichia coli*

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Summary

Verotoxin-producing *Escherichia coli* strains (VTEC) cause hemorrhagic colitis and hemolytic-uremic syndrome in humans. Laboratory diagnosis by conventional methods is slow and cumbersome. The results of a new rapid enzyme immunoassay (EIA Premier EHEC) for verotoxin detection both in isolated strains and in clinical samples are presented, and they are compared with cell culture (CC) and polymerase chain reaction (PCR) techniques. Fifty-four strains have been analyzed by both EIA and PCR, and 33 by all three methods. The kit has also been evaluated for experimentally infected stool samples directly and after their enrichment on MacConkey broth. Nineteen, out of the 54 strains, were positive by EIA and 20 by PCR. The results of the 33 strains evaluated by the three techniques were coincident with one exception. The latter was uninterpretable by CC, negative by EIA and positive by PCR. The sensitivity of the kit for experimentally infected stool samples was approximately 5×10^7 bacteria/ml in the direct test, and 5×10^4 bacteria/ml after broth enrichment. EIA sensitivity and specificity were similar to those of CC and PCR. The diagnostic times were 18h for EIA, 3 days for PCR and 5 days for CC. Sensitivity, rapidity and ease of performance make this technique especially valuable for clinical diagnosis.

Key words: *Escherichia coli*, verotoxins, enzyme immunoassay, cell culture, PCR

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Resumen

Las cepas de *Escherichia coli* productoras de verotoxina (VTEC) causan colitis hemorrágicas y síndrome hemolítico urémico en los seres humanos. El diagnóstico por métodos convencionales resulta lento y engorroso. En este trabajo se presentan los resultados de un inmunoensayo rápido con enzimas (EIA Premier EHEC) para la detección de la verotoxina en cepas aisladas y en muestras clínicas, y se comparan con los resultados obtenidos con células de cultivo (CC) y con la reacción en cadena de la polimerasa (PCR). Se analizaron 54 muestras mediante EIA y PCR, y 33 aplicando los tres métodos. También se evaluó el "kit" en muestras de heces infectadas experimentalmente, directamente y tras su enriquecimiento en caldo MacConkey. De las 54 muestras analizadas, 19 resultaron positivas en el inmunoensayo (EIA). Los resultados de las muestras evaluadas mediante las tres técnicas coincidieron, excepto en un caso, que no pudo interpretarse mediante CC, y que resultó negativo en EIA y positivo en la PCR. La sensibilidad del "kit" en las muestras de heces infectadas experimentalmente fue de aproximadamente 5×10^7 bacterias/ml en la prueba directa y de 5×10^4 bacterias/ml después del enriquecimiento en el caldo de cultivo. La sensibilidad y especificidad de EIA eran similares a las de CC y de PCR. Los tiempos de diagnóstico fueron 18 horas para EIA, 3 días para PCR y 5 días para CC. La sensibilidad, rapidez y facilidad de empleo hace que esta técnica sea especialmente valiosa para el diagnóstico clínico.

Introduction

Verotoxin-producing *Escherichia coli* O157:H7 strains (VTEC) have been traditionally associated with hemorrhagic colitis (3, 20), the hemolytic-uremic syndrome (8) and thrombotic thrombocytopenic purpura syndromes (7).

Other verotoxigenic serotypes have also been associated with these clinical symptoms, although less frequently, such as O4:H^-, O26:H11, O45:H2, O111:H^-, O128:H^- and O145:H^-, as well as O157:H^-, that may correspond to non-motile variants of O157:H7, among others (2). VTEC strains produce two types of verotoxin: verotoxin 1 (VT1) and verotoxin 2 (VT2), that can be synthesized simoultaneously or independently, and are codified by different bacteriophages. VT1 is similar to the Shiga toxin of *Shigella dysenteriae* type 1 (16, 22). Four different VT2 variants are currently known. VTEC strains that cause hemorrhagic colitis are also known as enterohemorrhagic *E. coli* (EHEC) strains.*

A conventional method for the detection of *E. coli* O157:H7 serotype (usually associated with verotoxin production) is the coproculture isolation of the microorganism, followed by the use of biochemical markers, such as sorbitol or β-glucoronidase (which are characteristically negative in these strains) (12,

* See Blanco et al., at pp. 385–394, this issue. (Editor's note.)

13), and by the determination of the serotype by specific antisera.

Production of verotoxin (11) is determined by cell culture, the "gold standard" technique. However, this method is usually slow and cumbersome. Besides, cell lines sensitivities are variable, the interpretation of the results is subjective, and the loose of verotoxin production by subcultures has been demonstrated.

Taken together, the diagnosis of *E. coli* O157:H7 infection and the confirmation of verotoxin production require 5 days approximately and, besides, it cannot detect other verotoxigenic serotypes.

Polymerase chain reaction (PCR) has been used for some years as a quick method for the detection of verotoxin-codifying genes in bacterial strains, both from culture (17) and directly from feces, with excellent results (14). This technique has the disadvantage of the non-availability of commercialized reagents, together with the general limitations of the method (4, 21, 24, 25).

As far as serological tests are concerned, they are not rapid and cross-reactions with other microorganisms have been described (5, 6, 9, 26).

Alternative tests are necessary to establish a rapid diagnosis from direct sample. In the last years enzyme immunoassays (EIA) have been experimentally developed for the detection of both verotoxins, altogether or each one independently (1, 15, 18). Similar results have been obtained by EIA with respect to cell culture, even though some difficulties have arisen for the detection of some VT2 variants (15).

Recently, a kit (Premier EHEC) for the detection of both verotoxins, directly from clinical samples or from isolated bacterial strains, has been commercialized (Acheson, D. W. K. et al., 1994. 2nd International Symposium on Verocytotoxin Producing *Escherichia coli* Infections. Bergamo, Italy). The aim of this study was to compare the results for the detection of VT1 and VT2 by EIA with those of cell culture and PCR from bacterial strains and stool specimens.

Materials and methods

Bacterial strains. *(i) Problem strains.* We studied 39 *E. coli* strains obtained from patients with enterocolitis from different hospitals in Spain (19). Thirteen of them belong to O157:H7 serotype, 8 to *E. coli* O157:H$^-$, 1 to *E. coli* O128:H$^-$, 5 to *E. coli* O26:B6, 2 to *E. coli* O111:B4, 1 to *E. coli* O157:H2, 1 to *E. coli* O157:H26, 1 to *E. coli* O157:H39 and 7 to *E. coli* of undetermined serotype. Besides, ten *Citrobacter freundii* strains were included because the presence of VT2-related gene sequences had been described in this species.

(ii) Control strains. E. coli 933 (O157:H7 VT1 and VT2 producer) and a *S. dysenteriae* type 1 strain were included as positive controls. A2 (*E. coli* O157:K88:H19 non-verotoxigenic) and 2 *S. dysenteriae* strains, that agglutinated with polyvalent A2 antiserum, were used as negative controls.

Detection of verotoxins by Cell Culture and Polymerase Chain Reaction (PCR). Verotoxin production was assessed by the method described by Konowalchuk et al. (11), in which ATCC CCL 81 Vero cell line was used. Thirty-two *E. coli* and one *S. dysenteriae* type 1 strains were studied by this method.

VT1 and VT2 codifying genes were analyzed independently by the method described by Olsvik (17), which amplifies a specific 895 bp VT1 sequence and a specific 479 bp VT2 sequence.

The confirmation of PCR results was done by restriction enzyme digestion. Our experience with this method has been published previously (14). All 54 strains described in the materials and methods section were studied by this procedure.

VT1 and VT2 verotoxin detection by Enzyme Immunoassay (EIA). The Premier EHEC kit (Meridian Diagnostics, Inc., Cincinnati, OH, USA) was used for the detection of both verotoxins in culture isolates and in stool specimens. The kit, based on a capture EIA, is composed of plastic microwell strips coated with a mixture of two monoclonal antibodies: HD3, directed against the VT1 B subunit, and HD1, which recognizes both verotoxin B subunits. Afterwards, a specific polyclonal antibody was added. Positive reaction was visualized by incubation with an enzyme conjugate (polyclonal anti-IgG antibody conjugated to horseradish peroxidase) and the following addition of a chromogenic substrate (urea peroxidase). The reaction was read by spectophotometric determination at the wavelengths 450/630 nm.

Sample preparation. Two methods were used to treat bacterial strains in order to compare the sensitivity and specificity of both procedures: solid medium and broth culture. Fourteen strains were analyzed by both methodologies and the other 40 were only evaluated by cultivation on solid medium. Twenty-one of these 40 strains were checked on duplicate to determine the test reproducibility.

(i) Plate culture. Two bacterial colonies were transferred from a MacConkey agar overnight culture into a tube containing 200 µl of the kit sample diluent and was subsequently homogenized with a mixer.

(ii) Broth culture. 50 µl from a Luria Bertani (LB) overnight culture was put into an Eppendorf tube containing 200 µl of the kit sample diluent and was also homogenized.

The evaluation of the kit Premier EHEC with stool samples was done through experimental contamination of the specimens. Stool samples with different characteristics were selected to check the efficiency of the commercial reagent: liquid stools, pasty stools, mucous stools and stools containing erythrocytes and leucocytes. Different concentrations (10^6–10^8) of VT1, VT2 and VT1 and VT2 positive bacterial strains were added respectively to different characteristic stool samples. All non-fluid stool samples had to be diluted previously to obtain valuable results. As negative controls, the same stool samples were analyzed without the addition of VT-producing strains.

A mixture of stool specimens and bacterial suspensions at ca. 5×10^4 bacteria/ml concentration was tested directly and with a previous enrichment on MacConkey broth for 4 and 18 h, respectively.

Enzyme immunoassay technique (Premier EHEC). The EIA technique was performed following the manufacturer's instructions. The isolated bacterial strains were analyzed by adding 100 µl of every bacterial suspension, obtained from solid medium or broth culture, to each microwell of the microtiter plate.

The stool samples were tested by the addition of 200 µl of the diluent sample to150 µl and 50 µl, respectively, of the mixtures obtained by the direct and enrichment method. 100 µl of each sample were transferred to each corresponding microwell. A positive and a negative control were included on each run. Results were read between 5 to 15 min of the incubation with the stop solution. The optical density (OD) of the negative control should

TABLE 1. Detection of verotoxins on culture isolates. Results obtained by Cell Culture (CC), Polymerase Chain Reaction (PCR) and Enzyme Immunoassay (EIA) techniques in cultures isolates of several species and bacterial serotypes

Species and serotypes	No. strains	CC[1]		PCR[2]		EIA[3]	
		+	−	+	−	+	−
Control strains:							
Escherichia coli O157:H7 VT1 and VT2 positive	1	1	0	1	0	1	0
Shigella dysenteriae type 1 VT1 positive	1	1	0	1	0	1	0
Escherichia coli O157:K88:H19 VT1 and VT2 negative	1	0	1	0	1	0	1
Shigella dysenteriae non type 1*, VT1 and VT2 negatives	2	ND[a]	ND	0	2	0	2
Problem strains:							
Escherichia coli O157:H7	13	11	2[b]	12	1	11	2
Escherichia coli O157:H⁻	8	5	3	5	3	5	3
Escherichia coli O26:B6	5	ND	1	0	5	0	5
Escherichia coli O111:B4	2	ND	ND	0	2	0	2
Escherichia coli O128:H⁻	1	1	0	1	0	1	0
Escherichia coli O157:H2	1	0	1	0	1	0	1
Escherichia coli O157:H26	1	0	1	0	1	0	1
Escherichia coli O157:H39	1	0	1	0	1	0	1
Escherichia coli (undetermined serotype)	7	ND	4	0	7	0	7
Citrobacter freundii	10	ND	ND	0	10	0	10

[1] CC, positivity refers to observation of morphologic alteration on cultured cells, compatible with the ones produced by verotoxins.

[2] PCR, positivity refers to the presence of specific sequences of VT1 and/or VT2, confirmed by restriction enzyme digestion.

[3] EIA, a result was considered positive when the optical density at 450/650 was ≥0.150.

[a] ND, not done. [b] One result by CC was uninterpretable.

* Positive agglutination with A2 polyvalent antiserum.

be <0.150 and the OD of the positive control ≥0.150. All samples with an OD ≥0.150, were considered positive and those with an OD <0.150 were considered negative.

Results

Detection of verotoxin production by CC and EIA and the presence of its codifying genes on bacterial cultures by PCR. All thirty-three strains described on the materials and methods section, including the controls, were studied by PCR and EIA. The results obtained by cell culture, PCR and EIA are shown on Table 1.

Control strains had the expected results in all runs. The data obtained from isolated strains, which are shown in Table 1, correspond to solid medium. The strains simultaneously studied from solid and liquid media by EIA produced the same qualitative results, although the average OD obtained from solid culture (OD = 2.661) was higher than

the one obtained from broth culture (OD = 1.814) (data not shown). The results obtained from the 21 strains, that were analyzed on duplicate by the plate method, were also coincident (data not shown).

Table 2 shows the results obtained for the 33 strains studied by using the three methods. Nineteen out of these strains were EIA Premier EHEC, cell culture and PCR positive, and thirteen were negative by using the three methods. One strain was negative by using the EIA technique, uninterpretable by cell culture and PCR positive.

Detection of verotoxin production directly from stools. *Stool samples experimentally infected.* Stool specimens experimentally infected were checked by using the EIA technique following two methods: the direct method and after their enrichment on culture medium (see materials and methods).

Results from the samples directly studied, and the controls incorporated (non-infected stools), are shown in Table 3. Positive results were obtained at concentrations higher than 5×10^7 bacteria/ml. The average $OD_{450/630}$ obtained from mixtures of feces with different VT1-producing strains concentrations was twice the one obtained from VT2-producing strains (O D = 0.532 for VT1 and OD = 0.244 for VT2), and this one was slightly higher to the one obtained from the mixtures with both VT-producing strains (OD = 0.187 for VT1+VT2).

Results obtained with experimentally infected samples incubated for their enrichment for 4 and 18 h are shown in Table 4. The three mixture types were negative after a 4 h MacConkey broth enrichment; on the other hand, they had positive results after their enrichment on this medium for 18 h.

Discussion

The EIA Premier EHEC method has been useful for the detection of VT from enterohemorrhagic *E. coli* and *S. dysenteriae* type 1 strains, both in plate and in broth cultures. However, the sensitivity of the technique has been higher by the first procedure. Besides, the reproducibility of the plate culture has been demonstrated. These results coincide with the preliminary data shown in the aforementioned International Symposium held in Bergamo (Italy), in 1994. We cannot compare these results with our data because the procedures used were different in each case.

The EIA Premier EHEC method is also suitable for the detection of verotoxin in stool samples of various characteristics. Those with

TABLE 2. Results obtained on comparison of the three evaluated techniques

	Cell Culture			PCR	
Premier EHEC-EIA	Positive	Negative	Uninterpretable	Positive	Negative
Positive	19*	0	0	19*	0
Negative	0	13	1	1	13
Total	19	13	1	20	13

This table shows the comparison of the results obtained for the 33 analyzed strains by the EIA, PCR and CC techniques.
* A *Shigella dysenteriae* type 1 strain is included.

TABLE 3. Detection of verotoxins by the EIA technique in stool samples. Results show the optical density values obtained by the EIA Premier EHEC technique on the detection of VT from the mixtures of stool samples and verotoxigenic bacterial suspensions

	Concentration of verotoxigenic bacteria per ml			Negative controls[a]
Stools(VT)[b]	10^6	5×10^7	10^8	
Liquid (VT1)	0.139 (–)[c]	0.408 (+)	0.610 (+)	0.106 (–)
Pasty (VT1)	0.157 (+)	0.592 (+)	0.918 (+)	0.094 (–)
Mucous (VT1)	0.144 (–)	0.568 (+)	0.978 (+)	0.093 (–)
Leucoc+Eryth (VT1)	0.127 (–)	0.559 (+)	0.920 (+)	0.082 (–)
Liquid (VT2)	0.107 (–)	0.207 (+)	0.327 (+)	0.106 (–)
Pasty (VT2)	ND[d]	0.287 (+)	0.449 (+)	0.094 (–)
Mucous (VT2)	ND	0.275 (+)	0.407 (+)	0.093 (–)
Leucoc+Eryth (VT2)	0.107 (–)	0.207 (+)	0.327 (+)	0.082 (–)
Liquid (VT1+VT2)	0.120 (–)	0.166 (+)	0.159 (+)	0.106 (–)
Pasty (VT1+VT2)	0.133 (–)	0.199 (+)	0.178 (+)	0.094 (–)
Mucous (VT1+VT2)	0.100 (–)	0.198 (+)	0.201 (+)	0.093 (–)
Leucoc+ Eryth (VT1+VT2)	0.106 (–)	0.185 (+)	0.157 (+)	0.082 (–)

[a] These results concern to the stool samples analyzed by EIA withouth the addition of verotoxigenic bacteria.
[b] Characteristics of the stool specimens used for EIA and type of verotoxin produced by the added strains.
[c] The interpretation of the results appears inside the parenthesis, next to the optical density, (–) negative and (+) positive.
[d] ND, not done.

pasty and mucous consistence needed a previous dilution in order to obtain positive results. The higher positivity obtained from the mixtures that contained VT1-producing strains versus those with VT2-producing strains is correlated with the sensitivity described by the manufacturer for each verotoxin (7 pg for VT1 and 15 pg for VT2), and is coincident with the fact that two monoclonal antibodies are used, which recognize the VT1 B subunit, HD1 and HD3, whereas only one, HD1, recognizes VT2 B subunit. This fact can be a disadvantage for the analysis of the VT2-producing strains, especially because the concern of the high prevalences of these infections in the studied area (19).

We can emphasize that OD results obtained with strains which produce both verotoxins were lower than those obtained with the strains that produce only one of them. This could be the consequence of some kind of competition between the two verotoxins for the binding to their specific receptors. Our data show a higher sensitivity (about 5×10^7 bacteria/ml) than those described on the kit (6×10^8 bacteria/ml), in order to obtain positive results in feces. Moreover, the commercial kit does not show the results obtained with a lower concentration of bacteria. According to our data, we did not obtain positive results with the concentration of 10^6 bacteria/ml. This emphasizes the value of performing a

TABLE 4. Verotoxin detection from enriched stool samples on broth culture. Results show the optical density values obtained by the EIA Premier EHEC technique on the verotoxin detection from the mixtures of the stool samples and the suspensions of verotoxin-producing bacterial strains after broth enrichment

Stools (VT)[a]	Concentration of the bacterial suspensions added to stool specimens per ml	
	5×10^4 MacConkey 4 h[b]	5×10^4 MacConkey 18 h[c]
Liquid (VT1)	0.045 (–)	0.167 (+)
Liquid (VT2)	0.042 (–)	1.933 (+)
Liquid (VT1+VT2)	0.041 (–)	2.488 (+)

[a]Characteristics of the stool specimens used for EIA and type of verotoxin produced by the added bacterial strains.
[b] Results obtained by EIA for the strains previously enriched on MacConkey broth for 4 h.
[c]Results obtained by EIA for the strains previously enriched on MacConkey broth for 18 h.

preliminary incubation of the sample in order to increase the initial inocula and to guarantee the reliability of the results. In fact, the presence of verotoxin has only been shown after the prolongation to 18 h, of the samples incubation time on MacConkey medium which limits the rapidity of the diagnosis by using this technique.

The optical densities obtained whith the mixtures containing VT2-producing strains, and on enriched MacConkey broth, were higher than those with VT1-producing strains. Those results might depend on a random enrichment, that should be favourable for VT2-producing strains, or might be related to pH variation (23) that can occur on 18 h incubation periods, which could alter VT1.

Premier EHEC method has shown a sensitivity similar to PCR technique, even though one strain was positive by PCR and negative by EIA. These contradictory results might be due to repression of the verotoxin-codifying genes or to structural differences of the expressed protein that could alter the binding to specific receptors. This hypothesis is consistent with the uninterpretable result obtained by this strain in cell culture.

Besides, this technique does not require skilled workers nor additional equipment to that available in any laboratory. The sample preparation is easy and does not need purifying methods that are sometimes necessary to remove PCR inhibition substances (25).

In contrast with PCR, the analyzed technique does not allow the identification of the type of verotoxin produced, although the importance of this is not determined.

Positive results obtained by cell culture and EIA Premier EHEC methodologies are concordant, although the theoretical sensitivity of the first method is higher for verotoxin detection (1 pg and 7–15 pg, respectively) (10). In comparison with cell culture, the results of EIA method are not subjective and we have not had indeterminate results.

Besides, the diagnosis time of enzyme immunoassay is outstandingly lower than that of cell culture (it takes 18 h and 5 days, respectively) and does not require special manipulation measures nor cabinet hoods to guarantee the sterility of cell cultures.

In conclusion, the technique analyzed in this study has been useful for the detection of verotoxin from isolated strains and from ex-

perimentally performed stool samples. Its rapidity, the objectivity of the interpretation of the results obtained, and its easy performance make it an advantageous method compared with cell culture and PCR.

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Buffering capacity and membrane H^+ conductance of *Halobacterium halobium*

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Summary

Buffering capacity and membrane H^+ conductance were measured in *Halobacterium halobium* suspensions in the light and in the dark over a wide range of external pH. The values of both variables for this archaeobacterium were significantly higher than those found for eubacteria in other reports. It appears from our results that the special chemical composition of the cell envelope and the movement of ions, mainly protons, may influence the magnitude of the buffering power and the H^+ membrane conductance of these cells.

Key words: *Halobacterium halobium*, buffering capacity, H^+ conductance, halophilic bacteria, membrane permeability

Resumen

En este trabajo determinamos la capacidad de tamponamiento y la conductancia de la membrana a los protones de suspensiones de *Halobacterium halobium* iluminadas y en la oscuridad en un amplio rango de pH externos. Los valores de ambas variables para esta arqueobacteria fueron significativamente superiores a los encontrados para eubacterias en otros trabajos. De nuestros resultados se desprende que la especial composición química de las envueltas celulares y el movimiento de iones, principalmente protones, puede influenciar la magnitud de la capacidad de tamponamiento y de la conductancia de la membrana de estas células a los protones.

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Introduction

The extremely halophilic bacterium *Halobacterium halobium*, which thrives in nearly saturated salt solutions (5), possesses a thin, very fragile cell envelope which is composed of an S layer and an inner membrane structure. The cell envelope consists of 75% of highly acidic proteins and 25% of lipids (2, 7). *H. halobium* also develops purple membranes on its cell envelope. The purple color of the membranes is due to the protein-linked chromophore *all-trans*-retinal. These membranes function as a light-activated proton pump to mediate photophosphorylation and they are composed of two rhodopsin-like proteins, bacteriorhodopsin and halorhodopsin, and various polar lipids derived from isopranyl diethers (18). Studies on bacteriorhodopsin and halorhodopsin indicate that they function as light driven proton and chloride pumps, respectively. Bacteriorhodopsin converts light energy into an electrochemical potential difference of protons across the membrane of the cells. This proton gradient directly drives ATP synthesis (Fig. 1) (1,19, 20).

The buffering capacity is the amount of protons that can be neutralized. It has two components: the buffering capacity of the cell surface (Bo) and the cytoplasmic buffering capacity (Bi). The membrane H^+ conductance is the rate at which proton can transverse a membrane. Since both the special chemical composition of the cell envelope and the proton translocating capacity of bacteriorhodopsin may influence the magnitude of buffering capacity and membrane conductance to protons, it was of interest to determine both values for *H. halobium* in the light and in the dark.

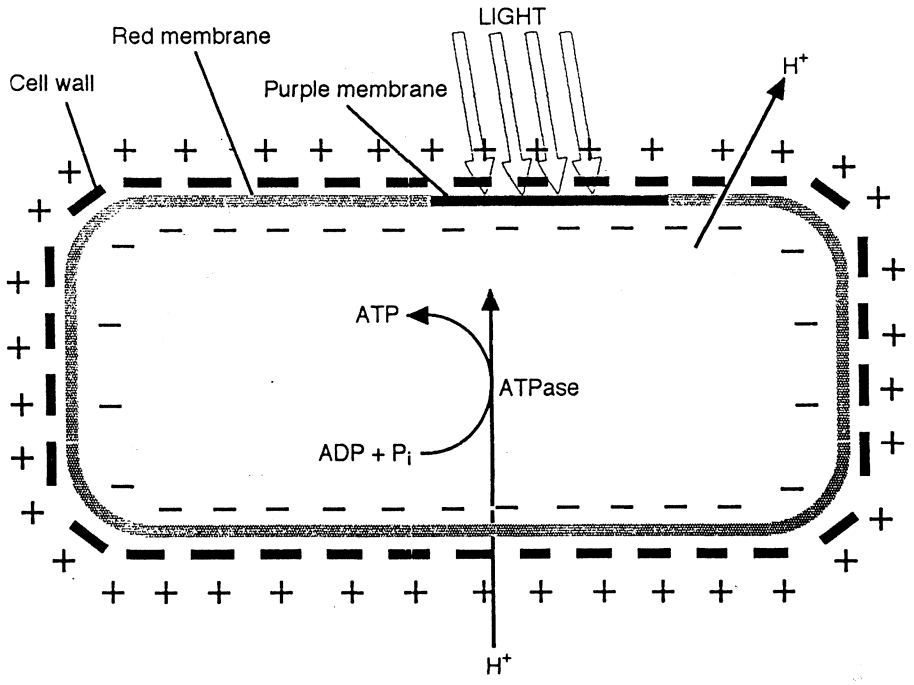

FIG. 1. Scheme of protons transport across the membrane of *Halobacterium halobium*. Based on Ronald M. Atlas (1988), *Microbiology: Fundamentals and Applications*, p. 142. MacMillan Pub. Co., New York.

The experiments reported here provide quantitative estimates of buffering capacities and membrane conductance to protons over a wide range of pH (5.3 to 8.3) of *H. halobium* suspensions. This bacterium, both in the light and in the dark, presented higher values of these variables than any of the bacterial species studied (3, 4, 10, 12, 13, 14, 15, 16, 17, 21).

Material and methods

Bacterial strain and growth conditions. *Halobacterium halobium* CCM 2090 was used in these experiments. Cells were grown with vigorous shaking in M_1 medium (8). They were kept as agar slope cultures stored at 4°C.

Chemicals. Valinomycin and carbonic anhydrase were from Sigma Chemical Co. All other chemicals were obtained from commercial sources. Valinomycin was used at a final concentration of 10 μM and added to cell suspensions as small volumes of concentrated stocks in acetone; final acetone concentrations did not exceed 0.2%. Carbonic anhydrase was prepared at 20 mg/ml in 3 M KCl.

Preparation of non-proliferating cell suspensions (NPC). Cells were harvested in the early stationary phase of growth and washed three times in 3 M KCl. The washed cells were treated with 3 mM EDTA in 3 M KCl (12) and resuspended in 3 M KCl to a final concentration of 0.5 to 2.5 mg of cell protein per ml. Light samples were illuminated with a 100 W lamp (light intensity 2×10^5 ergs cm^{-2} s^{-1}). Dark samples were kept in the dark for the time re-

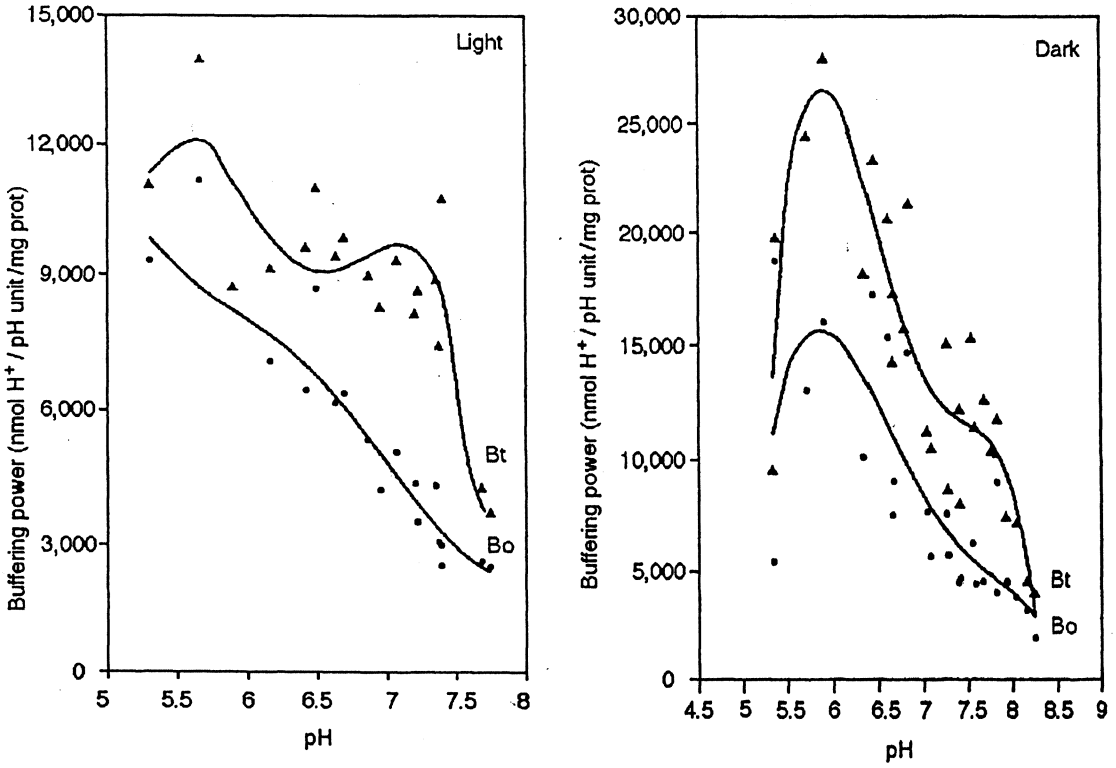

FIG. 2. Buffering capacity of the external surface (Bo) and total buffering capacity (Bt) of *Halobacterium halobium* suspensions in the light and in the dark.

quired for collection of data (several hours). The temperature of samples was 25–30°C, both in the light and in the dark.

Measurement of cell protein. Protein content was determined according to the method described by Lowry et al. (9).

Measurement of buffering capacity and membrane H^+ conductance. Experiments were conducted on 7-ml samples of cell suspensions in 10-ml glass vials, which were magnetically stirred. The buffering capacity and membrane conductance to H^+ of these bacteria were measured by an acid-pulse technique, as described and discussed elsewhere (10, 12, 13, 14, 15, 16, 17). Buffering capacity and membrane H^+ conductance are presented as functions of external pH. The smooth curves that describe the behaviour of these variables were obtained from a polynomic regression.

Results

Buffering capacity and membrane conductance to protons of *Halobacterium halobium* CCM 2,090 were measured in the region pH 5.3 to 7.8 in the light. The pH range studied for titrations of the cells in the dark was 5.3 to 8.3.

Fig. 2 summarizes measurements of Bo (buffering capacity of the cell surface) and Bt (total buffering capacity) as a function of pH for both suspensions studied. Illuminated cells presented a maximum value of Bo of 9,809 nmol H^+/pH unit per mg of protein at pH 5.3 and a minimum value of 2,352 nmol H^+/pH unit per mg of protein at pH 7.8. The Bt value of these suspensions at pH 5.7 was 11,920 nmol H^+/pH unit per mg of protein, and Bt at pH 7.8 it was 3,627 nmol H^+/pH unit per mg of protein. The suspensions of *H. halobium* in the dark presented higher values of Bo and Bt than those found for the illuminated suspensions. *H. halobium* in the dark had a maximum value of Bt of 26,514 nmol H^+/pH unit per mg of protein at pH 5.9 and a minimum value of Bt of 2,837 nmol H^+/pH unit per mg of protein at pH 8.3.

Values for the cytoplasmic buffering capacity (Bi) were calculated from smooth curves that describe the behaviour of Bo and Bt and are shown in Fig. 3. Cells in the dark exhibited Bi values that were 6-fold those of cells in the light in the acidic range of pH. Illuminated cells presented a maximum value of Bi of 5,473 nmol H^+ /pH unit per mg of protein at pH 7.35. Cells in the dark had a maximum value of Bi of 10,952 nmol H^+ /pH unit per mg of protein at pH 5.9.

Fig. 4 shows that illuminated suspensions of *H. halobium* exhibited values of membrane conductance to protons (C^H_m) from 20.9 to 27.9 nmol H^+ /s per pH unit per mg of protein in the region pH 5.3 to 7.4 (data from the smooth curve).

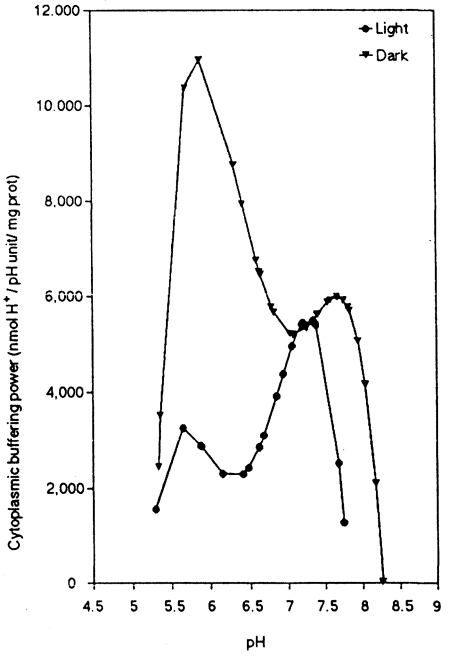

FIG. 3. Cytoplasmic buffering power of *Halobacterium halobium* cells.

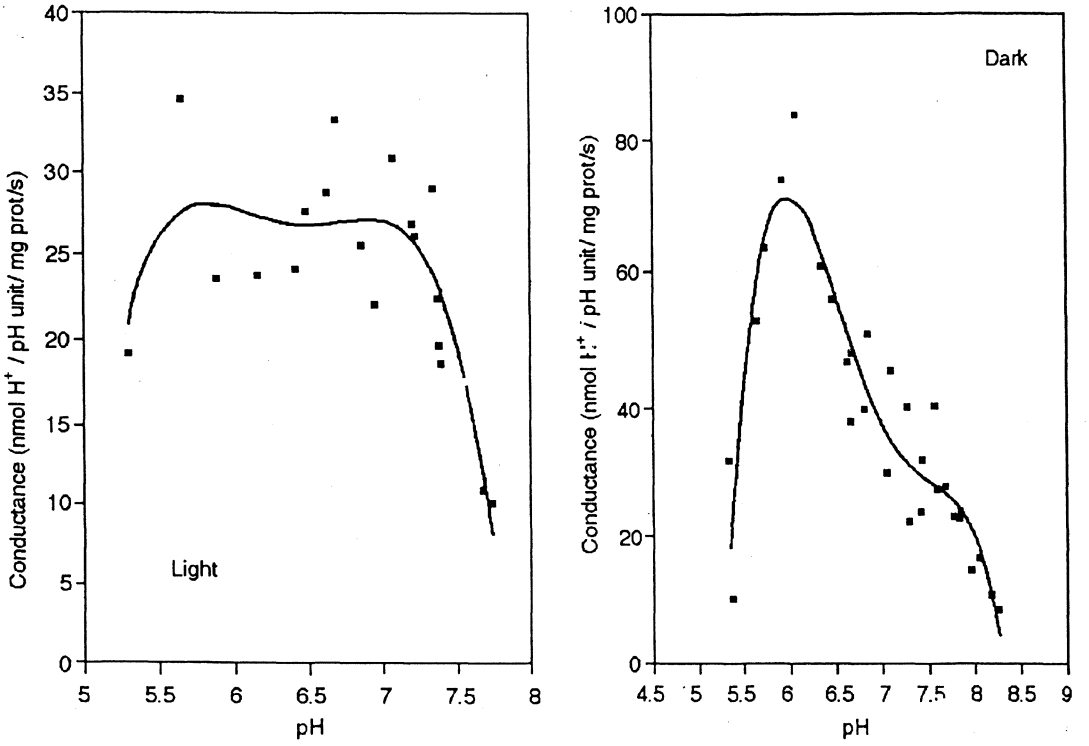

FIG. 4. Membrane H^+ conductance of *Halobacterium halobium* in the light and in the dark.

Above pH 7.4 C^H_m of these suspensions decreased to 7.9 at pH 7.8. Passive proton conductance of suspensions of *H. halobium* in the dark, as well as buffering capacity, was sensitive to the absolute external concentration of protons. These suspensions had a maximum value of C^H_m of 67.8 nmol H^+/s per pH unit per mg of protein at pH 5.9 and a minimum value of C^H_m of 4.4 nmol H^+/s per pH unit per mg of protein at pH 8.3.

Discussion

The results reported here give an estimate of the buffering capacity and membrane H^+ conductance of *H. halobium* under illumination and in the dark. The most important finding was the observation of significantly higher values of these variables in *H. halobium* than in any eubacteria studied (3, 4, 10, 12, 13, 14, 15, 16, 17, 21). These high differences could be due to different factors: (1) the special cell envelope structure which consists of 75% of highly acidic proteins and 25% of lipids; (2) the acidity of ribosomal proteins, and (3) the high intracellular salt concentration (2, 5, 7).

H. halobium cells in the dark exhibited higher values of buffering capacity and H^+ membrane conductance than illuminated cells. It has been shown that light induced a net outward proton translocation from the cells and that the release of protons from the cell in every photocycle in cell suspensions depended on pH values. Very closely related to proton translocation were the light-induced pH changes of the medium. The complex kinetics seen in cell suspensions may be due in part to the presence of another light-driven ion pump, halorhodopsin, and gated ion

channels (6, 11, 19). Our results suggest that the movement of ions, mainly protons, across the cell envelope of *H. halobium* influenced the buffering capacity and H^+ membrane conductance values for illuminated cells, diminishing them. Therefore, the results obtained for *H. halobium* cells in the dark reflected well the buffering power and the passive proton conductance of this archaeobacterium.

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Influence of medium composition on lactic acid production from dried whey by *Lactobacillus delbrueckii*

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Summary

The production of lactic acid, lactic acid dehydrogenase (LDH) and single cell protein (SCP) from dried whey by fermentation with *Lactobacillus delbrueckii* subsp. *bulgaricus 369* was investigated. A medium consisting of dried whey and supplements which enabled exponential growth of the organism was developed. Maximum lactic acid production was achieved using enriched whey (8% w/v), at pH 5.5 for 24 h. Addition of yeast extract plus $MnCl_2$ gave the highest production of lactic acid (1.71 g/l·h), specific activity of LDH (1.03 U/mg protein) and SCP (32.8%). In batch culture, the addition of 20 g/l glucose plus 0.75 g N/l, as $(NH_4)_2SO_4$, achieved the highest enzymatic activity. By contrast, when xylose and cellobiose were used as a source of fermentable carbohydrates all variables were repressed.

Key words: *Lactobacillus delbrueckii*, lactic acid, lactic acid dehydrogenase (LDH), dried whey, single cell protein (SCP)

Resumen

Se ha estudiado la producción de ácido láctico, ácido láctico deshidrogenasa (LDH) y proteína unicelular (PUC) a partir de suero de queso por fermentación con *Lactobacillus delbrueckii* subsp. *bulgaricus* 369. Se diseñó un medio de fermentación que contenía suero desecado y suplementos, el cual permitió el crecimiento exponencial del organismo. La máxima producción de ácido láctico se consiguió usando suero enriquecido (8% p/v), a pH 5,5 durante 24 h de incubación. La adición de extracto de levadura más $MnCl_2$ dio los valores más elevados de ácido láctico

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(1,71g/l·h), de actividad específica de LDH (1,03 U/mg proteína) y de PUC (32,8%). En cultivo discontinuo, la adición de 20 g/l de glucosa más 0,75 g N/l, en forma de $(NH_4)_2SO_4$, consiguió la actividad enzimática más alta. En contraste, cuando se utilizaba xilosa o celobiosa como fuente de carbohidratos fermentables fueron reprimidas todas las variables del cultivo.

Introduction

Lactic acid (LA) is considered to be an important product in pharmaceutical and cosmetic industries (19). Krische et al. (11) reported that LA is a useful compound in the food industry as a biologically produced acidulent and preservative. LA can be made from glucose, sucrose, cereals and agricultural wastes, which are all reducible to monosaccharides by lactic acid bacteria (i.e. *Lactobacillus delbrueckii*), with low productivity (10). An additional problem in LA fermentations is the requirement of the bacteria for several growth factors. Therefore, the productivity and substrate conversion of the process depend on the composition of the medium (1, 5, 17). Much effort has been put into the optimization of LA production. Melzoch and Konopaskova (14) have proposed that the highest LA productivity was that of 9.6 g/l·h by *Lactobacillus acidophilus,* in continuous cultivation under aerobic conditions, whereas by using batch culture system LA productivity was 4.0 g/l·h for *Lactobacillus amylovorus* (22). Whey powder from bovine milk is a convenient raw material for LA fermentation, since it contains a high concentration of lactose (65%) and protein (13%). The present research was a trial to improve the quality of dried whey for production of LA, lactic dehydrogenase (LDH) and single cell protein (SCP) by *L. delbrueckii.*

Methods

Organism and incubation. The bacterium used throughout the present work was isolated from whey and identified as *Lactobacillus delbrueckii* subsp. *bulgaricus* No. 369 by CRNZ (France). Stock cultures were maintained on nutrient agar slopes at 4°C. Active cultures for inoculation were prepared by growing the organism on MRS medium (13). This medium contained 10 g peptone, 10 g beef extract, 5 g yeast extract, 20 g lactose, 1 ml Tween 80, 2 g K_2HPO_4, 5 g sodium acetate·3 H_2O, 2 g tri-ammonium citrate, 0.2 g $MgSO_4$·7 H_2O, and 0.2 g $MnSO_4$·4 H_2O, in 1 liter deionized water. The initial pH was adjusted to 6.2. Culture flasks were agitated for 24 h at 37°C.

Culture medium. Dried whey from bovine milk was purchased from Sigma (St. Louis, MO, USA). It contained 65% (w/w) lactose, 13% protein and 2% LA.

Deproteinized whey solutions were prepared as described previously (8) and supplemented, unless otherwise indicated, with: yeast extract (Difco), 20 g/l; KH_2PO_4, 5 g/l; $(NH_4)_2SO_4$, 1 g/l; $MgSO_4$·7 H_2O, 5 g/l; and $MnCl_2$, 2 g/l. Cultivation was performed in 250 ml Erlenmeyer flasks (equipped with a rubber septum and screw caps) containing 100 ml of the cultivation medium, autoclaved for 20 min at 121°C. Cultures were inoculated with 2% v/v of a preculture at the final exponential phase, and incubated at 40°C.

Growth measurements. Cell concentrations were estimated by measuring optical density (OD) at 600 nm and relating the readings to biomass dry weight with a calibration curve.

Analyses. Lactose was determined enzymatically according to Bergmeyer (4). LA was measured by titration with NaOH, using bromethymol blue as the indicator.

LDH activity was estimated by measuring the rate of reduced nicotinamide adenine dinucleotide (NADH) oxydation at 340 nm. The decrease of absorbance (A_{340}) was read every minute. The standard assay mixture contained (in a total volume of 3 ml): 0.1 M phosphate buffer pH 6.9; 0.1 mM NADH; 8 mM sodium pyruvate; and 0.05 ml enzymatic extract. Enzymatic extract was diluted to get absorbances lower than 0.08/min. One unit of enzyme activity is defined as a rate of 1 mM of NADH oxidation per min. Specific activity was expressed as U/mg protein.

Protein content in the supernatant was determined by the method of Lowry et al. (12), with bovine serum albumin as standard, and in whole cells by the microkjeldahl method (3). Protein hydrolysate amino acids were determined by an amino acid analyzer (6).

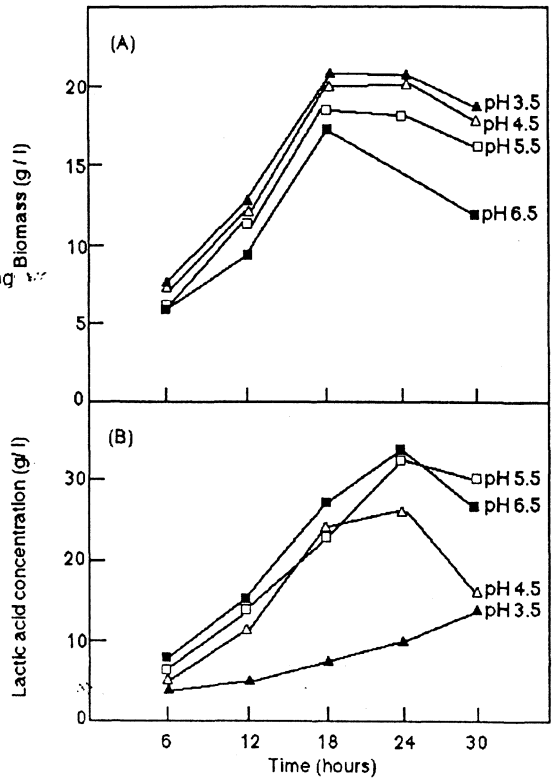

FIG. 1. Batch culture in the whey medium at different pH values. (A) Biomass production. (B) Lactic acid production. All data are average values from three repetitive experiments.

Results and discussion

Upon increasing the concentration of whey from 2 to 8% (w/v), the biomass of *L. delbrueckii*, (SCP), LA production, specific activity of LDH and lactose utilization increased. Above 8%, there was a drop in all these variables.

The growth and LA concentration by *L. delbrueckii* in batch culture, at controlled pH varying from 3.5 to 6.5 by using 1 N HCl or 1N NaOH, are shown in Fig. 1. It was found that pH affects the growth curve of *L. delbrueckii* only after the exponential growth phase (Fig. 1A). During this period, the death rate increased at higher pH values. The death phase occurred in culture media where LA concentration was higher than 27 g/l (Fig. 1B). This observation was found for all pH values. Therefore, the increase in death rate could be mainly due to autodigestion or to LA toxicity (17). It is also known that the concentration of products often exerts an influence on the fermentation pattern (23). Maximum yield of SCP is influenced by the pH of culture medium. All variables increased linearly with the increase in pH through the range 3.5 to 6.5 (Fig. 2). These results are in agreement with those obtained with *L. bulgaricus* 2217 (16), but differ from those of *L. helveticus* and *L. casei* (11, 17). An initial pH value of 5.5 was selected for subsequent experiments because death rate is higher with pH 6.5.

The type and concentration of fermentation products of homofermentative lactic acid bacteria is known to be highly dependent on culture conditions (7, 18, 20). In batch experiments several supplements for culture media were tested. Because nitrogen content of whey is low

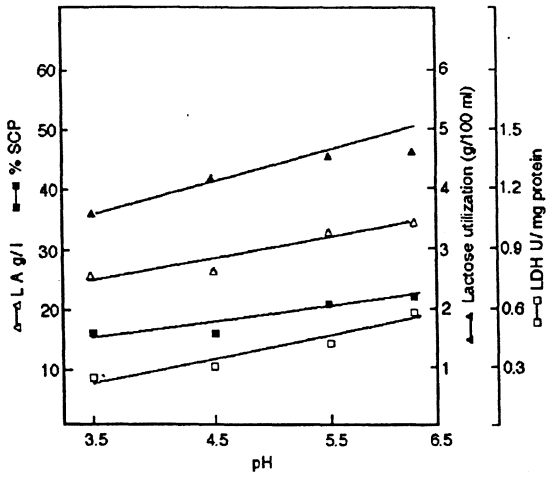

FIG. 2. Effect of pH on LA production, LDH synthesis, lactose utilization and SCP production in batch culture of whey medium. All data are average values from three repetitive experiments.

(approximately 2.1%) for producing sufficient biomass, different nitrogen sources were added to the whey medium, each one in a concentration of 50 mg N/100 ml medium; pH was adjusted to 5.5. It was found that $(NH_4)_2SO_4$ offered the best fermentation yields, with increases of LA productivity (1.5 g/l·h) and specific activity of LDH (0.93 U/mg protein), correlated with high SCP (23.9%; g/100 ml). Ammonium chloride, sodium nitrate and urea repressed LA production. None of the tested amino acids was able to enhance LA synthesis. These results are different from those obtained by Mistry et al. (15), who reported that non essential amino acids as a group were more stimulatory for acid production than essential amino acids. However, lactic acid bacteria are known as very fastidious concerning their nutritional requirements, especially for nitrogen (2). However, lactobacilli have a complex requirement for manganese, which was proved to be an essential growth factor because of its role as constituent of lactate dehydrogenase (11).

Addition of tryptone and casitone showed growth-stimulating activity. Yeast extract (5 g/l) and $MnCl_2$ (Mn^{2+}; 0.22 mM) proved to be an effective source of nitrogen/growth factor (LA, 41.0 g/l; specific activity of LDH, 1.03 U/mg protein; SCP, 32.8%). The growth-promoting effect of yeast extract has been investigated by Aeschlimann and Stockar (1), who reported on optimum concentration of 10 g/l for a strain of *L. helveticus,* while the best growth of *L. casei* in a short cultivation time occurred with a combination of yeast extract and hydrolyzed retentate (11).

The fermentation process was directed to improve the production of SCP, together with the improvement of whey assimilation for LA as well as LDH production. A complex medium was tested with increasing amounts (0.25; 0.50; 0.75 or 1.0 g/l) of $(NH_4)_2SO_4$ as a nitrogen source. Glucose, xylose or cellobiose were added at 10, 20 or 30 g/l concentration, respectively. Fig. 3 shows that *L. delbrueckii* produces the highest amount of LA (56 g/l) from glucose and $(NH_4)_2SO_4$, in a C/N ratio of 20 g/l to 0.75 g/l. However, the LDH activity increased markedly in accordance with the initial glucose concentration. When the glucose concentration changed from 10 to 20 g/l, the LDH activity increased from 0.84 to 1.24 U/mg protein, and a good yield (48.6%) of SCP was obtained. High concentrations of glucose (30 g/l) seem to repress the synthesis of LA and LDH during fermentation under the prevailing experimental conditions. These findings are in accordance with those found in *Clostridium thermohydrosulfuricum* (9) and *Lactobacillus amylovorus* (22).

On using xylose and cellobiose as carbon sources, the fermentation yields decreased. Westby et al. (21) confirmed that *Lactobacillus plantarum* NCIMB 8026 grows on pentose only in the presence of glucose.

The good nutritional quality of the tested bacterium proteins had been assessed through

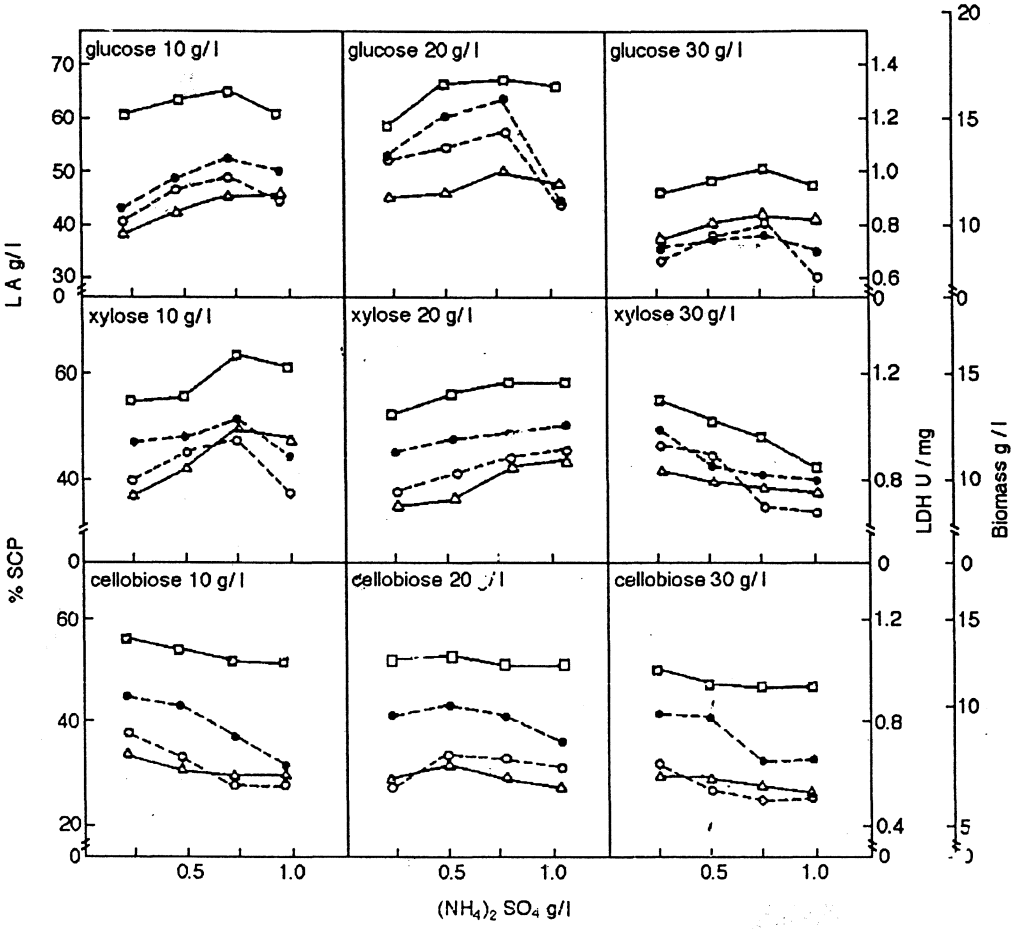

FIG. 3. Production of LA (○), LDH activity (●), SCP (Δ) and biomass (❑) by *L. delbrueckii* at different C/N ratios.

the chromatographic analysis of their amino acid constituents. Fifteen amino acids were detected in adequate levels. The tested proteins have a profile that compares with the FAO reference with regards to isoleucine (4.2 g/100 g). Relatively higher amounts of leucine (11.4; g/100 g) lysine (10.2 g/100 g), methionine (5.7 g/100 g), phenyl alanine (4.9 g/100 g) and threonine (5.3 g/100 g) were detected. The tested protein sample proved to be rich in the acidic amino acids: glutamic and aspartic acids (5.8 and 8.9 g/100 g, respectively). These results revealed a marked improvement in nutritional value of whey. In this respect, it should be emphasized that the first indicator of the nutritive potential of a SCP product is its chemical analysis, which is followed by biological testing with small animals and then large ones to ascertain non-toxicity, digestibility, fair energy output, desirable protein and fat contents, and inclusion of individual amino acids.

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Effect of heavy metals on soil denitrification and CO_2 production after short term incubation

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Summary

The toxicity of three heavy metals, Cd, Zn and Cu, has been tested in a Mediterranean soil. The soil was incubated (108 h) with mixed solutions of those metals before evaluating denitrification and CO_2 production, both by gas chromatography. These activities were used as biological indicators of heavy metal toxicity, and compared to non-treated control soil samples. Statistical analyses showed no significant differences in CO_2 production between treated and non-treated control soils. The lowest levels of respiration were observed in soils treated with the largest amounts of Zn and Cd. Denitrification increased significantly in soils treated with solutions containing 100 µg/ml of Cu and 1000 µg/ml of Cd or Zn.

Key words: heavy metals toxicity, denitrification, soil respiration, soil microbiota, statistical analysis

Resumen

Se ha ensayado la toxicidad de tres metales pesados (Cd, Cu y Zn) en un suelo mediterráneo. Tras la incubación (108 h) del suelo con soluciones mixtas de dichos metales, se evaluó, mediante

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cromatografía de gases, la desnitrificación y la producción de CO_2. Dichas actividades se utilizaron como bioindicadores de la contaminación, y se compararon con controles no tratados. En el caso de la producción de CO_2, no se han encontrado diferencias significativas entre los distintos tratamientos y el control. Los menores niveles de respiración edáfica se observaron en los tratamientos con mayores concentraciones de Zn y Cd. La desnitrificación aumentó significativamente en los tratamientos con 100 µg/ml de Cu y 1000 µg/ml de Cd o Zn.

Introduction

Heavy metals in high concentration are toxic to organisms. Soil microorganisms are not different in this respect; heavy metals affect microbial growth and survival, and can induce a natural resistance in bacteria (15). Nowadays, heavy metal contents in soil are the result of natural concentrations plus accumulated pollutants. The presence of those elements in fungicides affect agricultural soil management, which also concerns many industries that, as a matter of course, must handle their sewage sludges (1). Nevertheless, data of the maximum permissible limits for heavy metals concentrations in soil are still scant. Due to the noxious effects of heavy metals on crop yield, among other things, those limits should be determined according to a directive from the Commission of European Communities (11).

Different aspects of heavy metal toxicity to the microorganisms involved in soil dynamics have been reviewed earlier by Marks et al. (26), Brookes et al. (8, 9) and Lorenz et al. (25). An approach based directly on microbiological responses has been proposed for evaluating biological toxicity (23, 34). Since nitrogen cycling processes (22) are good biological indicators of heavy metal toxicity, they have been studied for this purpose, and nitrogen processes in soil have attracted particular interest. Previous studies focused on the effect of a single heavy metal in solution, when added at different times during a long term incubation. However, few studies performed short term incubation using a single mixed heavy metal solution that is added at one time to see its immediate effects in Mediterranean soils. This information would help to identify the most resistant bacteria to toxicity; CO_2 production and denitrification are considered to be good toxicity indicators in this kind of studies.

This work gives an experimental basis for the definition of the limits of tolerance of heavy metals in soil. Nowadays, this information is crucial for international organizations in changing the designing of prevention policies for environmental pollution. Both the heavy metals and the general variables selected to assess the heavy metals toxicity on the biological activity of soil were carefully chosen.

The aim of this paper is to study the effect of a single addition of a mixed heavy metal solution to a characterized Mediterranean soil, on denitrification and CO_2 production after a short term incubation.

Material and methods

Soil. Seven soil samples were taken from a *Quercus ilex* subsp. *ballota* grove near Madrid. Atmospheric deposition is the main source of heavy metals in these soils. Sampling was done to a depth of 15 cm in September 1993. All soil

samples were mixed, sieved (<2 mm) and then stored in the dark at 4°C. Texture was determined following the Bouyoucos method (7); pH was determined in distilled water:soil 1:1, with a Beckman 12 P/N 123134 pHmeter and a Radiometer GK 2401 electrode. Total nitrogen and ammonium were determined by the Jaworski colorimetric method (19, 20, 32), after a Kjeldahl digestion or with a KCl 2M extraction solution (33), respectively. Nitrates [following Milham et al. (27)] were determined with a Crison digit 501 mV/pHmeter and specific electrodes. Organic carbon was determined following the modified Walkley and Black method (38). As a result, the soil was characterized as having: 7.30 ± 0.01 pH, 1.84 ± 0.04 % organic carbon, 12.5 ± 0.22 total nitrogen mg/g of soil, 2.68 ± 0.31 N-ammonium μg/g, 1.53 ± 0.14 N-nitrate μg/g, and 1.6 (ml:g) water holding capacity (WHC).

Experimental design. Three heavy metals were assayed, (Cu, Zn and Cd) as $CuSO_4$, $ZnSO_4$ and $CdCl_2$. Mixed solutions were made in different proportions (in mg/ml, Cu:Cd:Zn): T_1, 10:100:1000; T_2, 100:10:1000; T_3, 10:1000:100; T_4, 1000:100:10; T_5, 100:1000:10; T_6, 1000:10:100. Each solution constituted one treatment. Each of the six treatments plus its control were performed in triplicate. 100 g of sieved soil (<2 mm) was brought to 100% WHC in 250 ml hermetic flasks with a silicone septum, with the corresponding treatment solution or with distilled water in the control sample. The incubation was carried out at 25°C, in the dark.

CO_2 and N_2O production, as respective indicators of total biological activity and denitrification, were determined by the acetylene inhibition method (39), under the conditions given in Vinther (36) and modified by Llinares et al. (24). Glucose (4 g/100 g of soil) was added as a source of carbon. Water saturation allowed the coexistence of aerobic and anaerobic microenvironments, while carbon was a substrate that optimized microbial denitrification (17, 28, 31, 37). A KNK-3000 HRGC gas chromatograph, equipped with a thermal conductivity detector (KNK 019-501), was used to determine CO_2 and N_2O production every 12 h for 108 h.

Statistical analysis. Regression curve slopes ANOVAs (35) were done for CO_2 and N_2O production for each treatment and control. A unidirectional ANOVA with replicates was done for the net total amounts of N_2O and CO_2 after 108 h incubation. If the ANOVA results showed significant differences, averages were compared with an Least Significant Difference (LSD) analysis.

Results and discussion

Many reports on heavy metal toxicity refer to soils of different climatic regions (21), but little is known about Mediterranean soils. The information from other soils can not be extrapolated to Mediterranean soils because they have different metal buffering capacities and, consequently, different metal bioavailabilities (16). Besides, most of the existing articles reported the effects of metals added, either as a single dose or in successive additions of small doses.

The basic difference between these two methods of addition has been discussed. Elsewhere soil pollution is a more or less continuous diffusion process, while metals are often added instantaneously in a single dose. Since our aim was to study the short term inhibition effects of metals, additions were made at one time in laboratory experiments. Other studies have shown that serial additions of heavy metals in small doses have a stronger inhibitory effect on soil respiration than addition at a single time (16). This suggests that our results probably underestimate the in vivo effect.

Another question is that metal pollution in the field is usually due to more than one metal (2). Combinations of three metals in different concentrations have been assayed in our experiments. These concentrations have been chosen in accordance with reports on sludges and soils studied by different authors (12, 29, 30). Nevertheless, and as Angle et al. (1) have pointed out, the effect of heavy metals depends not only on their concentrations but mainly on their availability; pH and cation exchange capacity play an important role (4). The experimental determination of the effects of adding specific concentrations of heavy metals to soils is a complex process, because any soil characteristics will modulate metal availability and toxicity.

There were significant differences in the ANOVA slopes for N_2O production ($F = 6.29$, $df = 6.42$, $P < 0.05$), and in the ANOVA for total N_2O production ($F = 14.75$, $df = 6.14$, $P < 0.01$), due to treatments. All LSD interactions for the ANOVA slopes are significant. The LSD results for the total N_2O production appear at the bottom of Table 1.

Little is known about the effect of heavy metals on denitrification, perhaps because the results are difficult to interpret (13). Our results show that only treatments T_2 and T_5 produced significant differences with respect to the controls (higher values than controls). Both treatments have the same Cu concentration (100 µg/ml). This suggest that Cu in concentrations around 100 µg/ml permits denitrification or biosynthesis of the enzymes involved in the process, and indicates that Cd and Zn are not toxic at those concentrations. It is important to point out that nitrate reductase of some denitrifying bacteria contains Cu^{2+} (10).

TABLE 1. N_2O values (means ± standard error) obtained each 12 h for 108 h of incubation (nmol per g of soil per hour). LSD tests did not show significant differences in treatments with the same letter (a or b) at the bottom line

Time (h)	Treatments						
	T_1	T_2	T_3	T_4	T_5	T_6	C
12	NA*	NA	NA	NA	NA	NA	NA
24	8.34 ± 0.00	10.42 ± 0.00	NA	NA	12.14 ± 0.00	9.06 ± 0.00	10.09 ± 0.71
36	10.20 ± 0.06	13.92 ± 0.35	2.20 ± 0.16	1.56 ± 0.15	14.30 ± 0.31	11.24 ± 0.00	11.17 ± 0.01
48	12.22 ± 0.38	15.96 ± 0.10	3.31 ± 0.23	16.00 ± 12	16.21 ± 0.21	12.94 ± 0.06	12.30 ± 0.01
60	48.45 ± 3.8	61.13 ± 12	50.74 ± 6.4	54.70 ± 3.3	90.00 ± 15	25.30 ± 2.6	39.00 ± 5.7
72	56.30 ± 4.0	80.39 ± 9.9	51.77 ± 2.2	60.43 ± 5.4	97.18 ± 15	32.30 ± 2.2	45.50 ± 6.3
84	72.24 ± 7.0	107.75 ± 16	52.98 ± 7.3	70.64 ± 4.8	132.64 ± 19	53.90 ± 7.9	47.90 ± 5.0
96	92.02 ± 2.7	115.11 ± 23	74.56 ± 2.1	78.80 ± 5.9	145.67 ± 24	56.22 ± 21	55.20 ± 6.7
108	104.60 ± 23	214.76 ± 24	110.80 ± 4.9	117.67 ± 29	223.30 ± 41	61.42 ± 0.00	92.43 ± 0.00
LSD	a	b	a	a	b	a	a

* NA, not available.

All the treatments produced significantly different ANOVA slopes. The T_2 and T_5 treatments have the highest growth rate; the T_1 and T_4 treatments the lowest; and T_3, T_6 and control treatments have similar intermediate rates. Due to the lack of literature on the effect of heavy metals on denitrification, it is difficult to compare our data with those of other authors. Bollag and Barabasz (6) assayed single doses of Cd, Cu, Zn and Pb on sandy soils at pH 6.9. They found Cd to be the most active inhibitor. In our study, treatments with the highest concentrations of this metal presented either no inhibition (T_3) or an activation (T_5) when compared with the non-treated controls. Perhaps this toxic effect of Cd on denitrification could be due to a drop in the production of a substrate necessary for the process. This could be related to alterations in other steps in the cycle, such as nitrogen fixation and nitrification; that is to say, to disfunctions in the cycle. For this reason, the effects on denitrification might be cancelled in long-term incubations. Modifications in the nitrogen circulation rates have been demonstrated: acceleration or deceleration depend on the concentrations of different heavy metals (2). Also the effects of soil characteristics on heavy metal solubilization could affect denitrifying populations in different ways.

The results of CO_2 production appear in Table 2. Neither the CO_2 production ANOVA slopes ($F = 2.61$, $df = 6.42$, $P < 0.05$) nor the total CO_2 ANOVA ($F = 2.26$, $df = 6.14$, $P < 0.05$) showed significant differences.

Soil respiration is one of the most studied variables in relation to heavy metal toxicity. In our experiments, three treatments (T_1, T_3 and T_5) seem to have an inhibitory effect on soil respiration, but there were no significant differences with respect to the controls. These treatments have the highest combination of Cd and Zn, while their Cu concentration is lower (10 or 100 μg/ml). Many authors such as Premi and Cornfield (29), Bhuiya and Cornfield (5) and Doelman and Haanstra (16), have pointed out that the inhibitory effect of this metal appears at higher

TABLE 2. CO_2 values (means ± standard error) obtained each 12 h for 108 h of incubation (nmol per g of soil per hour). Every value should be multiplied by a 10^{-2} factor

Time (h)	Treatments						
	T_1	T_2	T_3	T_4	T_5	T_6	C
12	0.80 ± 0.09	1.04 ± 0.12	0.96 ± 0.04	1.26 ± 0.10	0.79 ± 0.08	1.24 ± 0.10	0.98 ± 0.07
24	1.61 ± 0.01	2.31 ± 0.23	1.76 ± 0.15	2.32 ± 0.26	1.65 ± 0.20	2.37 ± 0.20	2.00 ± 0.20
36	3.17 ± 0.81	4.70 ± 1.2	1.84 ± 0.20	5.80 ± 0.46	3.31 ± 1.1	6.33 ± 0.95	4.00 ± 0.30
48	26.29 ± 4.1	43.31 ± 5.8	32.70 ± 0.86	42.14 ± 1.9	29.49 ± 3.4	44.26 ± 5.3	36.09 ± 3.2
60	45.73 ± 3.2	68.72 ± 10	54.81 ± 2.8	70.47 ± 1.6	51.10 ± 6.4	46.00 ± 3.6	61.92 ± 7.2
72	66.70 ± 3.1	95.73 ± 7	66.53 ± 0.78	75.98 ± 4.8	56.60 ± 8.8	80.93 ± 8.4	71.30 ± 9.0
84	105.37 ± 3.1	118.96 ± 6	102.40 ± 0.86	125.01 ± 6.4	87.16 ± 12	119.79 ± 13	104.12 ± 10
96	106.06 ± 2.4	134.31 ± 11	106.95 ± 3.2	126.38 ± 2.3	90.54 ± 18	132.00 ± 17	116.43 ± 16
108	111.92 ± 10	135.36 ± 11	124.62 ± 3.2	149.93 ± 6.3	105.43 ± 11	145.51 ± 20	153.92 ± 0.0

concentrations. That is why we conclude that Cd and Zn are responsible for the inhibition found here. Other authors found these metals not so toxic as our results suggest. Clay is an abiotic factor that was found to be the dominant one in decreasing the toxicity of Cd, and, to a lesser extent, that of Zn. This agrees with Babich and Stotzky (3), who reported that the toxicity of Cd (which was measured as the growth rate of several fungi in soil) decreased with increasing montmorillonite content. This conclusions were based on studies of soils with higher contents of clay than the soil used in our work. The secondary abiotic factor which decreased Cd toxicity was the organic matter content. Due to its extreme diversity, the effects of organic matter on heavy metals in soil are difficult to predict (18).

The CO_2 formation rates do not show any significant differences (ANOVA slope). Baath's results (2) seemingly contradict ours, but this difference could be related to soil characteristics, such as pH and cation exchange capacity, and the reactions of the microorganisms. One of the factors mainly affecting heavy metal soil toxicity is pH. The pH of the soil assayed by us is higher than that in the soils studied by Baath's, and favours metal binding. Additionally, heavy metal bioavailability can change in the course of an experiment, as a result of changes in soil pH or in microorganisms metabolic activity. Our results also differ from those reported by Doelman and Haanstra (16), who related a decrease in soil CO_2 emission to an increased incubation time. This findings could be related to two factors: (i) a fall in the amount of organic matter in the course of the incubation, and (ii) the survival of a few microorganisms able to live in the presence of a given concentration of heavy metals. Nevertheless, our results on CO_2 emission are similar to those results reported by Cornfield (14).

From our data, it may be concluded that the inhibitory effects of Cd, Cu and Zn affect more than one step of the nitrogen cycle. Modifications of denitrification rates may be the results of the effect of these metals on the first steps of the nitrogen cycle. The heavy metals that have been assayed do not clearly affect soil respiration. However, 108 h after adding heavy metals to the soils, respiration tends to drop. Abiotic factors probably affect the toxicity of heavy metals in these processes, but the interaction between the factors may have unpredictable effects on the global biological activity.

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Development of methanogenic consortia in fluidized-bed batches using sepiolite of different particle size

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Summary

The addition of support materials, such as sepiolite, to fluidized-bed anaerobic digesters enhances the methane production by increasing the colonization by syntrophic microbiota. However, the efficiency in the methanogenesis depends on the particle size of the support material, the highest level of methane production being obtained by the smaller particle size sepiolite. Because of the porosity and physico-chemical characteristics of these support materials, the anaerobic microbial consortia formed quickly (after one week of incubation). The predominant methanogenic bacteria present in the active granules, detected both by immunofluorescence using specific antibodies and by scanning electron microscopy, were acetoclastic methanogens, mainly *Methanosarcina* and *Methanosaeta.*

Key words: methanogenic bacteria, anaerobic digestion, sepiolite, fluidized-bed digesters, biofilms

Resumen

Se ha determinado que la adición de un soporte mineral, como la sepiolita, a digestores de lechos fluidificados incrementa la producción de metano debido a un favorecimiento de la colonización de la microbiota sintrófica. Sin embargo, la eficiencia de la metanogénesis depende directamente de la

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granulometría del material soporte (sepiolita); los mejores rendimientos y procesos de colonización se obtienen cuando el tamaño de partícula es menor. La porosidad y características físico-químicas de estos soportes permiten un rápido desarrollo de los consorcios microbianos metanogénicos (una semana). Las bacterias metanogénicas predominantes presentes en los gránulos activos, detectadas tanto por inmunofluorescencia con anticuerpos específicos como por microscopía electrónica de barrido, son metanobacterias acetoclásticas, principalmente *Methanosarcina* y *Methanosaeta.*

Introduction

Anaerobic digestion, using fluidized-bed digesters, has been considered one of the most efficient processes for the degradation of domestic sludges in wastewater treatment. This biological process involves the participation of several microbial groups, associated with support materials and forming a functional unit named microbial consortia (10, 18). These consortia may appear as biofilms or granules, which transform organic matter into carbon dioxide and methane (17).

Previous works have indicated the role of several bacterial species in the development of stable biofilms and granules (17). Generally, acetate is the main substrate for the acetoclastic methanogens (24); the two genera of acetoclastic methanogens most frequently detected in these anaerobic digesters are *Methanosarcina* and *Methanosaeta* (formerly *Methanothrix*).

However, the knowledge of the colonization of the support materials, stages of biofilm development, composition of methanogenic consortia and influence of different factors on microbial attachment have not been completely elucidated yet (10, 14, 29, 30). In addition, the cellular and molecular composition of consortia, as well as their functions, are poorly understood (25).

For these reasons, the scope of this work is to study the biofilm development in pilot-scale fluidized-bed batches, using as support material sepiolites with different particle size distribution.

Materials and Methods

Media and culture conditions. All the experiments were performed following the technique previously described by us (18), using 50-ml capacity serum vials (batches). The operations were carried out while the samples were gassed with a 70% nitrogen–30% carbon dioxide atmosphere. The composition of the culture medium was described by Sánchez et al. (23). The batches, stoppered with butyl rubber plugs and sealed with aluminium caps, were incubated at 37°C for 30 days.

Characteristics of the support material. The support material tested was the sepiolite supplied by Tolsa (Madrid, Spain): $[Si_{12}Mg_8O_{30}(OH_2)_4(OH)_4 \cdot 8H_2O]$. The particle size distribution selected was the following. Sepiolite 15/30: 8% humidity, 560 g/l of apparent density, specific surface of 270 m^2/g, and pH 8.5. Sepiolite 30/60: 8% humidity, 570 g/l of apparent density, specific surface of 240 m^2/g, and pH 8.8. Sepiolite 60/120: 8% humidity, 610 g/l of apparent density, specific surface of 240 m^2/g, and pH 8.6. Sepiolite 120, 10% humidity, 545 g/l of apparent density, specific surface of 240 m^2/g, and pH 8.5. And Pansil: 10% humidity, 60 g/l of apparent density, specific surface of 240 m^2/g, and pH 8.5.

Methane and volatile fatty acid analysis. The volatile fatty acids (VFA) and methane were monitored over time using a Perkin Elmer (Autosystem GC, Cheshire, UK) gas chromato-

graph. The methane in the gas phase was determined by thermal conductivity, using a 60/80 Carboxen 1000 column (Supelco, Bellafonte, PA, USA); and the temperatures of the injector and detector were 250°C, using helium as carrier gas. VFA in the liquid phase were extracted by centrifugation of the samples (24,000 × *g* for 15 min) and by addition of oxalic acid 1 M. VFA were determined by flame ionization chromatography with a Nukol column (Supelco). The temperatures of the injector and detector were 250°C, using nitrogen as carrier gas.

Analytical methods. The extracellular polymeric substances (EPS) of the microbial strains were extracted following the technique described by Fukuzaki et al. (9). The proteins and carbohydrates of these EPS were determined by the Bradford (2) method and by the phenol-sulfuric method described by Dubois et al. (7), respectively. Bacterial hydrophobicity was analyzed by the Bath test (adherence to n-hexadecane) (22).

Microscopic counts. Before filtering the samples, they were homogenized using the technique described by Camper et al. (3). Samples were filtered through 0.2 μm-pore size polycarbonate filters (GTTP type, Millipore Ibérica, Madrid, Spain). Samples were fixed with 2.5% of glutaraldehyde (Merck, Darmstadt, Germany), in 0.1 M cacodylate buffer (pH 7.2) , and centrifuged at 8,000 × *g* (30 min at 4°C). The pellet was resuspended in the following dead-sorption buffer: Zwittergent 3–12 (Calbiochem, La Jolla, CA, USA), 10^{-5} M; EGTA (ethylenglycol-bis-β-aminoethyleter) N´, N´, N´, N-tetraacetic acid (Sigma), 10^{-3} M; Tris-hydroxymethylaminoethane (Tris buffer) (Sigma), 10^{-2} M; peptone (Difco), 0.01%; pH, 7.0. The homogenization was achieved at 24,000 rpm for 3 min at 4°C (Ultra-Turrax T25, Janke & Kunkel, Ika-Labortechnic). Enumeration of total bacteria and total methanogenic bacteria were carried out using a Nikon Labophot-2 epifluorescence microscope (100 W Hg halogen lamp). Total bacteria were counted after staining with DAPI (4', 6-diamidino-2-phenylindole dihydrochloride) (Sigma, St. Louis, MO, USA), as described by Porter and Feig (20). Total methanogenic bacteria were determined by counting the number of natural fluorescent (due to cofactor F_{420}) cells without staining with DAPI

Scanning electron microscopy (SEM). Samples were prepared for SEM by a modification of the methods described by McCoy et al. (16), which consists in the fixation of the samples with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), for 12–24 h at 4°C. Samples were filtered through 0.2 μm-pore size polycarbonate filters (GTTP type, Millipore), previously treated with an aqueous 1% polyethylenimine solution (PEI, Sigma). Then, the filters were dehydrated in a graded ethanol series (50°, 70°, 80°, and twice 96°) for 10 min each, and two steps with absolute ethanol for 15 min each one. The dehydrated samples were critical-point dried, and then they were sputter-coated with gold for examination with a Jeol scanning electron microscope (JSM-840, Japan), at 20 kV accelerating potential. The micrographs were performed using Ilford HP4 film.

Indirect immunofluorescence. Polyclonal antibodies against glutaraldehyde-fixed whole cells of the microorganisms studied were obtained by the technique described by Castro et al. (4) using rats (Wistar race) supplied by Panlab (Madrid, Spain). For immunofluorescence assays, antisera were diluted in phosphate buffered saline (PBS), with 0.7% carrageenan (Sigma) and 0.01% sodium azide (Sigma), to achieve a 1:100 dilution. The indirect immunofluorescence was performed on 0.2 μm-pore size polycarbonate filters using a 1:40 dilution in PBS of rabbit anti-rat IgG FITC conjugate (Sigma) to detect the immunoreactivity following the technique described by Enger and Thorsen (8).

Results

The results shown in Fig. 1 indicate that there is no methane production in batches without support materials (control). In the batches supplemented with sepiolite of different particle size, the methane production starts after 4 days of incubation, and the maximum methane production is achieved after two weeks of incubation. At this moment, the highest methane production correspond to the sepiolites with lower particle sizes (Pansil and S 120).

The changing of VFA over time is very similar among the different support materials used, two peaks being observed on the 6th and 17th day of incubation, depending on the sepiolite considered. For all the support materials, acetate was the main component of the VFA produced, with values ranging between 4 g/l for S 30/60 and 6 g/l for S 15/30.

Methane and VFA productions over time, in the anaerobic fluidized-bed batches, were consistent with the formation of the microbial biofilm on the support surface, and with the bacterial counts by epifluorescence. Numbers of both total bacteria and methanogens increased after one week of incubation. The variation of the total bacteria/methanogens ratio was not significant in that incubation period (one week).

The predominant methanogens which colonized support materials at the end of the incubation period (one month) were *Methanobrevibacter smithii* and several strains from *Methanosarcina*, especially *M. mazei* S-6. They were detected by indirect immunofluorescence. The colonizing methanogens found in the lowest concentration were several strains of *Methanosaeta* (formerly *Methanothrix*) *soehngenii* (Fig. 2). However, it must be borne in mind that the counts refer to filaments and not to individual

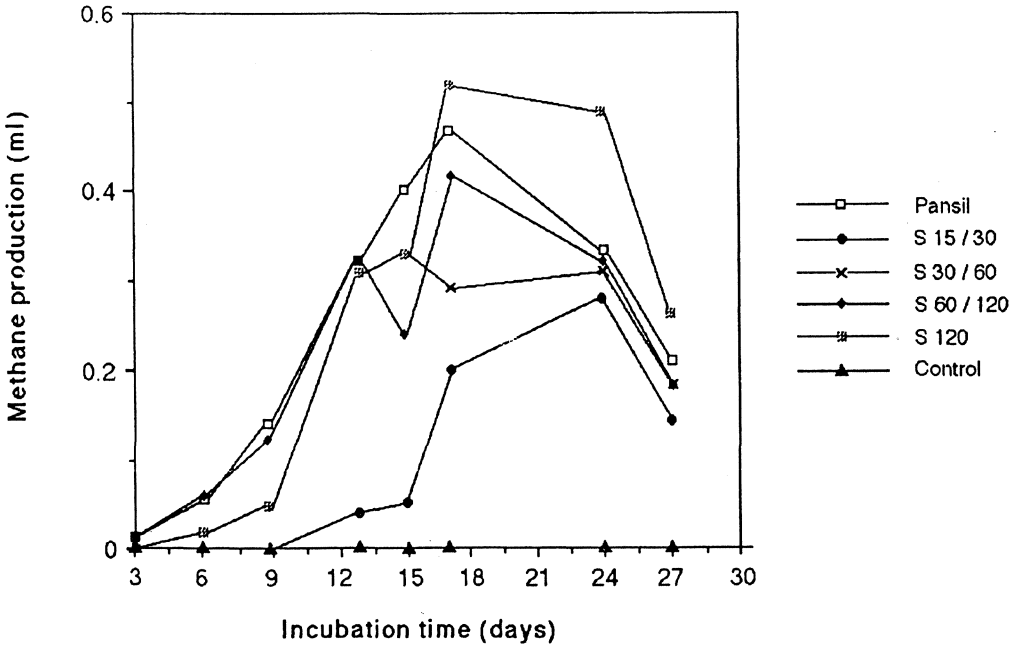

FIG. 1. Methane production, over time, in batch cultures with and without support materials.

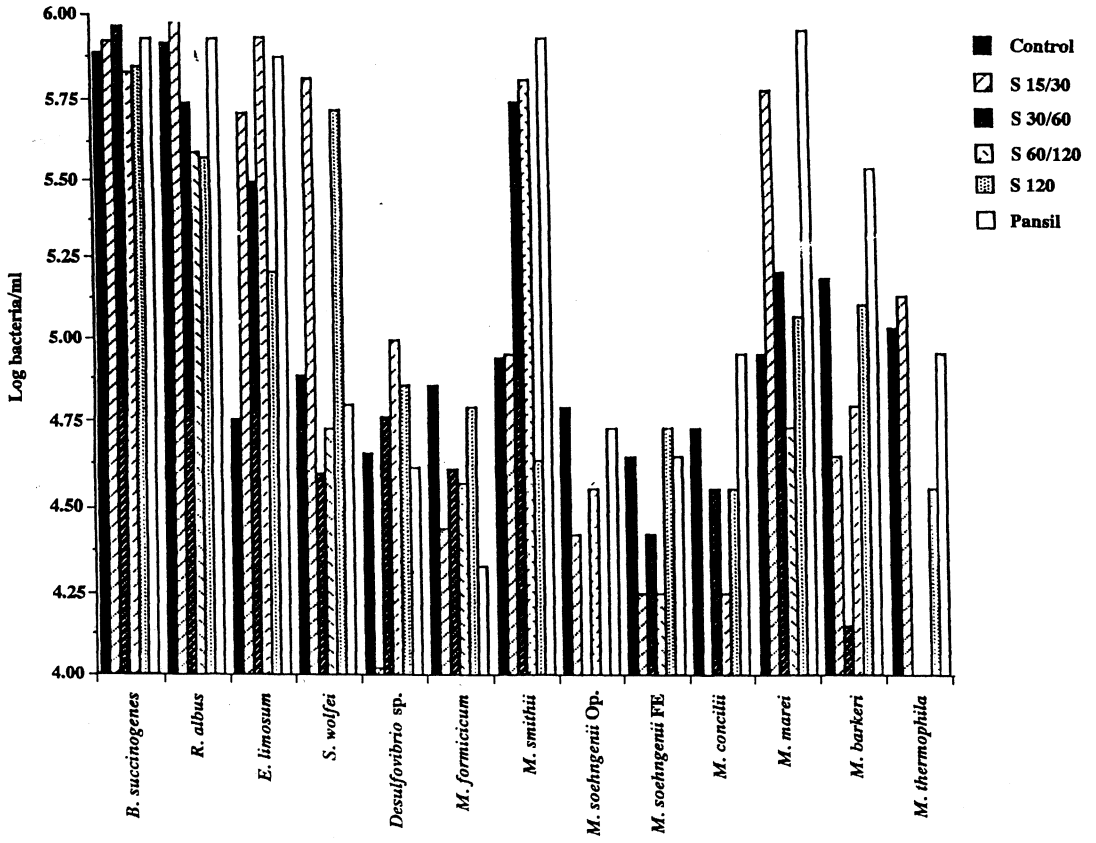

FIG. 2. Immunological counts obtained at the end of the incubation period (28 days) by indirect immunofluorescence on polycarbonate membrane filters (0.2 µm-pore size). Strains: *Bacteroides succinogenes* BL2[a]; *Ruminococcus albus* SY3[a]; *Eubacteria limosum*[b]; *Syntrophomonas wolfei* LYB (OCM 65)[c]; *Desulfovibrio* G11 (OCM 18)[c]; *Methanobacterium formicicum* MF (OCM55)[c]; *Methanobrevibacter smithii* PS (DSM 861)[d]; *Methanothrix soehngenii* Opfikon (DSM 2139)[d]; *Methanothrix soehngenii* FE (DSM 3013)[d]; *Methanosaeta concilii* GP6 (OCM 69)[c]; *Methanosarcina mazei* S-6 (DSM 2053)[d]; *Metanosarcina barkeri* MS (OCM 38)[c]; and *Methanosarcina thermophila* TM-1 (DSM 1825)[d].

Strains supplied by: [a] A. J. Richardson (Rowett Research Institute, Scotland, UK); [b] N. D. Lindley (Centre de Transfer en Biotechnologie et Microbiologie, UPS-INSA, Tolouse, France); [c] Oregon Collection of Methanogens (USA); and [d] Deutsche Sammlung von Mikroorganismen (Germany).

cells. SEM observations of different steps of support material colonization evidence an increase in the number of hydrolytic-fermentative bacteria in the first hours of incubation, mainly *Bacteroides succinogenes* and *Ruminococcus albus*; however, *Methanosaeta* was the most frequently observed methanogenic bacteria in this first step of colonization (Fig. 3A). In the consolidated biofilm, the predominant morphotypes observed were sarcina, small cocci and long filaments, corresponding to *Methanosarcina*, *Ruminococcus* and *Methanosaeta*, respectively (Figs. 3B–D).

The microbial colonization of support materials depends on the type and amount of polymeric substances released by the bacterial cells which form the anaerobic consortia. These polymeric materials, named extracellular polymeric

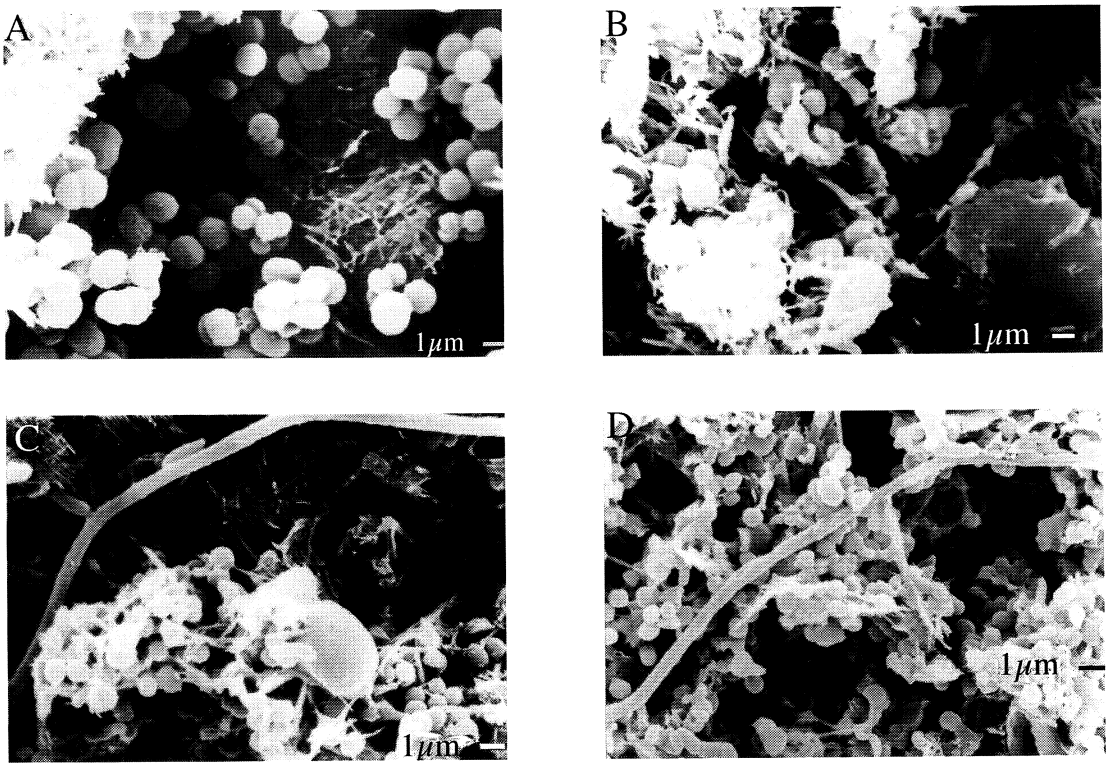

FIG. 3. Scanning electron micrograph of fluidized-bed batch: (A) supplemented with Pansil; (B) supplemented with Sepiolite 120 (S 120); (C) supplemented with Sepiolite 60/120 (S 60/120); and (D) initial colonization steps of *Methanosaeta* sp. on Sepiolite 60/120 (S 60/120).

substances (EPS), can serve as links between the support material and cells. According to previous research conducted in our laboratory (data not shown), these substances are synthesized in the first hours of incubation. The EPS produced enclose the bacterial nucleus of granules developed on the surface of the support, especially in the crevices and pores of the support materials. Therefore, the particle size of the support can affect both the efficiency and the time of bacterial colonization.

The analysis of the protein and carbohydrate contents of EPS produced under different experimental conditions is given in Table 1. The contents of carbohydrates and proteins in microbial EPS varied over time depending on the support material, the highest methane producers (Pansil and S 120) presenting a maximum in the protein content of EPS at the end of the studied period. A close and direct relationship (r = 0.999, p <0.0001) was achieved between protein/carbohydrate ratio and methane production in Pansil, although for other support materials this relationship was not statistically significant.

Besides, a significant relationship between the concentration of carbohydrates in the EPS and the methane production was found in the sepiolites S 30/60 and S 15/30 (r = 0.998 and p = 0.99, respectively). Only for Pansil the protein content of EPS was related to the methane production (r = 0.976, p <0.0001).

The hydrophobicity of the methanogenic consortia, measured as percentage of adherence to

TABLE 1. Chemical composition of the EPS (extracellular polymeric substances) synthetized by the bacterial consortia with different support

	Incubation time					
	Two weeks		Three weeks		Four weeks	
Support	Proteins[a]	Carbohydrates[a]	Proteins	Carbohydrates	Proteins	Carbohydrates
Control	64.21	40.95	51.48	52.62	77.59	220.34
S 15/30	92.24	107.43	61.49	47.72	59.82	62.73
S 30/60	96.91	123.58	73.50	111.00	28.73	41.91
S 60/120	68.88	64.24	81.51	79.16	25.41	68.67
S 120	89.90	196.45	85.51	107.73	120.11	110.31
Pansil	87.56	98.04	83.51	69.36	104.66	74.62

[a] Figures indicate μg/ml.

n-hexadecane over time, is shown in Fig. 4. Only sepiolite S 120, Pansil and S 60/120 showed high percentages (>70%) of adherence at the end of the incubation period, which indicates a functional and consolidated biological granule. However, hydrophobicity degrees of the consortia formed by the different support materials did not show a significant relationship (lineal and logarithmic) with the methane production of the fluidized-bed batches.

Discussion

Previous studies indicated that the addition of several materials as a support for bacterial biomass in anaerobic digesters promoted an increase in methane production (19, 23). Muñoz et al. (18) demonstrated that sepiolite and diabase were the support materials that produced the highest increase in the methane production in these types of pilot-scale anaerobic digesters. However, the factors that affect the colonization of these support materials over time have not been completely elucidated; only a few studies have been conducted on the dynamics of biofilm formation in anaerobic digesters (10, 21). In the present work, the microbial colonization of different particle sizes of sepiolite has been studied under laboratory conditions, using both indirect immunofluorescence and scanning electron microscopies.

The adhesion of bacteria to inert surfaces is required for the early steps of the biofilm formation. Bacterial adhesion depends on the electrical double-layer forces between the cell and the inert surface; London-van der Waals forces and chemical binding (hydrophobic and ionic bonds) are the predominant interactions that govern the attractive interaction between the surfaces. As it can be seen in Fig. 4, there were different hydrophobic interaction degrees depending on the particle size of sepiolite tested, these interactions being higher in the materials with lower particle size and higher methane production (S 120 and Pansil). Permanent adhesion usually requires de novo synthesis of an acid polysaccharide glycocalix that connects the adsorbed cell to the substratum (5).

As shown in Table 1, all the support materials tested possessed concentrations of proteins and carbohydrates higher than the control (batch without support materials) during the steps of biofilm formation (2 and 3 weeks of incubation). This increase is related to the enhancement of microbial adhesion and stability of the bacterial biofilm (1).

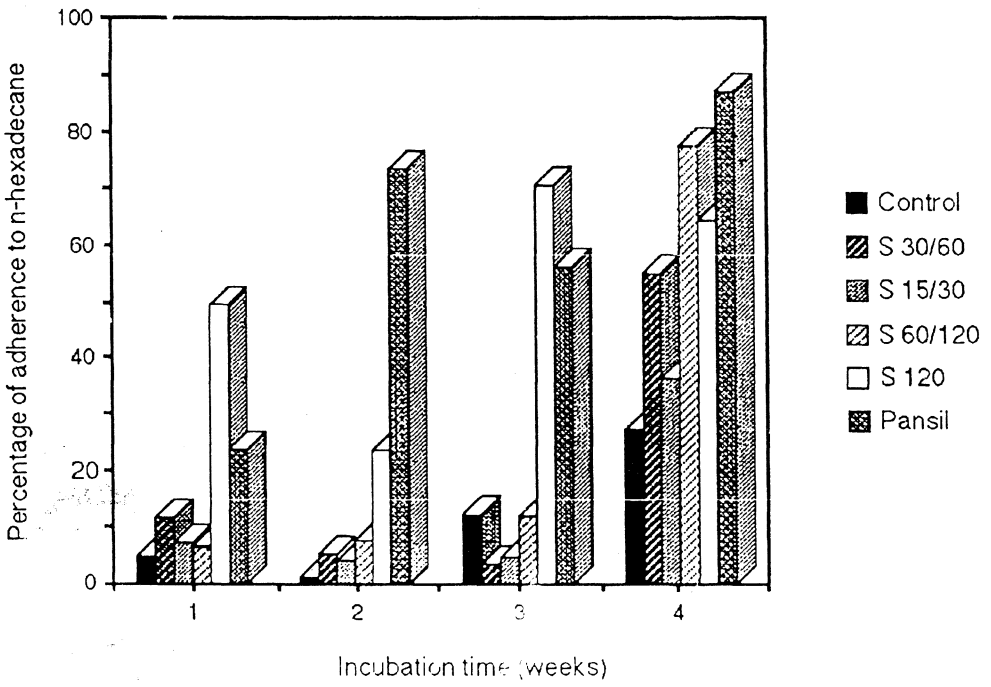

FIG. 4. Hydrophobicity of the methanogenic consortia measured as percentage of adherence to hydrocarbons (n-hexadecane).

The results obtained in this and previous studies (23), suggest that the bacterial biofilm was completely formed after 15 days of incubation in anaerobic batches (Fig. 1). This period is similar to the time required for the biofilm formation in other scale-pilot and industrial anaerobic digesters (16, 26).

In the present study, we have found a continous change over time of the ratio: total bacteria/methanogens. An enrichment of methanogens in the methanogenic granules was observed, in accordance with that reported by other authors (31). These facts are indicators of a well established syntrophic relationship among different microbial groups in the consortia for the degradation of propionate and butyrate, intermediaries of the acetoclastic anaerobic digestion. The high concentration of butyrate in the anaerobic batches is due to the specialized metabolism of the hydrolytic-fermentative bacterium *Eubacterium limosum* (32).

The association of different bacterial species in support materials and granules from anaerobic digesters has been reported previously (15, 28). In early steps of the microbial colonization of the support materials, filamentous forms are located on the outer layer of the support materials (Fig. 3D), and may establish links with the biofilm-forming bacterial types. Polysaccharide synthesis, mainly EPS, is a crucial mechanism at the steps in which bacteria grow and present metabolic activity, since the synthesis and arrangement of the EPS is an energy-dependent process (6).

After several days of incubation, anaerobic granules were formed, and due to the high ace-

tate concentration in the fluidized-bed digesters, the number of acetoclastic methanogens, such as *Methanosaeta* and *Methanosarcina*, increases regardless of the number of hydrogenotrophic methanogens (13, 25). At the end of the incubation period (30 days), *Methanosarcina* is the predominant methanogen in the digester (Figs. 3B and C).

Finally, the changing over time of the predominant methanogen in an anaerobic granule may be explained by the fact that *Methanosarcina* can decarboxylate acetate to produce methane; this capability produces a slower growth of *Methanosaeta* compared to *Methanosarcina* (12), although the former possesses a higher affinity for the substrate (K_M = 0.46 mM) (27).

It can be concluded that the differences detected in the support material which enhances methane production (see Fig. 1) may be caused by the mechanism and efficiency of the microbial colonization, although the hydrophobicity degree must be also considered. The filaments of *Methanosaeta,* constituted by several individual cells, are located into bacterial consortia and, in association with hydrolytic-fermentative bacteria, form the core or nucleus of the granule linked to the pores of the support material. For this reason, the bacterial adsorption to the granules depends on the available surface, i. e. the number and spatial disposition of pores or hollows of the support material. The highest efficiency in the granule formation depends on the porosity degree of the support material (11).

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The effect of O-antigen on transformation efficiency in *Serratia marcescens*

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Summary

Serratia marcescens is an enterobacterium that exhibits very low efficiency of transformation. According to previous work, neither the bacterium restriction system nor its nuclease production accounts for this low efficiency. Differences in the efficiency of transformation from plasmid DNA were found in wild type of *S. marcescens* and their O-deficient spontaneous mutant strains. This phenomenon seems to be independent of plasmid size. When electroporation was used, the survival of O^- mutants was much lower than those of their parental strains, but the frequencies of transformation among survivors were much higher. This suggests that the presence of the O-antigen is responsible for the low transformation frequencies observed.

Key words: *Serratia marcescens*, transformation efficiency, O-antigen, DNA uptake, electroporation

Resumen

Serratia marcescens es una enterobacteria que muestra unas eficiencias de transformación extemadamente bajas. Estas bajas eficiencias no pueden atribuirse, según trabajos previos, al sistema de restricción de la bacteria, ni a su producción de nucleasa. Se encontraron diferencias en las

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eficiencias de transformación por DNA plasmídico en cepas salvajes de *S. marcescens* y en mutantes espontáneos O^- derivados de ellas. El fenómeno es independiente del tamaño del DNA plasmídico. En el caso de la electroporación, la supervivencia de las cepas mutantes fue mucho menor, pero entre los supervivientes se observaron eficiencias de transformación mucho mayores. Esto indica que la presencia del antígeno O es la causa de las bajas frecuencias de transformación observadas.

Introduction

The introduction of foreign nucleic acid into bacteria by both transformation and transfection is widely used in genetic analysis. Some species are naturally competent for transformation. But in most cases, bacteria need some previous treatment to become competent ($CaCl_2$, or others). *Serratia marcescens* secretes a wide variety of proteins through its bacterial envelopes, which makes this bacterium a potentially useful microorganism to secrete foreign recombinant proteins. However, genetic manipulation of *S. marcescens* is more difficult than that of *Escherichia coli*. Some of these difficulties derive from the low efficiency of transformation of *S. marcescens*. Formerly, the powerful nuclease produced by *S. marcescens* was considered the main reason for the low transformation efficiency (7, 2), but it was demonstrated that this is not the case (6). In some Gram-negative bacteria, LPS contributes to the modification of transformation efficiency, e.g. the reduction of LPS O-side chains increases the efficiency of DNA uptake in *Klebsiella pneumoniae* (1). This article deals with the effect of O-side chain length on the ability of different *S. marcescens* strains to be transformed by plasmid DNA.

Materials and methods

Bacterial strains used in the present study are summarized in Table 1. Three of them were wild type, obtained from different sources, whereas the others were obtained by selection of clones resistant to different phages that have their primary receptor in the O-antigen. The plasmids used were pBR328 (8) and pSL001 (4).

Several transformation procedures were used. The first was the method of Cohen et al. (3). An alternative method was based on the techniques described by Merrick et al. (5). Both methods are based on the effect of divalent cations and thermal shock to induce the competent state. Lastly, the electroporation (9) was used, with the following procedure: 45 ml of fresh Trypticase Soy Broth (TSB, Difco, Detroit, MI, USA) were inoculated with 5 ml of an overnight culture and incubated with shaking until OD_{600} = 0.6, at 37°C. Bacteria were harvested by centrifugation, washed twice in cold water and finally suspended in 50 ml glycerol (10%). Bacteria were stored at –45°C until use. Electroporation

TABLE 1. Strains of *Serratia marcescens* used in this work

Bacterial strain	Characteristics	Source
ATCC 274	Wild type	ATCC
RM1	ATCC 274 O^- (kappa phage resistant)	Our laboratory
2170	Wild type	H.W. Ackermann
J92	2170 O^- (FSB55 phage resistant)	Our laboratory
NIMA	Wild type	R.P. Williams
NR1	O^- (FSB3 phage resistant)	Our laboratory

was carried out using a Invitrogen electroporator (Invitrogen, San Diego, CA, USA) and a Pharmacia LKB-ECPS 3000/150 power supply (Pharmacia, Uppsala, Sweden). We tested several combinations of capacitance/voltage; best results were obtained at 50 mF/1700 V. Cells were incubated for 1 h in TSB to allow phenotypic expression and transformants scored. The results shown are the average of three independent experiments.

Results and discussion

In all cases, the results were expressed as efficiency of transformation (ET, or number of transformants/μg of DNA) and frequency of transformation (FT, or ET/number of surviving bacteria).

The results of transformation efficiency obtained by using the classical methods are presented in Table 2. All strains (O^+ and O^-) showed low transformation efficiency, and in some cases no transformants were obtained using 0.1 μg of DNA. In *E. coli* the efficiency of transformation were about 7×10^5 transformants/μg of DNA. When plasmid pSL001 was used, no transformants were obtained in any of the strains. This plasmid is a 13.4 kb derivative of pBR328, with an insert which includes the gene *putA* of *S. marcescens*.

When electroporation was used to transform *S. marcescens,* increases in the transformation efficiency were observed. Table 3 shows the results of experiments of electroporation. By using this method, the yield of transformants were much higher than those obtained by the "classical" methods; in some cases (like strain J92), these increases were nearly 1000-fold. However, even when electroporation was used the efficiencies of transformation in *S. marcescens* were clearly lower than those obtained in *E. coli*. Besides, when efficiencies of transformation were compared in O^+ and O^- strains, no significant differences were appreciated. However, if the efficiencies of transformations were expressed as frequencies of transformants (i.e., when the percentage of survival was taken into account), the results clearly showed higher efficiencies in spontaneous O^- mutants. This means that fewer O^- mutants survived the electroporation procedure than the corresponding wild type strains; but among survivors, the frequency of transformation was much higher in strains that are defective in O-antigen. This contributes to demonstrate that restriction-mod-

TABLE 2. Results of the transformation of plasmid pBR328 by using two "classical" methods

	Cohen's method (ref. 3)		Merrick's method (ref. 7)	
Strains	ET	FT	ET	FT
2170	140	$3.25 \times 10^{-7} \pm 1.17 \times 10^{-6}$	11	$2.55 \times 10^{-8} \pm 2.23 \times 10^{-8}$
J92	32	$4.57 \times 10^{-7} \pm 2.41 \times 10^{-7}$	12	$2.85 \times 10^{-7} \pm 3.12 \times 10^{-7}$
ATCC 274	<10	$<1.1 \times 10^{-8}$	<10	$<1.1 \times 10^{-8}$
RM1	30	$3.3 \times 10^{-7} \pm 2.8 \times 10^{-7}$	12	$1.33 \times 10^{-7} \pm 4.0 \times 10^{-7}$
NIMA	<10	$<2.32 \times 10^{-8}$	<10	$<2.32 \times 10^{-8}$
NR1	40	$1.1 \times 10^{-7} \pm 3.40 \times 10^{-7}$	<10	$<2.9 \times 10^{-8}$

ET: Efficiency of transformation. FT: Frequency of transformation. (See text for explanation.)
Values of FT are given with standard deviation.

TABLE 3. Results of the transformation of two different plasmids by using electroporation

	pBR328		pSL001	
Strains	ET	FT	ET	FT
2170	2.7×10^3	$3.6 \times 10^{-4} \pm 4.6 \times 10^{-5}$	7.7×10^2	$9.8 \times 10^{-5} \pm 1.3 \times 10^{-5}$
J92	4.12×10^3	$1.8 \times 10^{-3} \pm 5.6 \times 10^{-5}$	3.06×10^3	$1.3 \times 10^{-3} \pm 1.0 \times 10^{-3}$
ATCC 274	4.8×10^3	$3.9 \times 10^{-4} \pm 6.7 \times 10^{-5}$	2.3×10^2	$2.3 \times 10^{-5} \pm 1.4 \times 10^{-5}$
RM1	1.65×10^4	$3.7 \times 10^{-3} \pm 8.1 \times 10^{-4}$	5.0×10^2	$1.27 \times 10^{-4} \pm 2.8 \times 10^{-4}$
NIMA	2.0×10^2	$1.56 \times 10^{-6} \pm 1.8 \times 10^{-6}$	8.0×10^1	$3.72 \times 10^{-7} \pm 4.0 \times 10^{-7}$
NR1	2.1×10^2	$1.27 \times 10^{-5} \pm 5.3 \times 10^{-6}$	4.0×10^1	$9.09 \times 10^{-7} \pm 3.0 \times 10^{-7}$

ET: Efficiency of transformation. FT: Frequency of transformation. (See text for explanation.)
Values of FT are given with standard deviation.

ification system of *S. marcescens* is not the reason for the low transformation efficiencies, since O-deficient mutants transform more efficiently, although their restriction-modification system is identical to that of the wild type strains. Moreover, as we pointed out (6), the use of a plasmid obtained from *S. marcescens* does not increase the transformation efficiency, nor the use of cured strains. As a conclusion, we can assume that the low transformation efficiency obtained in *S. marcescens* is a consequence of the different features of the bacterial envelope of this bacterium, as compared to that of *E. coli*.

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Growth of the fish pathogen *Renibacterium salmoninarum* on different media

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Summary

In the present study, the ability of a group of *Renibacterium salmoninarum* strains to grow in the presence or absence of the amino acid cysteine and other mineral and organic sources of sulfur and nitrogen has been evaluated. Most of the isolates tested were able to grow on a mineral media supplemented with L-cysteine-HCl or other organic compounds, such as the vitamin thiamine and a casein hydrolysate (Bacto Casamino Acids, Difco). Bacterial growth was also recorded on commercial and specific media not supplemented with L-cysteine-HCl, or in which this amino acid was replaced by the compounds cited above.

Key words: *Renibacterium salmoninarum*, in vitro growth, cysteine, nutrient requirements, culture media

Resumen

En el presente estudio se ha evaluado la capacidad de un grupo de cepas de *Renibacterium salmoninarum* para crecer en presencia o ausencia del aminoácido cisteína y de otras fuentes de azufre y nitrógeno. La mayoría de los aislados fueron capaces de crecer en un medio mineral suplementado con L-cisteína-HCl u otros compuestos orgánicos, como la vitamina tiamina y un hidrolizado de caseína (Bacto Casamino Acids, Difco). También se observó crecimiento tanto en medios comerciales como en medios específicos no suplementados con L-cisteína, o en los que este aminoácido se había sustituido por los compuestos citados anteriormente.

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Introduction

Bacterial kidney disease (BKD) was first described in Scotland more than fifty years ago; since then, it has caused serious mortality in salmonids in both fresh and salt water in Europe, America and Japan (1, 2, 10, 11, 12, 14). The causative agent, *Renibacterium salmoninarum*, is a very slow growing bacterium and fastidious in its requirements. This has limited the studies on the pathogenicity of BKD, as well as the identification of dominant antigens that confer protective immunity. It has been commonly accepted that *R. salmoninarum* has an absolute requirement of cysteine for growth (1, 4, 5, 13, 15) and, more recently, Daly and Stevenson (7) have reported that L-cysteine is essential for optimal growth. A better understanding of the pathogenesis of BKD and the development of effective programmes of control of the disease require the improvement of the growth conditions of *R. salmoninarum*. The present work describes the growth ability of a group of *R. salmoninarum* strains in presence or absence of the amino acid cysteine and other mineral and organic sources of S and N.

Material and methods

Bacterial strains. Eight *Renibacterium salmoninarum* strains were utilized in this study (Table 1) and kept frozen at –80°C until use.

Media. A basal medium (BM) (NH_4Cl, 5 g/l; NH_4NO_3, 1 g/l; Na_2SO_4, 2 g/l; K_2HPO_4, 3 g/l; KH_2PO_4, 1 g/l; $MgSO_4 \cdot 7H_2O$, 0.1 g/l; NaCl, 10 g/l; $MgCl_2$, 4 g/l; purified agar, 10 g/l), alone or supplemented with different amounts of the amino acid cysteine [0.01, 0.05 and 0.1% (w/v)], the vitamins thiamine and lipoic acid (both 0.1%), Bacto Casamino Acids (Difco) (0.1%) and dithiothreitol (6.3 mM) were utilized to determine the nutritional requirements of the isolates. In addition, the following commercial and specific media were employed to evaluate the growth rate of the different *R. salmoninarum* strains: Tryptic Soy Agar (TSA, Oxoid), Brain Heart Infusion Agar (BHIA, Oxoid), Mueller Hinton Agar (MHA, Oxoid and Difco), Kidney Disease Medium (KDM-2) (8) and Charcoal Agar (KDM-C) (6).

Growth tests. Serial 10-fold dilutions in Peptone Saline (PS) (0.85% NaCl; 0.1% peptone [w/v]) were prepared. Initial concentration of inocula was 1.2×10^9 cells/ml. Aliquots (20 µl) of each dilution were spotted onto each of the following media: BM, BM + 0.1% or 0.05% or 0.01% L-cysteine-HCl (cys), BM + 0.1% Bacto Casamino Acids (cas), BM + dithiothreitol (DTT), BM + 0.1% thiamine, BM + 0.1% lipoic acid, TSA, BHIA, MHA (Difco and Oxoid), KDM-2 and KDM-C lacking L-cysteine-HCl (KDM-2-no cys and KDM-C-no cys), MHA (Difco) plus 0.1% L-cysteine-HCl (MHA + cys), KDM-2, KDM-C, MHA and TSA plus 0.1% Bacto Casamino Acids (MHA + cas and TSA + cas) and KDM-2 and KDM-C in which L-cysteine-HCl has been replaced by 0.1% Bacto Casamino Acids (KDM-2 + cas, and KDM-C + cas). The Petri plates were incubated at 15°C and periodically examined for two months. Growth was recorded as the end-point dilution defined as the last dilution of culture at which growth was recorded on spot

TABLE 1. Origin of the *Renibacterium salmoninarum* strains utilized in the present study

Strain	Origin	Source[a]
ATCC 33209	*Oncorhynchus tshawytscha* (USA)	ATCC
ATCC 33739	*Salvelinus fontinalis* (USA)	ATCC
RS92	*Oncorhynchus kisutch* (Spain)	Our laboratory
K84	*Oncorhynchus mykiss* (England)	B. Austin
MT 251	*Salmo salar* (Scotland)	D. W. Bruno
MT419	*Salmo salar* (Scotland)	D. W. Bruno
MT422	*Salmo salar* (Scotland)	D. W. Bruno
MT 426	*Salmo salar* (Scotland)	D. W. Bruno

[a] ATCC, American Type Culture Collection, Rockville, MD, USA; B. Austin, Department of Brewing and Biological Sciences, Heriot-Watt University, Edinburgh, Scotland; D. W. Bruno, SOAFD, Marine Laboratory, Aberdeen, Scotland.

plates (6). Each test was carried out in triplicate.

Results and discussion

The mineral (basal) medium utilized (BM) did not support the growth of any *R. salmoninarum* strain regardless its content of inorganic sulfur (withdrawal of sulfates from its composition did not modify the growth results). Addition of L-cysteine-HCl to BM (0.05 to 0.1%) slightly stimulated the growth of most of the strains (end-point dilution ranging from 10^0 to 10^{-2}), while replacement of this amino acid by cystine resulted in the absence of growth of the isolates tested, which agrees with that observed by Daly and Stevenson (7) using KDM-C. Surprisingly, addition of other organic compounds which do not contain L-cysteine-HCl, such as the vitamin thiamine and Bacto Casamino Acids (an acid-hydrolyzed casein, usually employed as a source of nitrogen) favoured growth at similar levels or slightly higher than L-cysteine-HCl. The ability of bacteria to alter their metabolism in response to available nutrients could explain these unexpected results (3). None of the remaining compounds used as source of sulfur [dithiothreitol (6.3 mM) and lipoic acid (0.1%)] were able to support growth of *R. salmoninarum* strains.

The ability of *R. salmoninarum* strains to grow on different commercial and specific media was also evaluated. On MHA and BHIA (Oxoid) most strains grew only from a concentrated inocula or did not grow at all. However, on MHA (Difco) and on TSA (Oxoid) plates, colonies were recorded at considerably high dilutions (up to 10^{-6}, Table 2). In addition, KDM-2 and KDM-C media lacking L-cysteine-HCl in their composition gave end-point dilutions ranging from 10^{-1} to 10^{-5}. These results are in contrast with that reported by Daly and Stevenson (7), who indicated that *R. salmoninarum* does not grow when the medium is not supplemented with L-cysteine or other sulfur-containing amino acid.

TABLE 2. Growth of *Renibacterium salmoninarum* strains on different media

Media	Growth end-point dilution[a]							
	ATCC 33209	ATCC 33739	RS92	K84	MT251	MT419	MT422	MT426
Basal media								
BM	NG[b]	NG	NG	NG	10^0	10^0	NG	NG
BM + cys[c]	10^{-1}	10^{-1}	10^0	10^{-2}	10^{-1}	10^{-2}	10^{-1}	10^{-1}
BM + cystine[d]	NG	NG	NG	10^0	10^0	10^0	NG	10^0
BM + cas[e]	10^{-1}	10^{-3}	10^{-1}	10^{-2}	10^{-2}	10^{-3}	10^{-2}	10^{-1}
BM + thiamine[f]	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-2}	10^{-2}	10^{-1}
Commercial and specific media								
TSA	10^{-2}	10^{-3}	10^{-1}	10^{-4}	10^{-6}	10^{-3}	10^{-4}	10^{-5}
BHIA	10^0	10^0	10^0	NG	NG	10^0	10^{-1}	10^0
MHA (Oxoid)	10^{-2}	10^{-2}	10^0	10^{-3}	10^{-2}	10^{-3}	10^0	10^{-1}
MHA (Difco)	10^{-3}	10^{-4}	10^{-3}	10^{-5}	10^{-6}	10^{-3}	10^{-4}	10^{-5}
MHA + cys[c]	10^{-6}	10^{-7}	10^{-5}	10^{-7}	10^{-8}	10^{-8}	10^{-8}	10^{-5}
KDM-2	10^{-8}	10^{-8}	10^{-6}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}
KDM-C	10^{-8}	10^{-8}	10^{-5}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	10^{-8}
KDM-2 no cys[g]	10^{-3}	10^{-4}	10^{-1}	10^{-4}	10^{-4}	10^{-3}	10^{-3}	10^{-5}
KDM-C no cys[g]	10^{-3}	10^{-3}	10^{-1}	10^{-4}	10^{-4}	10^{-3}	10^{-3}	10^{-4}
MHA + cas[e]	10^{-4}	10^{-4}	10^{-3}	10^{-5}	10^{-6}	10^{-4}	10^{-5}	10^{-5}
TSA + cas[e]	10^{-4}	10^{-4}	10^{-3}	10^{-4}	10^{-6}	10^{-4}	10^{-4}	10^{-5}
KDM-2 + cas[h]	10^{-4}	10^{-5}	10^{-3}	10^{-5}	10^{-6}	10^{-4}	10^{-4}	10^{-5}
KDM-C + cas[h]	10^{-4}	10^{-5}	10^{-3}	10^{-5}	10^{-5}	10^{-4}	10^{-4}	10^{-5}

[a] The end-point dilution indicates the last dilution of the culture which produced visible growth. [b] Not growth. [c] Medium supplemented with 0.1% L-cysteine-HCl. [d] Medium supplemented with 0.1% cystine. [e] Medium supplemented with 0.1% Bacto Casamino Acids. [f] Medium supplemented with 0.1% thiamine. [g] Medium lacking L-cysteine-HCl. [h] Medium in which the L-cysteine-HCl has been replaced by Bacto Casamino Acids.

The differences observed between MHA supplied by Oxoid and Difco, could be due to the different casein hydrolysates present in their composition, since it has previously been reported that different batches of peptone used in preparing KDM-2 can determine the capacity of this medium to support the growth of *R. salmoninarum* (9).

The ability of most of the *R. salmoninarum* strains to grow on media not supplemented with L-cysteine-HCl, or on media in which this amino acid has been replaced by other compounds (thiamine and Bacto Casamino Acids), indicates that the presence of this amino acid in the medium is not indispensable for the in vitro culture of the bacterium. Therefore, the requirement of cysteine for growth can not be considered as a definitive criterion to differentiate *R. salmoninarum* from other Gram-positive bacilli.

Addition of Bacto Casamino Acids to basal medium gave growth similar or higher than that obtained with L-cysteine-HCl (Table 2). However, when this casein hydrolysate was added to commercial and specific media, bacteria grew less efficiently than with L-cysteine-HCl (Table 2). These differences could be due to the fact that TSA, MHA, KDM-C and KDM-2 have enough peptone in their composition to support the growth of *R. salmoninarum* to a certain extent, thus an extra supply of that compound would not improve much more the growth results.

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A *Candida albicans* gene expressed in *Saccharomyces cerevisiae* results in a distinct pattern of mRNA processing

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Summary

Two plasmids (derived from YCplac22 and YEplac112) carrying a *Candida albicans* gene (including the 5' non-coding promoter sequences) coding for a 30 kDa membrane-bound protein, were used to transform *Saccharomyces cerevisiae* cells. A 30 kDa protein was immunodetected by Western blot in the membrane fraction of transformants. Northern analysis showed the presence of three mRNA species (of about 1.1, 0.7 and 0.5 kb) hybridizing with the *C. albicans* gene as a probe. The same result was obtained using the 5' and 3' regions of the gene as probes, whereas only a 1.1 kb mRNA was found in *C. albicans* and none was detected in *S. cerevisiae* control transformants. Thus, heterologous expression of this gene in *S. cerevisiae* results in a distinct pattern of mRNA processing, either due to the location on plasmid vectors and/or to differences in the mRNA processing systems in the two microorganisms.

Key words: *Saccharomyces cerevisiae*, *Candida albicans*, heterologous gene expression, plasmids, mRNA

Resumen

Células de *Saccharomyces cerevisiae* fueron transformadas con dos plásmidos (derivados de YCplac22 y YEplac112) que portaban un gen de *Candida albicans* (incluyendo sus propias

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secuencias promotoras) que codifica una proteína de 30 kDa asociada a la membrana. El análisis de los transformantes demostró que el gen se expresa, pudiéndose detectar inmunológicamente la proteína de 30 kDa en la fracción de membranas. Mediante hibridación (Northern) se detectaron tres mRNA (de 1.1, 0.7 y 0.5 kb), empleando como sonda el gen completo de *C. albicans*. El mismo resultado se obtuvo cuando se utilizaron como sonda los fragmentos 3' y 5' del gen, mientras que en *C. albicans* sólo se detecta el mRNA de 1.1 kb. Estos resultados indican que la expresión heteróloga de este gen en *S. cerevisiae* determina un procesamiento distinto del mRNA, debido a su localización plasmídica y/o a diferencias en el procesamiento del mRNA entre los dos microorganismos.

Introduction

We had previously cloned a *Candida albicans* gene coding for a 30 kDa membrane-bound protein; this gene is transcribed in a 1.1 kb mRNA in *C. albicans* yeast and germ-tube cells (17). However, the function of this protein in *C. albicans* physiology has not yet been determined. In order to obtain information about the function of the protein, we studied the expression of the gene in *Saccharomyces cerevisiae* cells. *Candida* genes can be frequently expressed directly in *S. cerevisiae*, which suggests that sequences required for gene expression must be conserved, at least in some degree, between *C. albicans* and *S. cerevisiae* (10, 11, 16). In fact, many *Candida* genes are functional in *Saccharomyces*, and a large variety of *Candida* genes have been isolated by complementation in *Saccharomyces* (10, 11, 16).

In this work we describe the expression of a *C. albicans* gene in *S. cerevisiae* cells transformed with plasmid vectors in which the expression of the gene is controlled by its own 5' promoter sequences.

Materials and Methods

Strains used and growth conditions. The strain *S. cerevisiae* D3-7A (*MATα, ura3-52, trp1-289, leu2-3,112*) was used in this study. Yeast cells were cultured on YEPD (1% yeast extract, 2% peptone and 2% glucose). Transformants were selected, maintained and cultured in synthetic minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose, pH 7, 2% agar for plates) added with uracil (20 mg/l) and leucine (30 mg/l) (selective medium). *Escherichia coli* DH5α was used as recipient strain for transformation and plasmid amplification; transformants were selected and cultured in the presence of ampicillin (50 μg/ml).

Plasmid constructions. A 2 kb DNA fragment, which contains the transcribed and promoter regions of the *C. albicans* gene, was obtained by digestion with *Xho*I and *Bgl*II of the YRp7-derived plasmid, which contains a 9 kb of *C. albicans* genome DNA previously isolated (17). This fragment was subcloned into the multiple cloning site of YEplac112 and YCplac22 (7), digested with *Bam*HI and *Sal*I. The resulting plasmids pIB1 (episomal) and pIB2 (centromer) were used to transform *S. cerevisiae*.

Yeast transformation. *S. cerevisiae* cells were transformed with pIB1, pIB2, YEplac112 and YCplac22, using the lithium acetate/PEG method (14). Transformants were detected on selective medium. Tryptophan prototrophy of the transformants was found to be conferred by the presence of the plasmids, since auxotrophic colonies were recovered after growth on rich medium (YEPD). Transformants were also examined for all other genetic markers.

Northern blot. Total RNA from exponentially growing cultures of the transformants in selective medium was obtained as described by Denis et al. (4). RNA denaturation, gel electrophoresis and transfer to Hybond-N filters (Amersham) was performed according to standard methods (15). Labelling of DNA probes, as well as nucleic acid hybridization, were performed using the digoxigenin (DIG) nucleic acid labelling and detection kits (Boehringer-Mannheim), following the manufacturer's instructions. The whole 2 kb DNA fragment was used as a probe, as well as the 5' and 3' regions of the gene obtained by digestion with *Eco*RI, enzyme which cuts within the coding region, giving rise to two fragments of 1.2 (5') and 0.8 kb (3'), respectively (17). Actin mRNA levels were measured as controls, using as probe the *Kpn*I-*Bam*HI fragment carrying the actin gene from yeast (6).

Western blot techniques. Subcellular fractions (membranes and sodium dodecyl sulphate [SDS] cell wall solubilized material) were obtained as previously described (1, 5). Electrophoresis, blotting to Hybond-C filters (Amersham) and immunodetection with rabbit polyclonal antiserum raised against cell wall material from mycelial *C. albicans* cells (m-CWpAbs), were carried out as previously reported (1, 18).

Results

Immunodetection of the 30 kDa protein. Western blot of subcellular fractions from *S. cerevisiae* transformants cultures at 28°C in selective medium with m-CWpAbs allowed the detection of a 30 kDa polypeptide present in the membrane fractions (Fig. 1) and in the material solubilized from the cell walls by SDS-treatment (not shown). Several protein species were immunodetected in all cases, due to cross-reactivity of the *S. cerevisiae* proteins with m-CWpAbs. However, the 30 kDa polypeptide was absent in the control transformants, indicating that this molecule is encoded by the *C. albicans* gene. In addition, the amount of gene product correlates with the copy number of the plasmid, and it is more abundant in the pIB1 transformant. Similar results were obtained when subcellular fractions were isolated from transformants cultured at 37°C.

Northern blot analysis of the *Saccharomyces cerevisiae* transformants. The mRNAs transcribed from the *C. albicans* gene in the transformants were detected by Northern blot, using as probe the 2 kb fragment containing the

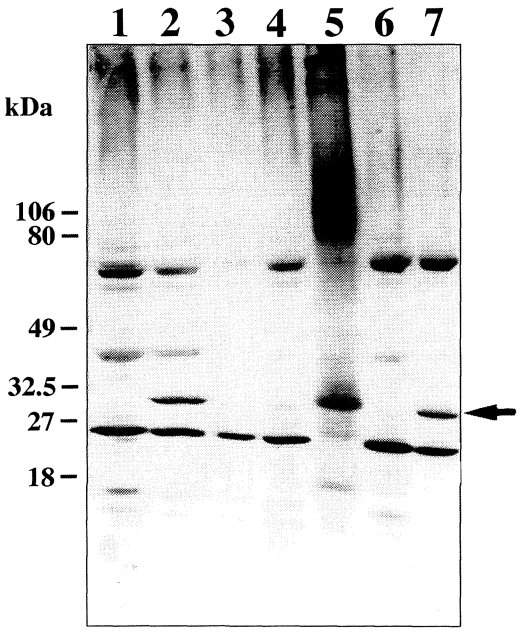

FIG. 1. Immunodetection by Western blot of the 30 kDa protein. Each slot was loaded with the membrane fraction (7–14 μg of protein) obtained from *Saccharomyces cerevisiae* cells transformed with YEplac112 (lane 1), pIB1 (lane 2), YCplac22 (lane 3) and pIB2 (lane 4), cultured on selective medium at 28°C, and from *Candida albicans* germ-tube cells (lane 5); lanes 6 and 7 correspond to YEplac112 and pIB1 transformants respectively, cultured at 37°C. Immunodetection was carried out with m-CWpAbs. The arrow indicates the position of the 30 kDa protein.

whole gene. As a matter of fact, three mRNAs were found: in addition to the 1.1 kb mRNA also present in *C. albicans* (17), two mRNAs of about 0.7 and 0.5 kb were detected in pIB1 and pIB2 transformants (Fig. 2), whereas no mRNAs were visualized in control transformants. The amount of transcripts correlates with the copy number of the plasmid. Similar results were obtained when RNA from pIB1 transformant was probed either with the 5' or 3' portions of the gene (Fig. 3).

Phenotype of the *S. cerevisiae* transformants. Transformants carrying pIB1 and pIB2 did not show any phenotypic difference with respect to the control transformants. Cell and colony morphology were unaffected, as well as growth rate on selective medium. Growth of colonies on selective medium containing sucrose or raffinose as fermentable carbon source were also similar to controls.

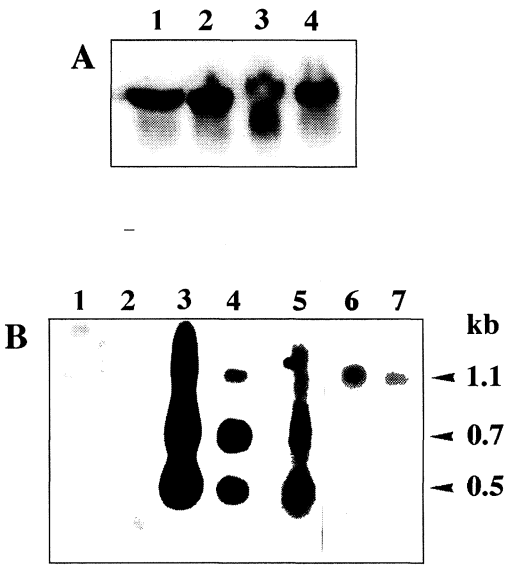

FIG. 2. Northern blot analysis of the *Saccharomyces cerevisiae* transformants. (A) Each slot contains 20 μg of total RNA from YEplac112 (lane 1), pIB1 (lane 2), YCplac22 (lane 3) and pIB2 (lane 4) transformants; the filter was hybridized with the fragment carrying the actin gene from yeast. (B) Each slot contains total RNA obtained from *Saccharomyces cerevisiae* transformed with YEplac112 (lane 1 and 2: 15 and 5 μg respectively), pIB1 (lane 3 and 4:15 and 5 μg respectively), pIB2 (lane 5: 15 μg) and from *Candida albicans* cells (lanes 6 and 7: 5 and 2 μg, respectively); the filter was hybridized with the 2 kb DNA fragment containing the *Candida albicans* gene; lane 5 is overexposed with respect to the other ones.

Discussion

Centromer and episomal plasmids carrying a *C. albicans* gene were transformed into *S. cerevisiae* cells. Expression of this gene, which is under the control of its own upstream promoter sequences, does occur in *S. cerevisiae* transformants, as reported for most *C. albicans* genes (10, 11, 16). The 30 kDa protein encoded by this gene is detected in the membrane fraction of transformants, as in *C. albicans* cells (17), although the presence of this protein does not confer relevant phenotypic features to the transformants, with regards to morphology and growth rate. This indicates that the cell physiology is not significantly affected.

When the mRNA transcripts from this gene were analysed, three molecular species were found: the 1.1 kb mRNA also detected in *C. albicans* cells (17), and two additional mRNAs of 0.7 and 0.5 kb. Although several causes may be suggested, the precise nature of the phenomenon is unknown. Regulation of gene expression is a complex mechanism involving the interaction of target DNA sequences with proteins which regulate distinct processes (initiation and termination of transcription, mRNA splicing) (2, 3, 12). Differences in both elements between *S. cerevisiae* and *C. albicans* may account for the results reported. Similar but not identical target sequences involved in mRNA processing are present in *S. cerevisiae* and *C. albicans* (10, 16). The fact that all three mRNAs are detected with the 5' and 3' parts of the gene as probes favors the hypothesis of the removal of internal se-

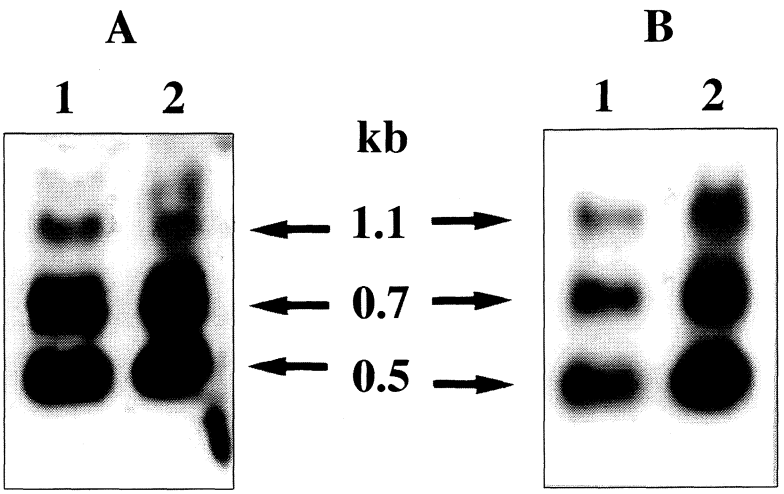

FIG. 3. Northern blot analysis using the 5' and 3' regions of the gene as probes. Total RNA from *Saccharomyces cerevisiae* pIB1 transformants was applied to each slot (5 μg, lanes 1, and 15 μg, lanes 2). Filters were hybridized with the 5' region (A) and 3' region (B) of the *Candida* gene as probes (see Materials and Methods).

quences. Other factors, such a mRNA stability, associated to translation and codon usage (9), seem to be less reliable.

In addition, expression of genes may be modified when located on plasmid vectors. The chromatin structure of the flanking regions may influence their expression (13), and regulatory factors may be limiting when the copy number of target sequences is increased (8). Nevertheless, in our case, this latter factor can be ruled out as the results reported are independent of the copy number of the plasmid. As a conclusion, our results point out that modifications in the pattern of gene expression may occur when heterologous genes are on plasmid vectors.

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Mapping of the *FLO5* gene in *Saccharomyces cerevisiae*

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Summary

The *FLO5* gene has been assigned to chromosomes I and VIII in *Saccharomyces cerevisiae*. According to the results obtained, flocculation of strain STX347-1D (reported as strain FLO5) is the result of two different genes: one of them is allelic to the previously reported *FLO1* and located at 4.7 cM from *PHO11* and 37 cM from *ADE1* on chromosome I. The other was mapped on chromosome VIII at 36.8 cM from *PET3* and 30.5 cM from *FUR1*. This second gene generated a constitutive flocculation phenotype that was not under the control of the *fsu3* suppressor. The latter has been reported to suppress certain *FLO1* genes.

Key words: *Saccharomyces cerevisiae*, *FLO1* and *FLO5* genes, yeast flocculation, surface proteins, gene mapping

Resumen

El gen *FLO5* ha sido asignado a los cromosomas I y VIII de *Saccharomyces cerevisiae*. De acuerdo con nuestros resultados, la floculación en la cepa STX347-1D (descrita como cepa FLO5) es el resultado de dos genes diferentes, uno de ellos alélico con el previamente descrito *FLO1* y localizado a 4,7 cM de *PHO11* y 37 cM de *ADE1* en el cromosoma I. El otro fue mapeado en el cromosoma VIII a 36,8 cM de *PET3* y 30,5 cM de *FUR1*. Este segundo gen dio lugar a un fenotipo floculante constitutivo que no está bajo el control de *fsu3*, descrito como supresor de floculación en distintos genes *FLO1*.

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Introduction

Flocculation in yeast describes a cell aggregation phenomenon by which cells form rapidly sedimenting clumps. Accordingly, flocculation is a suitable property for the separation of yeast from beer or wine (17, 18), and is also important in the biological production of ethanol (8). (For a review, see references 9 and 23.)

According to current hypotheses to explain yeast flocculation, surface proteins on flocculent cells (SPF) would bind to the cell wall mannan of neighbouring cells; the role of calcium ions being that to maintain these lectin-like proteins in active conformation (6). This theory is supported by the fact that protein denaturation (9) causes irreversible loss of flocculation but does not affect receptor sites. Moreover, flocculation can also be reversibly inhibited by the presence of certain sugars such as sucrose, mannose, maltose and glucose (7, 14, 20).

Flocculation in *Saccharomyces cerevisiae* is genetically controled. Several dominant genes affecting this character have been described: *FLO1* (allelic with *FLO2* and *FLO4*) mapped on chromosome I at 37 cM from the *ade1* marker (12) and 4.7 cM from the *PHO11* gene (15); *FLO5*, mitotically located on chromosome I (27), and *FLO8*, linked to *arg4* on chromosome VIII (33). The *FLO1* gene has been cloned (24, 29) and sequenced (25, 31), and *FLO5* was first cloned by Vezinhet et al. (27) and wrongly mapped on chromosome I (1). Recently, Teunissen et al. (26) reported the mapping of this gene on chromosome VIII and revealed the true localization of *FLO8* as allelic to *FLO1*. The sequences of chromosomes I and XI showed that another two putative flocculation genes are present in the *S. cerevisiae* genome, one of them located near the end of the left arm on chromosome I and the other one on chromosome XI. These genes have been named *FLO9* and *FLO10*, respectively (23). In addition, different semidominant and recessive genes, *flo3*, *flo6* and *flo7* (ref. 3), have also been reported and are now considered as possible alleles of dominant *FLO* genes that are subjected to suppression when expressed in different backgrounds (15). Mutations of different genes not directly related to flocculation (*TUP1*, *SSN6*, *sfl1* and *cka2*) may result in some pleiotropic effects, flocculation being one of them. Also, overexpression of *CHS2* gene and the expression in yeast of certain heterologous genes may induce flocculation identical to that exhibited by *FLO1*-containing strains (19).

Differences between FLO1 and FLO5 phenotypes have been reported in response to heat and proteinase treatments, flocculation intensity and mating type (2, 30). We report here the genetic and physiological characterization of a *FLO5* strain of *S. cerevisiae* and mapping of the gene on chromosome VIII by conventional mitotic and meiotic methods, thus confirming the results reported by Teunissen et al. (26).

Materials and Methods

Organisms and media. The strains of *S. cerevisiae* used in this study are shown in Table 1. YEPD medium, minimal medium (SD) and acetate-agar medium were prepared according to Sherman et al. (13). Adenine, uracil and aminoacids were added to a final concentration of 20 mg/ml when necessary.

Genetic techniques. Mating, sporulation and dissection of asci were performed according to standard procedures (13).

Mapping methods. *Mitotic mapping*. Mitotic mappings by the chromosome loss technique were carried out by using both the 2 μm mapping method (28) and the methyl benzimidazole-2-yl carbamate (MBC) method, according to Wood (32).

TABLE 1. Strains of *Saccharomyces cerevisiae* used in this study

Strain	Genotype	Source/Reference
STX347-1D	*MATa his2 ura3 gal1 FLO1 FLO5*	Y. G. S. C.[a]
IM1-8b	*MATa leu2 his4 sta^0 sta^{10} FLO1*	A. Jiménez[b]
IM1-8c	*MATα leu2 his4 sta^0 sta^{10} FLO1*	This study
CSH90L	*MATα spo11 ura3 ade1 his1 leu2 lys7 met3 trp5*	T. Benítez[c]
GM1-9B	*MATα ade2 leu2-3,112 pho11::LEU2*	H. Y. Steensma[d]
GT153-6A	*MATα ade1 gal7 leu2 ura3 his2 leu1 arg4 his6 ilv3 met14 asp5 lys7 lys9 met2 ade2 aro7*	Y. G. S. C.
NR9	*MATa leu 2-3,112 pho11::LEU2 trp1*	(11)
NR25	*MATa ade1*	(11)
CS1	*MATα leu2 ura3 trp5 met3 FLO1*	This study
CS2	*MATa leu2 ura3 trp5 met3 FLO5*	This study
CS3	*MATa ade1 his1 his2 trp5 FLO5*	This study
CS4	*MATα leu2 ade1 his1 lys7 FLO1*	This study
CS5	*MATα ade2 FLO5*	This study
LK	*MATα fur1 his3*	L. Kern[e]
X3117-1D	*MATa pet3 ade2 his6 ura1 lys2 arg4 gal2 met3 thr1 asp5*	H. Y. Steensma
Set of *cir^0* tester strains	*MATα his leu2-3,112 ura3-52 trp1-289 met2 his3-1* Cyh^R	Y. G. S. C.

[a] Y. G. S. C., Yeast Genetic Stock Center, Berkeley, California, USA; [b] Centro de Biología Molecular, Madrid, Spain; [c] Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Spain; [d] Department of Cell Biology and Genetics, Leiden University, Leiden, the Netherlands; [e] Laboratoire de Génétique Physiologique, Institute de Biologie, Strasbourg, France.

The first method requires a set of *cir^0* tester strains, each containing 2 μm plasmid DNA integrated into or near the centromere of a different chromosome. A recessive mutation in a *cir$^+$* strain can be assigned to its chromosome by crossing to the *cir^0* tester strains. At high frequencies, subclones of the *cir^0/cir$^+$* diploids will lose the integrated 2 μm plasmid DNA plus the chromosome into which integration had occurred, thus unmasking the recessive mutation.

In this case, flocculation is a dominant character. So, 16 strains, with the flocculent gene to be mapped, plus the 2 μm plasmid DNA (with the *URA3* gene) integrated into the centromere of a different chromosome, were obtained by crossing the strain CS3 (flocculent, *cir$^+$* and *ura3-52*) with the set of *cir^0* strains. Diploids were selected in minimal medium without uracil, sporulated, and flocculent *URA3* strains selected from the segregants.

These 16 strains were then crossed with strain 1645 (non flocculent and *cir$^+$*). *cir^0/cir$^+$* diploids from each of the 16 crosses were isolated on selective medium and then grown in YEPD. Dilutions of this culture were plated on a non-selective medium (50–100 colonies per plate), and the colonies screened for both *ura3* marker and the flocculent phenotype.

The second mitotic mapping was carried out by treatment of appropriate strains with MBC (E. I. du Pont de Neumours and Co., Inc.) prepared in a stock solution of 20 mg MBC/ml DMSO. Suitable amounts of the stock solution were added to autoclaved YEPD medium. A diploid heterozygous for one recessive marker on each of the chromosome to be tested, as well as heterozygous for the unmapped gene, was constructed. An exponential culture (8×10^6 cells/ml) of this diploid was treated with 100 μg MBC/ml for 24 h. Then, the cells were washed twice in TE

buffer, diluted and plated on YEPD medium at a concentration that resulted in about 50–100 colonies per plate. The colonies were then screened for both auxotrophic markers and the flocculent phenotype.

Meiotic mapping. Genetic linkage and chromosome location studies were performed as described by Sherman et al. (13). Map distances were calculated by Perkins' formula (10).

Measurement of flocculation. Cells were dispersed by washing twice with 0.25 M EDTA and twice with sterile water. Flocculation was initiated by resuspending the cells in 0.1 M sodium citrate buffer (pH 4.0), containing 0.01 M $CaCl_2$ (21). The degree of flocculation was then expressed on a subjective scale ranging from 0 (non-flocculent) to 4 (extremely flocculent), as reported by Johnston et al. (3).

Experimental assays. *Treatment with sugars*. Cells were harvested, dispersed in 0.25 M EDTA and heat-killed at 60°C. Flocculation in the strains used in this study was not affected by these treatments. The cells were then suspended in flocculation buffer containing mannose or maltose at different concentrations. When flocculation had been completed in the controls, the degree of flocculation was estimated as described above.

Treatment with proteases. Washed and dispersed cells were resuspended in 0.1 M sodium phosphate buffer (pH 7.5) containing different proteases according to Hodgson et al. (2). Suspensions were then incubated at 30°C with gentle shaking and samples (1 ml) were removed at the required intervals. The cells were washed, resuspended in 0.1 M sodium citrate buffer (pH 4), with 0.01 M $CaCl_2$, and flocculation was calculated as previously described.

Heat treatment. Washed and dispersed cells were resuspended in 0.05 M sodium acetate buffer (pH 4.5) containing 0.01 M $CaCl_2$ and then incubated at 70°C. Samples were removed at different times, and the degree of flocculation was estimated.

Results and discussion

Characterization of the FLO5 phenotype. Genetic characterization of strains harboring the *FLO5* gene was accomplished by crossing strains STX347-1D (reported as being FLO5) and CSH90L (non-flocculent); then, the segregants were tested for their ability to flocculate in liquid culture medium. Tetrad analysis revealed a non-monogenic segregation for flocculation (Table 2) suggesting that the flocculent strain STX347-1D might carry two unlinked flocculation genes. To gain deeper insight into this hypothesis, the four spores of an ascus exhibiting 4F:0NF segregation were micromanipulated and grown separately (strains CS1, CS2, CS3 and CS4). To check the possibility of one of the flocculation genes present in the four strains being allelic with the previously described *FLO1*, they were crossed with strains IM1-8b and IM1-8c (both *FLO1*). As shown in Table 2, the crosses of CS1 and CS4 with IM1-8b exhibited 4F:0NF segregation in 100% and 86% of the asci screened. This confirmed that the flocculation genes present in CS1 and CS4 were allelic with *FLO1*. However, the segregations of crosses CS2 and CS3 with IM1-8c included asci of the 2F:2NF type, thus indicating that the flocculation genes in CS2 and CS3 strains were not allelic with *FLO1*. Moreover, the crossing of strains CS1, CS2, CS3 and CS4 with each other strongly suggested the presence of two non-allelic flocculation genes. In this sense, the cross between CS2 ans CS5 (derived from CS3) always gave segregations 4F:0NF, and the cross between CS1 and CS3 mainly gave 3F:1NF and 2F:2NF segregations. In order to confirm whether the flocculation genes of strains CS1 and CS4 occupied the *FLO1*

TABLE 2. Segregation ratios of flocculation genes

Cross	Segregation (F:NF)					Number of tetrads
	4:0	3:1	2:2	1:3	0:4	
STX347-1D × CSH90L	5	15	7	0	0	27
CS1 × IM1-8b	20	0	0	0	0	20
CS2 × IM1-8c	1	11	11	0	0	23
CS3 × IM1-8c	4	14	3	0	0	21
CS4 × IM1-8b	18	3	0	0	0	21
CS1 × CS3	1	15	7	0	0	23
CS2 × CS5	33	0	0	0	0	33
CS4 × CS3	7	12	7	0	0	26
CS6 × GM1-9B	0	0	4	25	1	30
CS2 × GM1-9B	0	0	27	0	0	27
CS3 × GM1-9B	0	0	23	0	0	23

F, flocculent; NF, non-flocculent. Segregation of the other markers was 2+:2– in all cases.

locus, attempts to perform meiotic mapping were made. Strain CS1 was crossed with NR9 (harboring *pho11::LEU2* marker on chromosome I) and with NR25 (*ade1*). Tetrad analysis of parental ditypes/non parental ditypes/tetratypes revealed ratios of 58/0/6 with respect to the first marker and 16/0/38 for the second one (Table 3). This, in turn, allowed the mapping of the gene at 4.7 cM from *PHO11* and 35.2 cM from *ADE1*. Regarding strain CS4, the flocculation gene could be mapped following the same approach at 3.3 cM from *PHO11* and 37.8 cM from *ADE1*.

Mapping of the second flocculation gene. Mitotic mapping was accomplished by the 2 μm mapping method. The gene was chromosome-assigned by the construction of 16 cir^0/cir^+ diploids by crossing flocculent cir^0 and non-flocculent cir^+ strains. In growth in non selective media, the frequency of loss of each chromosome is seen as the result of the acquisition of uracil auxotrophy. This frequency was ca. 10–20%. Three hundred colonies of each diploid were tested for their ability to flocculate in liquid medium. Loss of the flocculation marker (and hence *ura3* auxotrophy acquisition) was apparent in only 22 colonies of the diploid formed by the cir^0 strain containing an integrate on the chromosome VIII centromere. These results, therefore, allowed us to assign the second flocculation gene to chromosome VIII.

The mitotic mapping of this new flocculation gene was also performed by the chromosome loss technique, using MBC as the inducer. A diploid was obtained by crossing strains CS3 and GT153-6A. The diploid was thus heterozygous for one marker on each chromosome in addition to the flocculation gene to be mapped. After 24 h in the presence of MBC, cell viability was 10%. Twelve colonies were non flocculent, 10 of them being aneuploid for chromosome VIII, since they were *arg4*. This confirmed that the *FLO5* gene was located on chromosome VIII. Besides, 20 out of 2000 colonies tested were aneuploid for chromosome I and flocculent. This led to the obvious conclusion that the *FLO5* gene was not located on chromosome I.

Meiotic mapping of *FLO5* was accomplished using the markers *pet3* and *fur1* of chromosome VIII. As shown in Table 3, the gene was mapped at 30.5 cM from *FUR1* and 36.8 cM distal from *PET3*, confirming the results reported by Teunissen et al. (26).

TABLE 3. Meiotic mapping

Gene pair	Cross	Number of asci			cM
		PD	NPD	T	
FLO1–PHO11	CS1 × NR9	58	0	6	4.7
FLO1–ADE1	CS1 × NR25	16	0	38	35.2
FLO1–PHO11	CS4 × NR9	42	0	3	3.3
FLO1–ADE1	CS4 × NR9	11	0	34	37.8
FLO5–PET3	CS3 × X3117-1D	10	0	28	36.8
FLO5–FUR1	CS3 × LK	21	0	33	30.5

Abbreviations: PD, parental ditype; NDP, non-parental ditype; T, tetratype; cM, centimorgans. Only tetrads with segregation 2:2 were analyzed.

Phenotype characterization. The phenotypes of strains harboring the *FLO5* gene were studied according to the method of Hodgson et al. (2). According to these authors, FLO1-based phenotypes are resistant to heat, but sensitive to chymotrypsin treatment, whereas the opposite happens in the case of FLO5-based phenotypes. Accordingly, strains CS1, CS2, CS3 and CS4 were checked for their response to heat and chymotrypsin in a comparative fashion with strains IM1-8b (FLO1 phenotype) and STX347-1D (FLO5 phenotype). Strains CS1 and CS4 (harboring the *FLO1* gene) clearly have a FLO1 phenotype; similarly, strain CS3 (harboring the *FLO5* gene) exhibited a FLO5 phenotype, whereas strain CS2 (genetically characterized as *FLO5*) did have an intermediate FLO1/FLO5 phenotype, and was therefore sensitive to both heat and chymotrypsin treatments.

The flocculation phenotypes of strain FLO1 and FLO5 were assayed for their ability to flocculate in the presence of mannose and maltose in order to typify them as the classical FLO1 or New Flo phenotypes (2). As may be observed in Fig. 1, strains STX347-1D, CS2 and CS3 behaved like IM1-8b and were affected only by the mannopyranosides, as reported for the FLO1 phenotype strains.

Effect of the *fsu3* suppressor on *FLO5*. The *fsu3* suppressor has previously been characterized (15) as being able to suppress certain FLO1-based phenotypes. In order to determine whether the suppressor had any effect on FLO5-based flocculation, strains CS2 and CS3, as well as CS6 (CS1-derived and hence FLO1), were crossed with GM1-9B (harboring *fsu3*). As shown in Table 2, all the diploids of CS2 and CS3 with GM1-9B displayed monogenic segregation for flocculation (2F:2NF), whereas the diploids of CS6 with GM1-9B showed non-Mendelian segregation for flocculation. Taking all these results in consideration, it may be concluded that the *FLO5* gene is neither controlled nor regulated by *fsu3*. It would be of interest to find out whether the other reported suppressors (*fsu1* and *fsu2*) are able to suppress the FLO5-based yeast flocculation.

In the light of the foregoing, *FLO1* gene has been mapped on chromosome I of *S. cerevisiae* at 35 cM from *ade1* (5) and 4.8 cM from *PHO11* (15). For years, the *FLO5* gene remained unmapped. In 1994 Bidard et al. (1), after using cytoduction techniques, claimed that the gene mapped on chromosome I. Recently, Teunissen et al. (26) remapped the gene on chromosome VIII at 34 cM from *PET3*.

Our results with strain STX347-1D (formerly reported to be FLO5) clearly show the presence of two flocculation genes, one being allelic to the previously reported *FLO1*, and the second

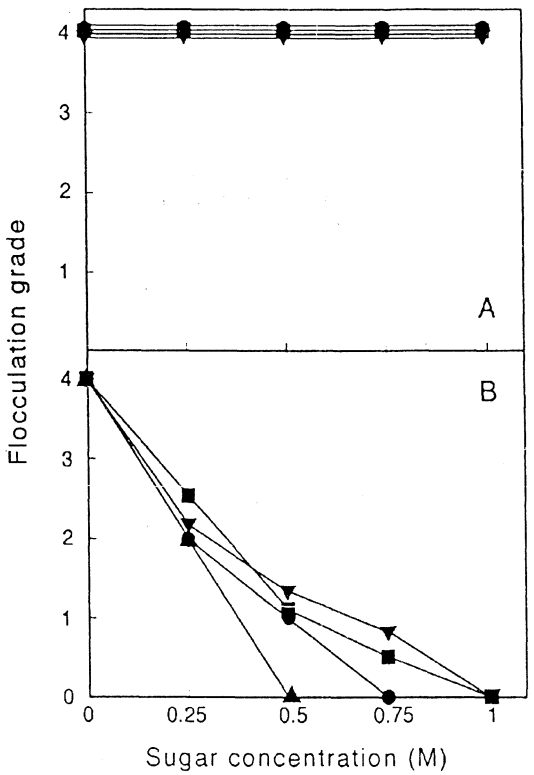

FIG. 1. Effect of different maltose (A) and mannose (B) concentrations on the flocculation of strains IM1-8b (●), STX347-1D (■), SV1 (▲) and SV3 (▼).

one located on chromosome VIII at 35.8 cM from *PET3* and 30.5 cM from *FUR1*. The presence of either of the genes is sufficient to originate a flocculation phenotype. The results reported here confirm those found by Teunissen and co-workers. While sequencing chromosome VIII, Johnson et al. (4) found a flocculation gene similar to the previously described *FLO1*. Besides, Teunissen et al. (26) were able to disrupt the *FLO5* gene by the cloned *FLO1*. From all this, we can conclude that both genes must be very similar, although the former gene is not subjected to regulation by the *MAT* locus (30). Furthermore, according to our results, *FLO5* is not suppressed by *fsu3*. On the contrary, the *FLO1* gene present in STX347-1D is suppressed by *fsu3*. This latter aspect of differential regulation by different suppressors of two otherwise related genes is currently being studied at our laboratory.

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MICROBIOLOGÍA SEM **12** (1996), 457–464 **PERSPECTIVAS**

Breve historia de la Sociedad Española de Microbiología, V. De 1987 a 1991

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Summary

In this fifth chapter of the short history of the Spanish Society for Microbiology (SEM), the major activities carried out from 1987 to 1991 are described. During that period, the 11th, 12th and 13th SEM National Congresses took place in Gijón (1987), Pamplona (1989) and Salamanca (1991), respectively. The Specialized Groups of the Society organized their own meetings. Courses on the introduction to research in microbiology, for undergraduate students, started in that period. The President of SEM was elected FEMS Vice-president during the Council Meeting of the Federation of European Microbiological Societies (FEMS), held in Madrid in September 1989.

Resumen

El quinto artículo de la serie sobre la historia de la Sociedad Española de Microbiología (SEM) abarca el período de 1987 a 1991, durante el cual se celebraron los Congresos Nacionales undécimo (Gijón, 1987), duodécimo (Pamplona, 1989) y decimotercero (Salamanca, 1991). Los Grupos Especializados celebraron las reuniones correspondientes y empezaron a organizarse los Ciclos de conferencias de iniciación a la investigación en microbiología, dirigidos a estudiantes de último curso de licenciatura. En 1989 tuvo lugar en Madrid la reunión del Consejo Directivo de la Federación de Sociedades Europeas de Microbiología (FEMS), donde el Presidente de la SEM fue elegido Vicepresidente de FEMS.

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XI Congreso Nacional de Microbiología

El XI Congreso Nacional de Microbiología, organizado por Carlos Hardisson Rumeu, tuvo lugar en el Centro de Enseñanzas Integradas de Gijón (antigua Universidad Laboral), los días 24 al 27 de junio de 1987. Pronunció la conferencia inaugural Eugenio Santos, y la de clausura Severo Ochoa. Enrique Herrero, ganador del segundo premio de la SEM, impartió la conferencia correspondiente que, en esta ocasión, estuvo dedicada a David Vázquez, anterior presidente de la Sociedad, recientemente fallecido. Se recordaba así al científico y amigo. El congreso contó con tres simposios internacionales, con intervención de especialistas nacionales y extranjeros, quince mesas redondas y más de quinientas comunicaciones, presentadas en forma de panel. Participaron más de mil microbiólogos españoles, además de los invitados extranjeros. Esta edición del congreso repitió la experiencia de editar todas las contribuciones por invitación en forma de artículos completos.

Período 1987–1989

Durante el XI Congreso en Gijón tuvo lugar la Junta General Ordinaria, donde se expusieron las actividades llevadas a cabo en el período 1986–1987. Como actividades futuras, se aprobó la realización de cursos de iniciación a la investigación en microbiología, destinados a estudiantes de los últimos cursos de carrera. También se aprobó, a propuesta del Editor-Coordinador de la revista, Rubens López, la ampliación del Consejo Editorial, incorporando dos editores extranjeros. El objetivo era conseguir la inclusión de la revista en *Current Contents*, una vez que se había logrado que apareciese reseñada en *Chemical Abstracts*. En relación con los Grupos Especializados, la mayoría mostraba una trayectoria ascendente, a excepción de los de Virología, Microbiología Clínica y Noroeste. Se decidió tratar el problema de cada grupo por separado en las sucesivas Juntas Directivas de la SEM. Respecto a la sede del siguiente Congreso Nacional, se consideró la propuesta de Pamplona, bajo la dirección de Ramón Díaz, quien confirmaría tal extremo tras discutir la propuesta con sus colaboradores.

La Junta General Extraordinaria, celebrada a continuación, discutió la enmienda de los siguientes artículos de los Estatutos de la Sociedad: 1, 4, 8, 10, 11, 15 y la disposición final. Las propuestas fueron aprobadas por los asistentes. Con la nueva redacción quedaban definidas las atribuciones de los cargos directivos de la SEM y se introducían los cargos ya citados de Presidente y Secretario Electos.

Una de las primeras actividades de la Junta Directiva durante el período que nos ocupa fue la creación del Grupo de Biodegradación y Biolixiviación, promovido por Fernando Laborda; este grupo se denominaría de Biodeterioro. Pendiente de discusión el estado de los grupos prácticamente inactivos de Virología, Microbiología Clínica y Noroeste, se propuso consultar a los socios de los dos primeros mediante una carta o encuesta para decidir sobre el futuro del grupo. El Grupo de Virología, que con la creación de la Sociedad Española de Virología había sufrido una baja importante de socios, conservaba todavía más de sesenta inscritos. Sin embargo, el de Microbiología Clínica, con Luis Arcalís como presidente en funciones, estaba más mermado. La problemática del Grupo del Noroeste era de otra índole, de manera que se decidió instar a su Presidente, José Ángel García Rodríguez, a que reanudara las actividades. En 1988, el Grupo de Taxonomía organizó en Madrid su III Reunión, bajo la dirección de Guillermo Suárez. Seguiría a continuación el Simposium FEMS sobre "Ar-

FIG. 1. Delegados de las distintas Sociedades de Microbiología Europeas y Junta Directiva de la SEM, con motivo del FEMS Council Meeting celebrado en Madrid en 1989.

queobacterias", celebrado en Troya (Portugal). Por su parte, el Grupo de Microbiología de Alimentos estaba intentando que su próxima VI Reunión, a celebrar en Madrid, contase con la participación de la Comisión Internacional de IUMS. Igualmente, intervendría en la reunión sobre "Modern microbiological methods", que se iba a celebrar en Santander en 1989, organizada por comisiones de la SEM, FEMS, IUMS, IDF (International Dairy Federation) y AOAC (Association of Official Analytical Chemists, EE. UU.).

Finalmente, las gestiones de la Secretaria ante la administración central habían dado su fruto con la obtención de la exención del pago del IVA en todas las cuotas (socios, congresos, etc.). La SEM era la primera sociedad científica que conseguía este tratamiento. Por otra parte, la enmienda de los Estatutos definió las competencias de los cargos directivos. Fue necesario, además, otorgar poder notarial a los cargos de Secretario y Tesorero, que facultara la disposición conjunta de los fondos de la Sociedad (práctica que, por otro lado, se venía realizando desde la fundación de la SEM).

Las relaciones entre la SEM y la FEMS se habían afianzado, de modo que se acordó proponer al Presidente, César Nombela como Vicepresidente o Secretario General de FEMS durante el FEMS Council Meeting que tuvo lugar en Madrid en 1989. Esta reunión fue organizada por la entonces delegada de la SEM en FEMS, y Secretaria de la Sociedad, Concepción García Mendoza. Era la primera vez que la reunión general de FEMS se celebraba en España. Igualmente, se había conseguido el apoyo de FEMS para el "Workshop" sobre "Bacterias halófilas", organizado por Francisco Rodríguez Valera, en Alicante (1989).

Una ayuda a la expansión de la revista de la SEM vino con una financiación complementaria obtenida del Fondo Nacional para la Investigación Científica y Técnica. Se empezó a distribuir a las bases de datos internacionales y bibliotecas de renombre sugeridas por el entonces ICYT (Centro de Información Científica y Técnica del

CSIC). Anteriormente, su distribución se limitaba a las sociedades de microbiología de FEMS y latinoamericanas, junto con los intercambios de revistas establecidos por el CSIC con la anterior publicación *Microbiología Española*. Por su parte, el *Boletín Informativo*, a cargo de Rosalina Pomés, empezó a incluir información sobre cursos de doctorado y de postgrado organizados por las distintas universidades españolas, siguiendo los acuerdos de la reunión monográfica de Sigüenza. En la misma línea, el Presidente propuso la puesta en marcha de un programa sobre enseñanza de la microbiología, con objeto de tratar la homologación de esta materia en las diferentes facultades y escuelas donde se impartía. Se constituyó una comisión, con Andrés Chordi como coordinador y representante de las facultades de Farmacia, José Prieto por Medicina, Enrique Montoya por Biología, Ezequiel Cabrera por Ingeniería Agronómica y Elías Rodríguez Ferri por Veterinaria.

La CECT, por su parte, había conseguido incorporar por un período de dos años a Cristine Dawson, procedente de la NCTC (National Collection of Type Cultures, Gran Bretaña). Además, después de la reunión de Budapest, estaba planeando participar en el siguiente encuentro en Baarn (Holanda).

Como resultado de la encuesta enviada a los socios del Grupo de Virología, se acordó su mantenimiento temporal, a título testimonial y con exención del pago de la cuota. Al contar con más de 30 integrantes, de acuerdo con los Estatutos, no procedía la disolución. Por su parte, y después de consulta, el Grupo de Microbiología Clínica decidió su continuidad. Fernando Baquero se encargaría de su revitalización y se le daría un enfoque más básico. El Grupo de Micología preparaba su próxima V Reunión, en Barcelona en 1990, conjuntamente con la Asociación Española de Especialistas en Micología (AEEM), bajo la dirección de José M. Torres y de José Guarro. El Grupo de Microbiología de Alimentos también pensaba celebrar su VII Reunión en Barcelona, en 1990, a cargo de Benito Oliver-Rodés Clapés. El nuevo Grupo de Biodeterioro anunciaba su primera reunión en Madrid en 1989, organizada por Fernando Laborda.

Continuando con el tema abordado previamente por las Sociedades integrantes de FEMS en Londres, sobre "Organismos manipulados genéticamente", el Presidente, César Nombela, propuso a la Junta Directiva la creación de un grupo de trabajo que tratara sobre el tema, atendiendo así a la creciente importancia de esta línea de investigación. Rafael Sentandreu, que formaba parte de la comisión integrada por diversos organismos como CDTI, CICYT, MOPU, INIA, y auspiciada por la CEE, fue propuesto, junto con Tomás Ruiz Argüeso, Francisco Ruiz Berraquero, Fernando Laborda y Francisco Rodríguez Valera, para la formación de ese grupo de trabajo, dedicado al "Manejo y liberación al medio ambiente de microorganismos manipulados genéticamente".

La respuesta de los socios para incluir sus palabras clave en los ficheros de la SEM llegó casi al 60%. Por otra parte, para impedir la utilización de las siglas SEM por la Sociedad Española de Mesoterapia, se realizaron las gestiones pertinentes. El problema quedó resuelto por sí solo, ya que la mencionada sociedad no estaba correctamente registrada como asociación. A raíz de este hecho, se acordó introducir las siglas SEM en los Estatutos, mediante una enmienda que se presentaría en la siguiente Asamblea Extraordinaria, en el Congreso de Pamplona. En el mismo se contemplaría la inclusión de socios eméritos y estudiantes, así como la reducción del número de firmas necesario para la presentación de candidaturas.

Correspondiendo la renovación parcial de la Junta Directiva, y de acuerdo con la última en-

FIG. 2. Presidencia de la Sesión "Morfología y Estructura" del XII Congreso Nacional de Microbiología de Pamplona, en 1989. Moderadores: Concepción García Mendoza y Juan Antonio Leal.

mienda de los Estatutos, se solicitó por primera vez la candidatura de Presidente Electo, así como la renovación de Secretario, Tesorero y tres Vocales. Tras la votación resultaron elegidos: Presidente Electo, Francisco Ruiz Berraquero; Secretaria, Concepción García Mendoza; Tesorero, Juan Antonio Leal Ojeda, y Vocales, Julio Casal Lombos, Ricardo Guerrero Moreno y Bernabé Sanz Pérez, cubriendo Jorge Lalucat Jo la vacante de Vocal producida por el paso de Francisco Ruiz Berraquero a Presidente Electo.

La CECT, que continuaba su actividad internacional, participó en la reunión de Colecciones de Cultivos Tipo de la CEE, celebrada en Braunschweig (Alemania). Se había conseguido, de la propia CEE, financiación para su total integración informática en MINE (Microbial Information Network Europe), lo cual permitía establecer nudos informáticos especializados entre las distintas colecciones y la sede central de Alemania. A la vez, la CECT realizaba gestiones ante el Ministerio de Industria para la obtención del título de Autoridad de Depósito Internacional de Patentes.

Se atendió la petición de la IUMS de renovar el nombramiento de representante de la SEM en la División Internacional de Virología, refrendando por acuerdo la anterior designación de Rafael Nájera, pese a las diferencias surgidas en su día con motivo de la fundación de la Sociedad Española de Virología. Debido a la renuncia de Rosalina Pomés como Coordinadora del Boletín Informativo, se acordó nombrar a Josefina Rodríguez de Lecea para tal cargo. Igualmente, habiendo finalizado el plazo de vigencia, según los Estatutos, para el cargo de Editor–Coordinador de la revista, fue propuesto para el siguiente período Juan Antonio Ordóñez Pereda.

El curso para estudiantes de último año de carrera se denominaría finalmente "Ciclo de conferencias de iniciación a la investigación en Microbiología". Su primera edición iba a realizarse en Segovia, bajo la dirección de Ernesto García López, y sería financiado conjuntamente por la SEM y por la Fundación Ramón Areces. Se anunciaría oficialmente en el Congreso Nacional de Pamplona, donde Ernesto García recibiría el tercer Premio de la SEM, dedicado

esta vez a Lorenzo Vilas. Con motivo de la celebración del Consejo de FEMS en Madrid, con el Comité Ejecutivo y la reunión de Delegados, donde se decidiría sobre la candidatura de César Nombela como futuro Vicepresidente, se acordó celebrar conjuntamente una Junta Directiva de la SEM, para que todos pudiesen participar de este acontecimiento. La candidatura de César Nombela fue aceptada y, según el procedimiento habitual, sería sometida a votación en el siguiente Consejo de FEMS, en París en 1990. Una vez aprobada, el nombramiento se haría efectivo en 1991.

XII Congreso Nacional de Microbiología

El XII Congreso Nacional de Microbiología se celebró en la Facultad de Medicina de Pamplona durante los días 24 a 27 de septiembre de 1989, y fue organizado por Ramón Díaz García. La conferencia inaugural fue pronunciada por Ernesto García, ganador del Premio de la SEM, y estuvo dedicada a Lorenzo Vilas, uno de los fundadores de nuestra Sociedad y que, además, desempeñó el cargo de Secretario durante veinte años. La conferencia de clausura estuvo a cargo de Jesús Guinea Sánchez-Uzábal. El congreso contó con tres simposios internacionales, dieciocho mesas redondas y más de seiscientas contribuciones en forma oral y en paneles, distribuidas en doce secciones temáticas. Hubo una asistencia de cerca de mil microbiólogos españoles y destacados microbiólogos extranjeros.

Período 1989–1991

En la Junta General Ordinaria celebrada en Pamplona con motivo del XII Congreso Nacional, se describieron las actividades realizadas a lo largo del bienio anterior, se hizo entrega del título de Socio de Honor a Julian Davies y se anunció la propuesta del Presidente, César Nombela, como Vicepresidente de FEMS. También se comunicó el relevo del anterior Editor–Coordinador de la revista, que a partir de ese momento sería Juan Antonio Ordóñez. El Presidente, César Nombela, se despidió en su última intervención ante la Asamblea de la SEM. Finalmente, se acordó la celebración del siguiente Congreso Nacional en Salamanca, aceptando la propuesta de Julio R. Villanueva.

La Junta General Extraordinaria aprobó, tras votación, la enmienda de los artículos 1, 8 y 14 de los Estatutos.

En su andadura ordinaria, la Junta Directiva trató de la celebración del cincuentenario de la Sociedad Francesa de Microbiología, la cual había invitado a participar a todos los Presidentes de las Sociedades Europeas. Se estaba considerando, a la vez, la proximidad del cincuenta aniversario de la SEM. Los Grupos Especializados continuaban organizando sus reuniones periódicas . Así, el Grupo de Taxonomía Bacteriana celebraría su III Reunión (Murcia, 1990), bajo la dirección de Francisco Torrella; el Grupo de Aragón, Rioja, Navarra y Soria, su II Reunión, (en Huesca), organizada por Antonio Rezusta. El Grupo de Microbiología Industrial realizó una intervención en el III Congreso Nacional de Biotecnología (Murcia, 1990), a cargo de José Luis Iborra, que estaba organizando cursos sobre "Empleo de los ordenadores en Microbiología" y sobre "Genética de levaduras principalmente vínicas" en Valencia, en colaboración con José Martínez Peinado. El Grupo de Microbiología Clínica había emprendido sus actividades y Fernando Baquero estaba organizando una reunión en Leganés (Madrid) sobre "Microbiología ambulatoria", a la que seguirían otras en el Colegio de Médicos de Madrid. El Grupo de

Biodeterioro había comenzado a preparar una reunión internacional, también en Madrid, para 1991. Se haría en colaboración con la Sociedad Inglesa de Biodeterioro, y se dedicaría a "Biodeterioro en España" y "Biodeterioro en obras de arte". Por su parte, el Grupo de Micología planificaba su correspondiente reunión, conjuntamente con la AEEM, en Tenerife, a cargo de Luis Rodríguez. Esa reunión se denominaría I Congreso Nacional de Micología. El Grupo de Microbiología de Alimentos preparaba una reunión en Cáceres, bajo la dirección de Miguel Ángel Asensio. Ambas reuniones se celebrarían en 1992. Por otra parte, el Grupo del Noroeste no alcanzaba a reiniciar su gran actividad anterior, por lo que urgía darle una solución. Ésta se haría, como en otras ocasiones, mediante consulta a sus miembros.

La CECT había finalizado la tercera edición del Catálogo de Cepas, esta vez en inglés, para poder realizar intercambios internacionales. La última reunión del Proyecto MINE se había celebrado en Santa Maria Ligure (Génova, Italia), y la siguiente sería en Valencia, organizada por Federico Uruburu, junto con el Grupo de Taxonomía Bacteriana. La CECT se encontraba adscrita a la Universidad de Valencia como servicio oficial de la misma, contando con sus propios estatutos, y, próximamente, con nuevos locales en un edificio, también nuevo, dedicado a servicios de la Universidad. A su vez, el CSIC había concedido una plaza de ayudante científico.

El primer Ciclo de conferencias de iniciación a la investigación en Microbiología, organizado en Segovia bajo la dirección de Ernesto García, había alcanzando un gran éxito. Ricardo Guerrero se ofreció para organizar el segundo en Sitges (Barcelona), para el siguiente año. La revista, bajo la dirección de Juan Antonio Ordóñez, continuaba adelante y, para facilitar su inclusión en *Current Contents*, había incorporado a varios científicos extranjeros en el Comité Editorial. El *Boletín Informativo*, bajo la dirección de Josefina Rodríguez de Lecea, introducía novedades como "Opinión de los socios" y ofertas de trabajo en la industria, investigación, etc.

La secretaría de la SEM había instalado un fax y se estaba confeccionando un tríptico y un póster para difundir las actividades de la Sociedad. Este material se enviaría a los Departamentos de Microbiología de las universidades y del CSIC, hospitales, y demás centros. Se había recibido la candidatura de J. Farmer III (Atlanta, EE. UU.) como Socio de Honor de la SEM, presentada por Rafael Gómez-Lus.

Las relaciones de la SEM con la IUMS eran cada vez más intensas. El Presidente, César Nombela, participó en la reunión ejecutiva en Berlín en 1990, coincidiendo con el Congreso Internacional de Virología, mientras que los respectivos Congresos de Bacteriología y Micología tuvieron lugar en Osaka (Japón). Nuestra Sociedad estuvo representada por Antonio Ventosa, quien fue nombrado miembro del Comité Internacional de Sistemática Bacteriana (ICBS). En la Asamblea General se aprobó la publicación de la nueva revista *World Journal of Microbiology and Biotechnology*, que se enviaría a todas las sociedades integrantes de la Unión Internacional.

En el Consejo Ejecutivo de FEMS, celebrado en París en 1990, al que asistió la delegada de la SEM, Concepción García Mendoza, se presentó la propuesta de organizar en España, en 1992, un nuevo Simposium FEMS, esta vez sobre "Bacterial growth and lysis: metabolism and structure of the sacculus", a cargo de Miguel Ángel de Pedro. Una vez más, la SEM contó con una beca FEMS para investigadores jóvenes. César Nombela fue elegido Vicepresidente de FEMS.

El CSIC, por su parte, había comunicado a la SEM la posibilidad de cambio de local de nuestra secretaría. El nuevo estaría en Hortaleza, 104. Además, gracias a la inclusión de las pala-

bras clave en los ficheros de socios, se pudieron poner en contacto especialistas españoles con los grupos extranjeros que así lo solicitaban. De nuevo las siglas SEM fueron utilizadas indebidamente por otra sociedad científica, en este caso la de Malacología. Se procedió en consecuencia, y la citada sociedad admitió y enmendó su error. El cuarto Premio de la SEM correspondió a Antonio Ventosa, quien pronunciaría la correspondiente conferencia plenaria durante el XIII Congreso Nacional, en Salamanca. Para atender a la petición del FIS de un representante de la SEM especialista en tuberculosis, se designó a Vicente Ausina.

Correspondiendo una vez más la renovación parcial de la Junta Directiva, y tras haber solicitado las correspondientes candidaturas, se incorporaron a la nueva Junta, después de la votación preceptiva, las siguientes personas: Vicepresidente, Rafael Rotger Anglada; Secretario Electo, Juan Antonio Leal, y Vocales, Enrique Montoya Gómez, Jorge Lalucat Jo y Sara Isabel Pérez Prieto, a la vez que se hacía el relevo del Presidente. Tomó posesión de este cargo Francisco Ruiz Berraquero, hasta ese momento Presidente Electo. A César Nombela se le nombró invitado permanente en la Junta Directiva de la SEM, durante el tiempo que permaneciese como Vicepresidente de FEMS.

Próxima la celebración del XIII Congreso Nacional, Ricardo Guerrero presentó el programa del segundo Ciclo de conferencias de iniciación a la investigación en Microbiología, que se celebraría en Sitges en 1991. También anunció la preparación del 6th International Symposium on Microbial Ecology (ISME-6), del cual había sido nombrado presidente por el ICOME (International Committee on Microbial Ecology), y que se celebraría en Barcelona en septiembre de 1992. Seguidamente, presentó la candidatura de Moselio Schaechter (Boston, EE. UU.) como Socio de Honor de la SEM, nombramiento que, tras la consulta preceptiva a los socios, tendría lugar durante el Congreso de Salamanca.

XIII Congreso Nacional de Microbiología

El XIII Congreso Nacional tuvo lugar en las Facultades de Ciencias Biológicas y Físicas de Salamanca, durante los días 15 al 18 de julio de 1991, organizado por Julio R. Villanueva. Pronunció la conferencia inaugural Paul Nurse y la de clausura Manuel E. Patarroyo. El Premio de la SEM volvió a estar dedicado a la figura de Jaime Ferrán, como homenaje a su labor en la vacunación anticolérica. La conferencia correspondiente al Premio estuvo a cargo de Antonio Ventosa. Además, hubo otra conferencia plenaria, que fue impartida por Emilio Bouza Santiago. Se organizaron doce simposios y cinco mesas redondas. Las más de quinientas comunicaciones libres fueron expuestas en forma de paneles. Asistieron más de mil microbiólogos españoles y prestigiosos invitados extranjeros.

Al alcanzar ya con este capítulo el año 1991, queda cubierta la mayor parte de la historia de la Sociedad. En el próximo número de la revista registraremos los principales acontecimientos de la etapa más reciente, la que va desde los años 1991 a 1995, con lo cual daremos fin a esta serie de artículos.

MICROBIOLOGÍA SEM **12** (1996), 465–468 **PERSPECTIVAS**

José Luis Cánovas Palacio-Valdés (1934–1995)*

Gonzalo Giménez Martín

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José Luis Cánovas Palacio-Valdés (14.10.1934–05.09.1995) nació en Totana (Murcia) y residió la mayor parte de su vida en Madrid. Quién sabe si por esa tendencia humana a recordar el propio origen, su fallecimiento se produjo en la localidad que le viera nacer. Alumno aplicado, realizó el bachillerato en el Colegio del Pilar de Madrid; finalizado éste, comenzó sus estudios de abogacía atraído, en parte, por la profesión paterna. Sin embargo, una intensa vocación científica le obligó a modificar su trayectoria académica: en 1954 comenzó la Licenciatura en Farmacia en la Universidad Complutense de Madrid. Ello fue clave para su total dedicación posterior. Un hito importante lo marca la figura del Prof. José María Albareda, entonces Catedrático de Geología Aplicada de la Facultad de Farmacia y secretario general del Consejo Superior de Investigaciones Científicas (CSIC). El Prof. Albareda seleccionaba entre sus alumnos aquéllos que consideraba más idóneos y sobresalientes para una posible dedicación a la investigación científica. De esta manera, José Luis Cánovas fue uno de los escogidos, pasando a integrarse en un grupo conocido como "Club Edaphos", dentro del Instituto de Edafología y Fisiología Vegetal. Estaba formado por científicos jóvenes: unos, recién licenciados, y otros, cursando aún sus estudios académicos. Dentro de la promoción de 1960 de la Facultad de Farmacia había tres componentes del Club Edaphos, tres íntimos amigos y compañeros: José Luis Cánovas Palacio-Valdés, Jorge Fernández López-Sáez y Emilio Muñoz Ruiz, que obtuvieron los expedientes académicos más sobresalientes, galardonados con Premios Extraordinarios de Licenciatura. Los tres iniciarían, a raíz de la finalización de sus estudios universitarios, la conexión con científicos del CSIC, a fin de realizar la tesis doctoral.

En 1961, José Luis Cánovas fue el primer becario del Prof. Manuel Losada, que acababa de regresar de la Universidad de California, en Berkeley, donde había trabajado con grandes

*Al cumplirse el primer aniversario del fallecimiento de nuestro compañero José Luis Cánovas, a quien muchos socios de la SEM conocieron y admiraron, *Microbiología SEM* quiere recordar su figura publicando este breve artículo como homenaje a un gran científico y a una persona de inolvidables cualidades humanas.

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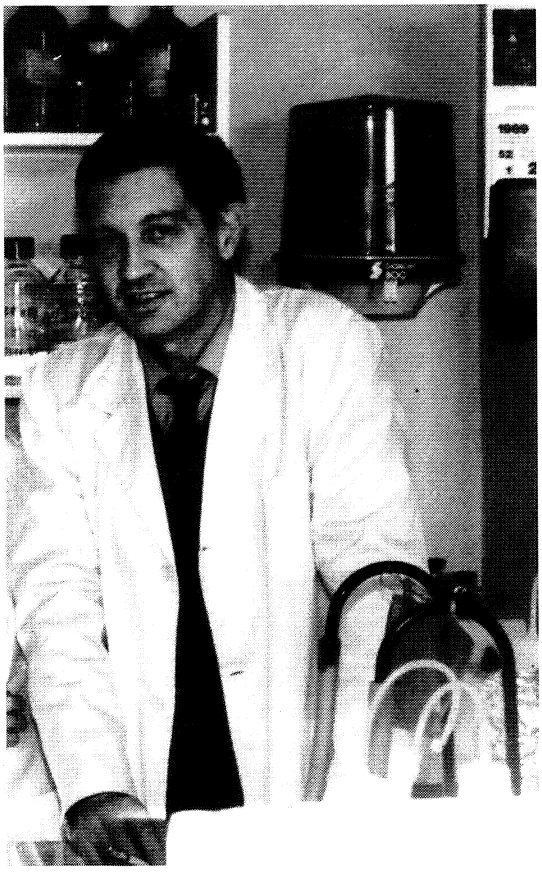

FIG. 1. José Luis Cánovas Palacio-Valdés, en su laboratorio del Centro de Investigaciones Biológicas, CSIC, en la calle Velázquez, 144, Madrid (hacia 1989).

maestros de la biología moderna: David Arnon, Roger Y. Stanier, Melvin Calvin, y otros. Bajo la dirección del Prof. Losada, extraordinario maestro, José Luis Cánovas inició su andadura por la investigación científica a través de su tesis doctoral,"Metabolismo del acetato y piruvato en bacterias y levaduras de panadería". Con ella obtuvo, en 1964, el título de Doctor en Farmacia, con Premio Extraordinario, y publicó varios trabajos en tres de las revistas internacionales más prestigiosas del momento: *Biochimica et Biophysica Acta*, *Biochemische Zeitschrift* y *Archives of Microbiology*.

A finales de 1964 José Luis Cánovas se traslada, como becario del British Council, a la University of Leicester, Gran Bretaña, a fin de ampliar su formación en el Departamento de Bioquímica, bajo la dirección del Prof. Hans L. Kornberg. José Luis Cánovas volvió a demostrar su valía científica personal; fruto de su estancia en Gran Bretaña fue la publicación, como primer autor, de dos trabajos sobre la fosfopiruvato carboxilasa, uno en *Biochimica et Biophysica Acta* y otro en *Proceedings of the Royal Society of London.*

De vuelta a España, recibe un contrato de la University of California, Berkeley, en calidad de "Assistant Research Bacteriologist", adscrito al Department of Bacteriology and Immunology, bajo la dirección del Prof. Stanier. Se va a California a principios de 1966, donde desarrolla una intensa actividad científica, fruto de la cual son cinco nuevos trabajos en los que figura como primer autor, uno en *Science*, tres en *European Journal of Biochemistry* y otro en *The Biochemical Journal.*

José Luis Cánovas vuelve a España a finales de 1967. Por entonces se le propone trasladarse al Instituto Pasteur, pero Cánovas no se decide por ir a París, aunque sí realiza una visita al Pasteur ese mismo año. Su idea era no ausentarse de España, sino trabajar en el recién creado Instituto de Biología Celular y volcarse en la investigación científica, para comunicar todo lo que había aprendido en el extranjero en pro de la ciencia española y en la formación de nuevos postgraduados de nuestro país...

...Y reinicia su labor en España. Ya desde 1965 era Ayudante científico, adscrito al Instituto de Edafología y Fisiología Vegetal. En 1967 fue nombrado Colaborador Científico en el Instituto de Biología Celular; en 1971, Investigador Científico; y en 1974, Profesor de Investigación adscrito a dicho Instituto. Durante estos años dirige varias tesis doctorales y colabora con los

Drs. Manuel Ruiz-Amil y Gertrudis de Torrontegui. Como todo investigador con ganas de trabajar, solicita ayudas económicas para realizar sus estudios. Unas veces, en España, con los fondos provenientes de entidades privadas: la División Farmacéutica Lepetit (los años 1968-1969) y la Fundación Rodríguez Pascual (los años 1975-1976). Otras veces, de organismos públicos, como el Fondo Nacional para el Desarrollo de la Investigación Científica y Técnica. Entre 1970 y 1991 esas diversas entidades patrocinaron nueve proyectos de investigación en los que participó. También recibió ayudas de instituciones extranjeras, como los National Institutes of Health (EE.UU.), entre 1971 y 1973.

Recibió en 1980 la encomienda de la Orden del Mérito Civil, ocupó diversos cargos en la dirección del Instituto de Biología Celular, así como en el organismo central del CSIC, alcanzando en 1978 la vicepresidencia del mismo. Científico de excepción, investigó durante una primera etapa (1963–1977) los organismos procariontes, preferentemente fisiología y bioquímica bacterianas con relación al análisis de rutas metabólicas. Entre 1977 y 1983 desarrolla su investigación sobre la proliferación celular en bacterias. Y en su última etapa (1983–1995), siguió estudiando el ciclo celular proliferativo en células eucariontes, contando en este período con la colaboración de Jorge Fernández López-Sáez, Aurora González Fernández, Matilde Hernández-Navarrete, Gonzalo Giménez Martín y Consuelo de la Torre y García Quintana.

La labor científica de José Luis Cánovas ha recibido justo reconocimiento, tanto en el ámbito nacional como en el internacional: miembro del jurado del Premio Bernardo Houssay, de la Organización de Estados Americanos; vocal de la European Science Foundation; vocal del Comité PNUD-Unesco; miembro electo de la International Cell Resarch Organization; miembro del Comité Planificador de la Academia Europea, de la que se le nombró Académico Fundador; miembro de la Real Academia de Farmacia de Madrid, etc.

Con el fallecimiento del Prof. José Luis Cánovas Palacio-Valdés la ciencia ha perdido un gran partícipe de la misma; sus amigos, un extraordinario cultivador de la amistad; y todos, un entrañable hombre bueno. Que Dios lo tenga en su gloria.

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MICROBIOLOGÍA SEM **12** (1996), 469–472 **PERSPECTIVAS**

Indicadores de la actividad científica*

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Entre los indicadores específicos de la actividad científica figuran los bibliométricos, que se basan principalmente en las características de su producto, que es claramente distinto de los bienes y servicios de carácter tecnológico, ya que solamente se realiza cuando una aportación al conocimiento se comunica a toda la comunidad científica mediante una publicación. No hace falta decir que deben ser manejados en estrecha relación con el resto de los indicadores sociales y económicos, así como sometidos a las mismas condiciones que éstos, en especial un riguroso examen crítico de su validez y fiabilidad. Consisten en datos numéricos acerca de los fenómenos sociales de producción, transmisión y consumo de la información en el seno de las comunidades científicas determinadas, teniendo en cuenta las funciones desempeñadas en ellas por los distintos tipos de publicaciones y los diversos patrones vigentes en cada una, sin referencia precisa a los cuales no pueden ser interpretados.

Todas estas condiciones son ignoradas por los recuentos acríticos de publicaciones manejados como puntuaciones supuestamente bibliométricas. El más difundido en la España de los últimos veinte años ha sido el llamado *factor de impacto*, resultante de contar el número de citas

* El presente texto corresponde a los tres últimos apartados del artículo "Una ciencia independiente", publicado por el autor en el número 241 de la revista *Historia 16* (pp. 183–192). Se publica aquí con el permiso del autor y de la revista citada. José M. López Piñero es catedrático de Historia de la Medicina de la Facultad de Medicina de la Universidad de Valencia y trabaja en el Instituto de Estudios Documentales e Históricos sobre la Ciencia, centro mixto del Consejo Superior de Investigaciones Científicas y de la Universidad de Valencia. Es ampliamente conocido en el ámbito nacional e internacional por sus trabajos sobre historia de la medicina y la biología. Ha desarrollado una extensa investigación acerca de la influencia del descubrimiento de América sobre la ciencia y la medicina europeas. También destacan sus estudios sobre científicos valencianos, particularmente del período de la Ilustración. Es autor y coautor de numerosos libros y artículos. Entre los primeros, podríamos destacar *Diccionario histórico de la ciencia moderna en España* (Península, 1983). Bajo su dirección se han formado numerosos investigadores que, a su vez, dirigen otros grupos de trabajo en diversas universidades y centros del CSIC.

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recibidas en las revistas incluidas en el repertorio *Science Citation Index*, del que resulta obligado ocuparse, debido a las cada vez más perniciosas consecuencias de su empleo. En diversas ocasiones hemos recordado que en los estudios sociales sobre la ciencia se ha abandonado por completo el modelo simplista acerca de las funciones desempeñadas por las citas en la comunicación científica que hace un tercio de siglo sirvió de fundamento al *Science Citation Index*. En el debate en torno a la cuestión, importantes especialistas han negado toda validez a los indicadores basados en las citas, aunque la mayoría considera que deben ser manejados con extrema cautela y después sometidos a rigurosas críticas en cada ocasión. Por otra parte, se ha demostrado que las deficiencias técnicas de este repertorio conducen a una proporción media de errores cercana al 25 por ciento. A lo sumo, el *factor de impacto* obtenido a partir del mismo es una tosca tasa de la repercusión en un conjunto de revistas fundamentalmente angloamericanas, que de ninguna manera es representativa de la comunidad científica internacional. Su manejo al margen de todo criterio, sin conocimiento de la estructura y la dinámica de esta última, es un lamentable ejemplo de lo que Pitirim Sorokin, el gran sociólogo de Harvard, denominó *cuantofrenia*.

Resulta alarmante que continúe utilizándose de este modo en nuestro país para la valoración de los aspirantes en concursos y oposiciones o para la del rendimiento del personal docente e investigador, como cómoda coartada que hace innecesarias las estimaciones de los especialistas de cada área científica, evitando el compromiso y los enfrentamientos que éstas plantean. Un hecho reciente refleja la gravedad de la desorientación: un interesante artículo sobre las penosas condiciones de servidumbre económica que debe cumplir una publicación periódica de un país *periférico* para ser incluida en el *Science Citation Index* ha sido complementado, en la versión castellana de una importante revista de síntesis, por una nota en la que un prestigioso bioquímico emite un diagnóstico de la actividad científica española exclusivamente basado en el mismo y con el tono terminante propio de la respuesta de un oráculo.

El uso generalizado de este *factor de impacto* se ha convertido en un poderoso estímulo para la aberrante conducta de acumular *papers* y en un grave peligro para el desarrollo normal del periodismo científico español. Su principal presupuesto básico es, en efecto, que debe publicarse exclusivamente en revistas extranjeras de elevado *factor de impacto* y, en consecuencia, que no son necesarias las revistas científicas españolas, cuya desaparición o marginación ha sido explícitamente propugnada. El triste final de la revista de Cajal durante la pasada década es todo un símbolo. Las razones que invalidan este presupuesto son evidentes. No puede ignorarse la subordinación a los intereses de las editoriales que publican las revistas extranjeras en cuestión y al poder de los *mandarines* académicos que las controlan, ni tampoco son vehículos adecuados para multitud de funciones, entre ellas, todas las relacionadas con las cuestiones científicas planteadas por nuestra sociedad. Por otro lado, ésta es una de las raíces de la degradación en España del castellano como lengua científica, en contraste con su creciente importancia internacional. Convendría difundir las denuncias, algunas serias y otras burlescas, que desde Latinoamérica y los propios Estados Unidos vienen a decir que el idioma de los científicos españoles actuales es un *spanglish* paralelo al de los grupos latinos marginales de las ciudades norteamericanas.

Ante la imposibilidad de exponer en este lugar un acercamiento bibliométrico sistemático a la actividad científica española de las dos últimas décadas, nos limitaremos a ofrecer a continuación algunos datos sencillos que pueden resultar orientadores.

El ámbito de las publicaciones científicas

Durante las dos últimas décadas, los libros y folletos de tema científico editados en España han pasado de cerca de cuatro mil a cifras en torno a seis mil títulos anuales. En relación con los publicados sobre estas materias, han oscilado entre el diez y el quince por ciento, porcentajes semejantes a los de países como Francia, Italia, Alemania, Holanda y Estados Unidos, algo inferiores a los de Gran Bretaña, Japón y Suecia, y muy por debajo de los de Suiza, Dinamarca y Finlandia. Por razones obvias, la comparación con los que tenían regímenes comunistas no pueden hacerse directamente.

Varios patrones se han mantenido con escasas oscilaciones a lo largo de estos veinte años. En la distribución por áreas de dichos títulos, una cuarta parte ha correspondido a las ciencias de la naturaleza, otra a las ciencias médicas y otra a la ingeniería: la restante se ha dividido entre las matemáticas y la agronomía, en proporciones que ahora son semejantes debido al crecimiento de los títulos agronómicos. Los manuales didácticos han significado aproximadamente el quince por ciento del conjunto, aunque con diferencias muy acusadas por áreas, ya que han sido la mitad en matemáticas y porcentajes inferiores al dos por ciento en medicina y agronomía. Los textos infantiles y juveniles apenas superan el uno por ciento, proporción muy inferior al de los países más avanzados de la Unión Europea. Los libros traducidos han sumado en torno a un diez por ciento en todas las áreas, con la excepción de las matemáticas, en las que no llegan a cinco, y las ciencias médicas, en las que se duplica dicho porcentaje.

Las revistas circulantes en los repertorios y bases de datos nacionales de bibliografía científica no han aumentado de modo significativo a lo largo del período, oscilando en torno a trescientas, de las cuales más del cuarenta por ciento están dedicadas a la medicina. Tampoco lo han hecho las incluidas en los internacionales, que han sido aproximadamente la mitad de las anteriores. Ello contrasta con el notable crecimiento que, como vamos a ver, ha experimentado el número de trabajos científicos españoles circulantes a nivel internacional. La debilidad del periodismo científico español se manifiesta en su baja tasa de revistas de larga pervivencia, con una proporción desmesurada de las de aparición prácticamente ocasional, y sobre todo en su distribución por instituciones editoras.

Dicha distribución constituye uno de los desajustes más evidentes del panorama que estamos considerando. Las universidades y el Consejo Superior de Investigaciones Científicas, de donde proceden más de tres cuartas partes de la producción científica española, como anotaremos a continuación, publican solamente una cuarta parte de las revistas circulantes tanto nacional como internacionalmente. Por el contrario, las asociaciones profesionales y las editoriales privadas publican más del sesenta por ciento de ambas. Esta situación no se produce en ningún otro país de rango científico. Además, hay que tener en cuenta que en su inmensa mayoría las editoriales comerciales son multinacionales.

Aumento de la actividad científica

Como hemos adelantado, los trabajos científicos procedentes de las instituciones españolas incluidos en repertorios y bases de datos nacionales e internacionales han experimentado globalmente un notable crecimiento a lo largo de las dos últimas décadas. En los nacionales casi se han duplicado, pasando de cifras inferiores a ocho mil anuales a más de quince mil. En los internacionales, el crecimiento ha sido desigual en las distintas áreas. El más reducido ha corres-

pondido a las ciencias médicas, cuyos trabajos solamente han aumentado en torno a un cincuenta por ciento, mientras que los de las ciencias químicas se han multiplicado por tres y los de ingeniería, por más de cuatro. En su conjunto, la producción española ha pasado a significar entre el uno y el uno y medio por ciento de la circulante en la comunidad científica internacional, que supera el millón de trabajos anuales.

Este crecimiento no tiene una interpretación sencilla, aunque de forma esquemática puede decirse que se debe principalmente al aumento efectivo del rendimiento de la actividad científica española. Sin embargo, conviene añadir que también se ha incrementado la proporción de trabajos publicados en revistas extranjeras. Entre los circulantes internacionalmente, dicha proporción no llega a la mitad de las ciencias médicas y agrarias, oscila alrededor del sesenta por ciento en las químicas y se acerca al ochenta en las físicas, pero es superior al noventa y cinco en las biológicas y la ingeniería, lo que constituye otra señal de la debilidad del periodismo científico español.

En la distribución de dichos trabajos por instituciones, las universidades suman más de la mitad y el Consejo Superior de Investigaciones Científicas, una cuarta parte, seguidos de los hospitales de la Seguridad Social, de los que procede en torno al treinta por ciento de los dedicados a las ciencias médicas. En la de los circulantes a través de repertorios y bases de datos nacionales, la participación del Consejo desciende por debajo del diez por ciento. Ambas distribuciones muestran una acusada centralización de la producción científica en Madrid y Barcelona, que reúnen la mitad de los trabajos.

Los datos cuantitativos disponibles acerca del consumo de la información científica son los relativos a las ciencias médicas, obtenidos a través de las referencias bibliográficas de los trabajos publicados en las revistas españolas del área. El índice de aislamiento (porcentaje dedicado a publicaciones nacionales) oscila alrededor del quince por ciento, resultando adecuado para un país de producción científica de segundo rango. También se ajusta a los patrones normales el hecho de que ascienda a cifras cercanas al cincuenta por ciento en las revistas de atención primaria.

Por el contrario, la distribución por países de dichas referencias refleja una situación anormal, ya que Estados Unidos suma más del cincuenta por ciento de las mismas, porcentaje que casi duplica el que corresponde a las publicaciones norteamericanas en el conjunto de las circulantes en la comunidad médica internacional que utiliza el inglés como *lingua franca*. Esta desmesurada proporción, que supera incluso en torno a diez puntos la que Estados Unidos tiene en las referencias bibliográficas de las revistas médicas británicas, se produce a costa de que los porcentajes de Alemania, Francia, Suiza, Italia, Países Bajos, etcétera sean muy inferiores al peso de estos países en la literatura médica del mundo occidental, a un aislamiento casi completo de la importante producción japonesa y a la práctica ausencia de la europea oriental y de la latinoamericana. La desconexión con los países americanos de lengua castellana es un fenómeno relativamente reciente. Resulta paradójico que se produzca en un período de creciente importancia del idioma, a pesar de la retórica hermandad y de las diversas iniciativas de cooperación existentes en todos los órdenes.

MICROBIOLOGÍA SEM **12** (1996), 473–475 **OPINION**

Help! Latin! How to avoid the most common mistakes while giving Latin names to newly discovered prokaryotes

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Most of the mistakes commonly made in naming newly discovered species (and genera) of prokaryotes (i.e. Bacteria and Archaea/ Eubacteria and Archaeobacteria) of transferring species from one to another genus can be avoided by following this advice:

(i) Gender of specific epithet

A common mistake is that, while forming new combinations (*nov. comb.*) by transferring a species to another genus, authors forget to change the gender of the specific epithet if that of the accepting genus differs from that of the former genus. The epithet has to follow the genus in gender!

Adjectival endings (suffixes) in Latin are in Table 1.

Hypothetical examples: If the species "*Blocus limosus*" would be transferred to the genus "*Leucobacterium*", it would become "*L. limosum*" (*nov. comb.*). If "*Ercobacter mobilis*" would be changed to "*Aurigenium*", it would have to become "*A. mobile*".

Note that names ending on *-bacter* are treated as masculine!

TABLE 1

Masculine (m.)	Feminine (f.)	Neuter (n.)	Example*
-us	-a	-um	albus, alba, album
-is	-is	-e	facilis, facilis, facile
-er	-eris	-ere	celer, celeris, celere

*Meaning: white, easy, rapid.

(ii) Genus names formed from personal names

These may be formed directly, or, alternatively, as a Latin diminutive, and are always feminine. Such Latin names depend on the ending of the respective personal name. Proceed according to Table 2.

Note that for names ending on -e and on -o different alternatives have been—and thus must be—used!

Some personal names in Europe were already latinized in the 18th century and earlier. If they end on -us, replace -us by -a or by -ella (diminu-

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TABLE 2

Personal name ending on	Add ending	Person	Example	Diminutive ending
Any consonant	-ia	Farmer	*Farmeria*	-ella or -iella
-a	-ea	Rochalima	*Rochalimaea*	Skip a, add -ella
-e	-ia	Burke	*Burkeia*	-lla
-e	-a	Benecke	*Beneckea*	-lla
-i	-a	Nevski	*Nevskia*	-ella
-o	-nia	Cato	"*Catonia*"	-nella
-o	-a	Beggiato	*Beggiatoa*	-nella
-u	-ia	Mbutu	"*Mbutuia*"	-ella
-y	-a	Deley	*Deleya*	-ella

tive form). Example: personal name Bucerius, organism's name "*Buceria*" or "*Buceriella*".

Examples for diminutive names are, e.g. *Salmonella*, *Klebsiella*, *Shigella*, "*Catonella*", "*Mbutuella*", "*Deleyella*".

(iii) Specific epithets formed from personal names

In principle there are two possibilities to proceed in either choosing the adjectival form (a) or the substantival form (b):

(a) Latinize the personal name according to column 2 of the preceding table and add the ending -nus (m.), -na (f.) -num (n.) according to the gender of the genus name. Thus you have formed an adjective that has the meaning of "pertaining, belonging to the person...".

(b) Latinize the name according to the sex of the person to be honored and form the genitive. To do this correctly, use the advice given in Table 3.

The problem with names ending on -a is that they may be latinized in four different ways (for example: the name MacKenna):

(i) Treat MacKenna as if it were a classical Latin name like Seneca. Then it follows the a-declination, and the genitive for Ms. or Mr. MacKenna would give the same specific epithet, namely *mackennae*, meaning "of MacKenna".

(ii) The other three possibilities allow to recognize the sex of the person the new organism is to be named after. Mr. MacKenna is latinized

TABLE 3

Name ending	Add for female person	Add for male person
-a	-e, -eae, or -iae	-e (classic), -i, -ei, or -ii
-e	-ae	-i
-i	-ae	-i
-o	-niae	-nis (classic) or -nii
-u	-iae	-ii
-y	-ae	-i
-er	-ae	-i
Any other	-iae	-ii

to Mackennaus, which results in the specific epithet *mackennaei* (only m.)

(iii) The name is latinized to Mackennaeus (m.) (like Linnaeus) or Mackennaea (f.) with the consequence that the specific epithet is *mackennaei* (m.) or *mackennaeae* (f.), respectively.

(iv) The name is latinized to Mackennaius (m.) or Mackennaia (f.) so that the specific epithet would be *mackennaii* (m.) or *mackennaiae* (f.), respectively.

The reader will understand that the latter two possibilities, although permissible, look and sound rather awkward and are due to produce a lot of misspellings. Therefore I strongly recommend to use the first two versions only.

Examples (partly hypothetical) for epithets derived from personal names are: *smithii, maxwelliae, ottonis, ottonii, catoniae, novyi, daleyae, nealsonii, verdii, milleri, carpenterae, micdadei, postgatei, stanieri, dorotheae.*

(iv) Specific epithets derived from names of localities

These are used to indicate the place of origin or occurrence of organisms. They are constructed by adding to the locality's name the ending *-ensis* (m. or f.) or *-ense* (n.) in agreement with the gender of the genus name. Only if the name of the locality ends on -a or -e, these vowels are dropped before the addition of *-ensis/-ense*. Examples: *Thiospirillum jenense* (from Jena).

Do not form epithets by use of the latinized locality's name in the genitive! (e.g. *londoni* instead of the correct *londonensis*).

Exceptions are those cases where there exist regular Latin adjectives that have been in used for countries, continents, rivers, cities, etc. at least since the middle ages, such as: *europaeus, africanus, asiaticus, americanus, italicus, romanus* (Rome), *germanicus, britannicus, gallicus, polonicus, hungaricus, graecus, hispanicus, rhenanus* (Rhine), *frisius, saxonicus, bavaricus, bohemicus, mediterraneus* (Mediterranean Sea), etc...

(v) Names combined from words of Greek and Latin origin

When names are combined from two or more words of Greek or/and Latin origin, there is an easy rule to follow:

a) If the first compound is Latin, the connecting vowel is an *-i-*, no matter whether a Greek or Latin compounds follows.

b) If the first compound is Greek, the connecting vowel is an *-o-*, no matter whether a Greek or Latin compound follows.

c) If the second compounds starts with a vowel, no extra connecting vowel is required.

Examples:

a) *rectivirgula, lactilyticus, avipneumoniae, omnivorans, Aquifex.*

b) *Halobacterium, chromofuscus, Pseudomonas, Leuconostoc.*

c) *acetoxidans, salexigens.*

General advice: Consult the **International Code of Nomenclature of Bacteria (1990 Revision)**, published in 1992 by the American Society for Microbiology, 1325 Massachusetts Ave. N. W., Washington, D. C. 20005, USA.

This consists of the Code itself plus 10 Appendices, of which especially Appendix 9 relates to the matters listed above.

If you really get a problem with a name, send me a fax (+49-228-737576). I promise to help.

V ENAMA / I Encontro Nordestino de Microbiologia Ambiental

Fortaleza - Ceará (Brasil) 2–5 de diciembre de 1996

Del 2 al 5 de diciembre de 1996 tendrá lugar en Fortaleza-Ceará (Brasil), el V ENAMA / I Encontro Nordestino de Microbiologia Ambiental. El congreso está patrocinado por las Universidades Federales de Ceará y de Paraíba, y contará con la participación como conferenciantes invitados de:

—Erko Stackebrandt, Deutsche Sammlung von Mikroorganismen, Braunschweig, Alemania
—Takor Patel, Memorial University Newfoundland, Canadá
—Ricard Guerrero, Universidad de Barcelona, España
—Johana Dobereiner, URRJ-EMBRAPA, Brasil
—Jan Dirk van Elsas, Institute for Soil Fertility, Holanda

Se organizarán sesiones de pósters sobre microbiología acuática y microbiología terrestre, así como mesas redondas y simposios sobre diferentes aspectos de la microbiología ambiental. Entre ellos:

—Biotecnología Ambiental. Coord: Rosana F. Vazoller, UFSCarlos, Brasil
—Ecosistemas Acuáticos. Coord: Victoriano Campos, Univ. de Valparaíso, Chile
—I Encontro Regional de Microbiol. Ambiental. Coord.: José Siqueira Jr., UFPb, Brasil
—Ecosistemas Terrestres. Coord: Allen Norton Hagler, UFRJ, Brasil

Los días 2, 3 y 4 de diciembre se celebrarán reuniones de trabajo que estarán coordinadas por diferentes especialistas, tanto invitados como de la organización del congreso: Biotecnología Ambiental (P. Sánchez, Brasil), Ecosistemas Acuáticos (R. B. de Oliveira, Brasil), Ecosistemas Terrestres (M. A. Rezende, Brasil).

Puede solicitarse información a:

Profa. Dra. Regine H. S. dos Fernandes Vieira
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MICROBIOLOGÍA SEM **12** (1996), 477–480 **OPINION**

The ins and outs of scientific writing

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Scientific writing is an integral part of any scientific career. Proposals must be written to secure research funding, then progress reports must be written to maintain that funding and, finally final reports must be written to present the results. A professional scientist's writing obligations do not diminish as his or her career advances; in fact, they increase. It has been calculated that research scientists spend at least one-third of their time at their desks writing. Because very few European institutions of higher learning offer courses in technical writing, many scientists never have the opportunity to learn that scientific, or technical, writing is a genre in itself with its own idiosyncrasies.

Scientific writing is not easy; there are no shortcuts. Like all effective writing, it is hard work. Even the best writers, writing in their own language, struggle with every paragraph, every sentence, and every phrase. They write and rewrite. The task becomes even more difficult for non-natives. Styles change. What is good scientific discourse in certain societies is not necessarily considered good style by other scientific communities. Times change. What was acceptable one hundred years ago is not considered strong scientific writing today. Because 80% of all scientific writing today is done in English, this paper will try to highlight some of the problems that arise and will try to help you to make your writing more effective. It will center around two basic difficulties which must be faced when writing an article: language and structure.

Communicating results is just as important as obtaining results. While most scientists find criticism to their theories and experiments challenging, they become defensive when they are asked to rewrite their reports. The time and care spent on the research is seldom reflected in the writing. Obviously the intellectual demands for writing a scientific paper are not, and never will be, those for splicing genes, nonetheless much scientific effort can be wasted if those who must read about it find the reading tedious. The general attitude that if scientists could write they would be writers, and not scientists, is an excuse no scientist who considers himself or herself a professional can afford. Hopefully the brief guidelines that follow will be of help the next time you sit down to write a scientific article.

Language

Scientific language is precise, clear and familiar. It should be straightforward and concise. When you are clear and simple, conciseness follows;

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when you are precise and familiar, clarity follows. Clarity is the most important quality of good writing, and clarity is largely a result of proper organization. Being precise, on the other hand, does not mean presenting every single detail; it means selecting only the relevant details.

Always keep your reader in mind. Do not overburden him or her with non relevant minutia. The purpose of scientific writing is to inform, not to impress. Keep your sentences simple. Prepositional phrases are important because they provide transition for incorporating details, but too many of them can confuse the reader. Abstract nouns that precede the word «of» should also be avoided. Instead of saying, for example, «it is used for the detection of» say «it detects», or instead of «it is for the production of» say «it produces», or instead of «it has a tendency to» say «it tends to». These wordy constructions not only waste reading time, but also attract superfluous words that cloud up important details.

Another misconception is the exaggerated use of passive verbs, Latin based words, and verb phrases (not phrasal verbs). That type of writing belongs to the formal style of the 17th century. It weakens scientific writing. Winston Churchill once said: Short words are the best and old words when short are the best of all.

Active verbs are much more forceful than passive verbs. Do not be afraid to use «I» or «we». After all, you did the research, not some unimportant, invisible, or unknown agent, and you deserve the recognition. Why then recur to a sentence structure that focuses on the predicate at the expense of the agent? The preference for passive verbs over active verbs has its parallel with the preference for Latin-based words over simple Anglo-Saxon words. Three or four syllable Latin-based words do not make your writing sound more scholarly; in any case, they may make it sound somewhat pretentious, which is something you really want to avoid. Contrary to what many non-natives think, using «start» instead of «activate», «part» instead of «component», «made up of» instead of «consists of», «show» instead of «demonstrate» or «use» instead of «utilize» in no way downgrades their writing; it may, in fact, upgrade it in the eyes of the modern reader.

Two final suggestions to avoid wordiness include being aware of redundancies and staying away from needless verb phrases. By redundancy we mean the needless repetition of words that add nothing to the meaning of a main word. Examples of redundancies in scientific writing are «already existing», «a blue color», «still persists», «continue to remain», «mix together», and so on. Simply say «existing», «blue», «persists», «remain», or «mix». Examples of verb phrases to be avoided in good scientific writing are «make a decision», «make a suggestion», «be capable of», etc. These should be reduced to «decide», «suggest» or «can».

Structure

Just as choice of vocabulary is important for efficient communication, so is the way in which you structure your paper or report. Structuring means much more that organizing the paper into the basic sections of Summary, Introduction, Materials and Methods, Results, Discussion, and Conclusions. It involves developing an effective strategy, deciding what to put in and what to leave out. These decisions will obviously depend on the type of communication, the type of research, and the particular audience it is addressed to. Nevertheless, the presentation must follow some predevised plan.

The Summary

The summary is the single most important section in a scientific paper. It tells what the re-

search is about; it can either state the results of the research or it can state the questions the research answers. While the title tells the reader what the basic topic is about, the summary supplies the details that do not fit in the title. It gives the reader just enough information to know whether he or she wants to go on reading.

Some scientists are a bit reluctant to summarize all their work in just a few sentences at the very beginning. They feel people may not take the trouble to read through the paper. This is not true. A scientific paper is not like a mystery story. The summary is not to entice all audiences to read to the end, but to entice interested audiences to continue reading. Once they know what happened interested audiences will want to continue reading to learn how it happened.

The Introduction

The introduction gives details about the research that could not fit in the title or in the summary. A strong introduction tells the reader why the research is/was important. It is a mistake to assume that the reader will implicitly understand the importance of the research just from the title. Background information is important. The amount of background information will depend on the audience addressed. References to other papers not only provides background information but also shows where your research stands with relation to other research in the same field.

The introduction is also the place where the reader will find information concerning the basic criteria, or steps, followed in the discussion of the research. How is it presented? Is there a sequenced order in the presentation? It helps the reader feel comfortable to know from the start what direction the discussion will take. It also shows that there is a preconceived strategy, or plan, in the presentation. The last thing you want is for your reader, or reviewer, to get lost!

Material and Methods

This section, also referred to by some authors as the *scope* of the report (Blicq, 1981), defines the techniques used. It describes the area covered and outlines the method of investigation used. Should a glossary of terms be required, it would probably appear separately in this section. Methods must be clearly described, so that other researchers can repeat them and obtain the same results.

The Results

The Results section of your report should not be bulky. Only the main facts and figures should appear here. The details of these findings will either appear in the Discussion or in appendices. A clear report needs carefully selected facts. Remember, the main objective of your report is not to overwhelm the reader but to enlighten the reader.

Because the results of the research have already been stated briefly in the Summary and will again be dealt with extensively in the Discussion, some writers prefer not to repeat them in a separate section. This will, of course, depend on the how important they are to the overall report. In any case, unnecessary repetition should be avoided.

The Discussion

The Discussion is usually the longest part of a scientific report. It includes as much evidence as the reader will need to understand the subject: facts, arguments, details, and results. In the Discussion section the reader expects to find an explanation of how the results were (or will be) obtained. In other words, what the writer(s) set out to do, how he/she went about it, and what was actually done or found out as a result of this effort.

Because this section includes a number of details it is important to present these details in an organized, logical order to avoid confusing the reader. There are times, however, when the material cannot be easily sequenced. In that case, it might be convenient to break the material down into subsections according to importance. Subtitles can act as markers, facilitating reading. When introducing subtitles, however, it is important to differentiate them from the main titles. Usually the titles of the basic parts (Introduction, Discussion, etc.) are centered and in capital letters while the subtitles begin at the left margin and are often in bold type. This of course may vary according to publishing norms.

The organization can be chronological or spatial, there are no set rules. Other organizing techniques are subject development or concept development. Reports using the chronological or subject method are usually more straightforward and allow less room for imaginative development. A strategy based on concept development is best when the writer wants to include persuasive arguments. Whatever the strategy, it is important for the reader to feel comfortable, to know where he or she is at. Your writing should move from sentence to sentence, paragraph to paragraph, without making the reader trip over words or ideas. Not only must your language be fluid, but your ideas must also be fluid!

The Conclusions

The conclusions bring together the loose ends of your research. They convey a sense of completion in that they view the results from an overall perspective. The conclusions are very similar both in length and in content to the summary. The most important aspects of your work are repeated, together with your personal conclusions. Recommendations for further research also belong in this section.

* * * * *

Although this article is not, properly speaking, a scientific report and must not necessarily conform to the norms, I would nevertheless like to conclude with a recommendation. It would be extremely useful for our science students if courses on scientific writing, and specialized language courses as well, were offered as electives of our universities. As I have tried to point out, scientific writing is much more than putting words on paper. You can not always blame the translator when you are asked to rewrite a paper; you can not blame yourself either if you did the best you could. A course on the basics of scientific writing could have solved the problem. Remember, good scientific writing is a craft not a gift; it is a skill that people develop through much practice.

Some books on scientific writing

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

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

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

Cash, P. (1988). How to Develop and Write a Research Paper. Prentice-Hall.

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

Jordan, R. R. (1989). Academic Writing Course. Collins.

Lee, M., Stephenson, G., Anderson, M., Lee, L. A. (1990). The Handbook of Technical Writing: Form and Style. Harcourt Brace Jovanovich.

Leki, L. (1988). Academic Writing: Techniques and Tasks. Macmillan.

Master, P. A. (1986). Science, Medicine and Technology: English Grammar and Technical Writing. Prentice-Hall, 1986

Miles, J. L., Bush, D. W., Kaplan, A. (1982). Technical Writing: Principles and Practice Science Research Associates.

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

Swales, J. (1971). Writing Scientific English. Nelson.

Turk, C., Kirkman, G. (1989). Effective Writing. E and FN Spon.

MICROBIOLOGÍA SEM **12** (1996), 481–484 **OPINIÓN**

Internet para microbiólogos

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El crecimiento de las redes de comunicación informática, la más conocida de las cuales es Internet, ha repercutido de manera notable en la sociedad en general y en la comunidad científica en particular. Al mismo tiempo se ha producido, en primer lugar, un aumento exponencial del número de secuencias de ácidos nucleicos y proteínas acumulados en bases de datos genéticos (GenBank, EMBL, SwissProt y otras) y, en segundo lugar, la disponibilidad de herramientas informáticas capaces de extraer información significativa de las meras secuencias de bases (DNA o RNA), o de aminoácidos, y de interaccionar eficiente y rápidamente con bases de datos genéticos de gran volumen. Todo ello ha determinado la apertura de nuevas vías de acceso a la información y de investigación en Biología Molecular y en Microbiología, a partir del análisis de la información depositada en dichas bases de datos.

La Tabla 1 presenta un listado de servidores de correo electrónico de interés para el microbiólogo. Las instituciones que disponen de servidores de correo electrónico pueden ofrecer información (bases de datos, programas,...) mediante un ftp anónimo (file transfer protocol), usando "anonymous" como "login name" y la dirección electrónica de uno mismo como "password". El primer paso para usar un servidor es obtener la información necesaria sobre cómo redactar el mensaje de correo electrónico y qué posibilidades ofrece el servidor; esto se consigue enviando un mensaje al servidor que contenga sólo la palabra "help" en el cuerpo del mensaje. El servidor enviará un mensaje (a veces de muchas páginas) con la información concerniente al formato y uso que permite dicho servidor. Las funciones de búsqueda de homologías de algunos servidores enunciados en la Tabla 1 pueden ser similares en algunos aspectos, pero los fundamentos matemáticos de la búsqueda pueden diferir sustancialmente; esto hace que los resultados de las búsquedas producidos por los distintos servidores puedan ser diferentes.

Un aspecto a considerar es la selección del banco de datos genéticos sobre el que se realizará la búsqueda, si se utilizará la secuencia de bases, o alguna pauta de lectura abierta en ella contenida, o todas la pautas de lectura posibles. En el caso de búsqueda de homologías de proteínas se ha de considerar que no todas las proteínas que pueden ser codificadas por las secuencias

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TABLA 1. Servidores de correo electrónico y ftp anónimos de utilidad para el microbiólogo

Servidor	Funciones
blast@ncbi.nlm.nih.gov	— Búsqueda rápida y detallada de homologías en secuencias de DNA o proteínas — Diferentes programas: blast, tblastn, blastn, blastx — Ofrece la posibilidad de búsqueda en el banco nr, "non redundant"
retrieve@ncbi.nlm.nih.gov	— Obtención de secuencias de DNA y proteínas a partir de su número
FASTA@EBI.AC.UK	— Búsqueda de homologías en secuencias de DNA y proteínas
NETSERV@EBI.AC.UK	— Obtención de secuencias de DNA y proteínas a partir de su nombre o número — Ofrece "software" público
QUICK@EBI.AC.UK	— Búsqueda de homologías en secuencias de DNA. Más rapido que FASTA
BLITZ@EBI.AC.UK	— Búsqueda de homologías de secuencias de proteínas
PHD@embl-heidelberg.de	— "PredictProtein" extrae características bioquímicas y estructura 2a. de una proteína. No produce resultados gráficos
Tmap@embl-heidelberg.de	— Predicción de segmentos transmembrana en proteínas
ftp ncbi.nlm.nih.gov	/repository — EcoSeq y EcoMap, secuencias y mapas de *E. coli* — Rebase, enzimas de restricción /toolbox — Programas de NCBI /pub — Programas públicos: BLAST, MACAW (alineamiento múltiple)

acumuladas en EMBL o GenBank se encuentran registradas en bancos de secuencias de proteínas como SwissProt. Un planteamiento incompleto de la búsqueda puede no detectar información sustancial que aparecería con un planteamiento diferente, por lo que es conveniente usar varios servidores y varias estrategias de búsqueda.

El servidor BLAST permite realizar muchos tipos de comparaciones mediante diferentes programas, tanto para ácidos nucleicos como para proteínas, frente a diferentes bases de datos: GenBank, EMBL, SwissProt y la base de datos nr (non redundant) resultante de la traducción en las 6 pautas de lectura de las secuencias de GenBanK-EMBL. El servidor FASTA realiza comparaciones de una secuencia (DNA o proteína) frente a varias bases de datos. El servidor BLITZ realiza una comparación rápida de una secuencia de aminoácidos frente a la base de datos SwissProt. El servidor QUICK realiza una comparación rápida de una secuencia de bases (DNA) frente a GenBank-EMBL. El servidor PHD (Predict Protein) realiza un análisis de las características bioquímicas de una secuencia de aminoácidos. El servidor RETRIEVE obtiene secuencias de las distintas bases de datos en NCBI (EMBL, GenBank, SwissProt, PIR y otras) a partir de una palabra clave, número o nombre de la secuencia.

Para algunas tareas es de utilidad disponer en ordenadores locales de programas adecuados que permitan extraer, editar y presentar de diferentes maneras la información contenida de una forma implícita en la secuencias. Uno de los paquetes informáticos más conocido y completo para realizar estos trabajos es el GCG de Genetics Computer Group, que requiere licencia de uso. El paquete de programas Staden es de utilidad en grandes proyectos de secuenciación (Tabla 2). Merece la pena considerar que un conoci-

TABLA 2. Servidores www de utilidad para el microbiólogo

Servidor	Funciones
http://www.embl-heidelberg.de	— Servicios de información biológica
	— Servicios computacionales
	— Supercomputación en Biologia Molecular
http://www.ebi.ac.uk	— Búsqueda de similitudes y obtención de secuencias
	— Entrada de nuevas secuencias
	— Acceso a EMBL y SwissProt
	— Catálogo de programas
http://www.ncbi.nlm.nih.gov	— Búsquedas en GenBank y otros bancos
	— Acceso FTP
	— BankIt: entrada de nuevas secuencias en GenBank
http://www3.ncbi.nlm.nih.gov	— Entrez: programa de búsqueda bibliográfica y de secuencias asociadas
http://kiwi.imgen.bcm.tmc.edu	— Diferentes programas de búsqueda en bancos
	— Alineamiento de secuencias y otras utilidades
	— Predicción de estructura secundaria en proteínas
http://dot.imgen.bcm.tmc.edu	— Diferentes búsquedas de homologías de proteínas en diferentes bases de datos, entre ellas nr
http://cbrg.inf.ethz.ch	— Acceso al banco de secuencias de proteínas Swissprot
	— Programa "predict genes"
	— Programa "Darwin" de análisis de DNA y proteínas
http://cgsc.biology.yale.edu	— Base de datos de cepas, genes, mutaciones, mapas *E. coli*
http://www.atcc.org	— Material biológico y otros recursos relacionados
http://expasy.hcuge.ch	— Predicción de estructura secundaria de proteínas, entre otros servicios
http://www.pdb.bnl.gov	— Base de datos de estructuras 3D de biomoléculas
http://www.gcg.com	— Paquete de programas GCG de análisis de secuencias de DNA, RNA y proteínas (requiere licencia)
http://www.mrc-lmb.cam.ac.uk	— Paquete de programas de R. Staden para proyectos de secuenciación
http://www.bio.net	— Grupos BIOSCI

miento básico del sistema operativo UNIX facilita el uso de los servidores de correo electrónico y de los paquetes informáticos como GCG.

Además de los servidores de correo electrónico, y el protocolo FTP, Internet ofrece otras vías y servicios de utilidad para la investigación: la WWW (World Wide Web) es una red de servidores en Internet que permiten su uso interactivo, de una forma dialogada (a diferencia de los servidores de correo electrónico). En la profusión de servidores que pueden contactarse por WWW, algunos son de especial interés para un laboratorio de Microbiología (Tabla 2). Destacan entre estos los servidores WWW del EMBL (Laboratorio Europeo de Biología Molecular), situado en Heidelberg (Alemania) y en Hinxton (Inglaterra), y los servidores del NCBI (National Center for Biotechnology Information, EE.UU.). Los servidores de modelado de proteína (por ejemplo, http://expasy.hcuge.ch) basan su utilidad en el conocimiento previo, obtenido experimentalmente, de la estructura secundaria de aquellas proteínas que presenten homología con la proteína de interés. Las posibilidades conjuntas de los servidores presentados en la Tabla 2 son muy extensas, por lo que la mejor forma de conocerlos es consultar con atención sus primeras páginas en la red.

El servidor de WWW "entrez" depende del NCBI. Se trata de un servidor de búsqueda de referencias en bases de datos bibliográficos (MEDLINE, National Library of Medicine's database of biomedical articles), que, al mismo tiempo, se cruzan con aquellas secuencias de DNA o proteínas asociadas a dichas referencias bibliográficas, contenidas en cualquiera de los bancos de secuencias existentes en NCBI. El servidor ofrece una gran diversidad en cuanto al campo de búsqueda de palabras clave (texto, autor, revista,...).

El servidor del "*E. coli* Genetic Stock Center", contiene la gran acumulación de datos genéticos de *Escherichia coli* disponibles, organizados en una forma fácilmente accesible. Permite también la solicitud de cepas directamente a través de WWW. Un servidor similar es el de la ATCC (American Type Culture Collection), que cubre un rango mucho más amplio de materiales biológicos, entre ellos las colecciones microbiológicas mantenidas en dicha institución.

Otro de los servicios gratuitos que ofrece Internet al microbiólogo es la posibilidad de mantenerse informado y en contacto con otros investigadores: la red BIOSCI es un foro de discusión, un entramado de grupos de noticias, cada uno de ellos sobre un tema específico, accesibles localmente si se dispone de "software" adecuado (nn, rn), o distribuidos mediante correo electrónico a cada uno de los suscriptores, y donde cualquiera de ellos puede participar. Entre los temas que son tratados en grupos BIOSCI se encuentran: "Métodos y reactivos", "Microbiología", "Biotécnicas" y muchísimos otros. Para recibir información de cómo acceder a los grupos BIOSCI, simplemente debe enviarse un "e-mail" con las palabras "info ukinfo" (desde España) en el cuerpo del mensaje al servidor biosci-server@net.bio.net. Desde América, las palabras deben ser "info usinfo". Existe un servidor WWW para los grupos BIOSCI (Tabla 2).

Las herramientas mencionadas permiten establecer relaciones taxonómicas a partir de diferencias y similitudes en secuencias de RNA ribosómico, de DNA o de proteínas. Pueden también deducirse las frecuencias de uso de codones en una determinada especie, y a partir de ellas la existencia de genes y su nivel de expresión (Borodovsky, M. et al., 1995, Nuc. Acids Res. **23**, 3554–3562), identificar nuevos genes por homología (Nieto, J. M. y Juárez, A., 1996, Mol. Microbiol. **19**, 407), y hallar secuencias consenso en promotores (Lisser, S. y Margalit, H., 1993, Nuc. Acids Res. **21**, 1507–1516) de unión a ribosomas o a diferentes factores transcripcionales, por citar sólo algunos ejemplos. La sección "computer corner" de la revista *TIBS* y la revista *Nucleic Acids Research* tratan frecuentemente temas relacionados con los expuestos en este artículo.

Finalmente, aunque el potencial de acceso y análisis de la información que se abre con estas nuevas herramientas informáticas es enorme, es bueno recordar que se trata sólo de eso, herramientas, y que su uso debe ser supeditado al conocimiento de la Microbiología y al buen sentido del investigador que se sirve de ellas.

MICROBIOLOGÍA SEM **12** (1996), 485–501

Revisión de libros

Microbiología y genética molecular

Josep Casadesús (ed.)

Coedición de la Sociedad Española de Microbiología y la Universidad de Huelva.
Publicaciones de la Universidad de Huelva, Huelva, 1995. 2 volúmenes. 898 pp.
Precio: 6.000 PTA. ISBN 84-88751-24-9.

La crítica de una obra como la que nos ocupa resulta, para quien la emprende, una tarea sencilla, porque todos los aspectos en los que se quiere incidir al comentarla están relacionados con la utilidad que resulta de su lectura y uso. Entre esos aspectos hay algunos mejorables, pero vaya por delante que se trata de un excelente libro, con muchas virtudes y algún defecto.

En relación con esto último, me referiré en primer lugar al título. Considero que induce a confusión, al menos, cuando lo vi por primera vez. Bajo ese título, *Microbiología y genética molecular*, yo esperaba encontrar uno más de los muchos libros de texto existentes sobre microbiología. Mi sorpresa fue muy grata cuando descubrí que se trataba de algo muy distinto. En efecto, esta obra resulta ser una amplia revisión de muchos de los aspectos más importantes de la fisiología y genética, tanto de bacterias como de levaduras, así como de la virología a nivel molecular. Queda, pues, lejos de lo que es un libro de texto básico, tanto por su planteamiento y su estructura como por la profundidad del tratamiento dado. Y esto es algo en lo que vale la pena insistir porque, aparte de sus valores didácticos, se trata de una obra de gran utilidad para el profesional y para el estudiante avanzado. No quiere decir eso que resulte de difícil lectura, sino que el nivel con el que se abordan la mayoría de los capítulos supera los límites de una obra de divulgación y, por tanto, requiere de unos conocimientos y una base previa de microbiología y de genética molecular.

Le falta, a mi juicio, un mejor ordenamiento lógico de los capítulos. Este aspecto aumenta particularmente la necesidad, antes indicada, de tener un cierto nivel de partida para poder asimilarlos independientemente unos de otros. Por lo que respecta a las cuestiones editoriales, al mismo tiempo que se aprecia una magnífica edición, tipografía y calidad del papel, puede decirse que la reproducción de algunas de las fotografías, aunque aceptable, podría ser mejor. Y con esto doy prácticamente por acabados los defectos o aspectos mejorables que la rápida lectura de la obra me ha sugerido.

Quisiera destacar muy especialmente la amplitud temática del libro, que le confiere un indudable interés general. Las contribuciones están tratadas con un detalle y una meticulosidad propios de las "reviews" de las revistas especializadas. Son muy de agradecer las numerosas figuras y esquemas que ilustran acertadamente los diferentes capítu-

los. La confección de cada uno de ellos va más allá de una mera recopilación de información, ya que suelen incluir, como base principal de la argumentación, resultados de los mismos autores. Estos resultados acaban convirtiéndose en el hilo conductor de la explicación.

En total son 112 personas las que han contribuido, con los datos de los trabajos más recientes de sus propios grupos de investigación, a este enfoque moderno de la obra. Gracias a ello, en sus 43 capítulos o artículos de revisión, se presentan aspectos realmente muy novedosos en todas las materias tratadas. Los distintos capítulos ofrecen una interesante visión del futuro desarrollo que cabe esperar de los diferentes temas. Todo ello proporciona actualidad y frescor al libro. Como consecuencia de este último aspecto, la obra se convierte en una excelente guía de la investigación española actual en microbiología. Además, proporciona una detallada información sobre los intereses de trabajo de los diferentes grupos que han contribuido a su elaboración.

No obstante, estos 112 autores representen sólo una parte de la gran cantidad de investigadores españoles que trabajan hoy en una área que, como todos sabemos, es de las que más implicaciones tiene a todos los niveles, y cuya tecnología se emplea, además, en otros campos científicos. Resulta evidente que, en la selección de los autores, el editor ha buscado principalmente tratar un máximo de temas, para mostrar la diversidad actual del trabajo sobre genética molecular de microorganismos en nuestro país.

En los dos tomos de *Microbiología y genética molecular*, como ya he comentado, los temas están discutidos en profundidad, pero, al mismo tiempo, gracias a unas buenas introducciones y a una exposición clara y didáctica, se consigue una lectura fácil y amena. En mi opinión, éste es un libro ideal para todos aquellos profesionales que, con años de experiencia, quieran actualizar ideas —cosa, por cierto, muy sana y necesaria—, sobre materias colaterales a las de su foco de interés directo.

Otro de los aspectos que muestra el cuidado con que se ha redactado este libro es la bibliografía citada. Ésta resulta con frecuencia descuidada en las revisiones que se suelen publicar, pese ser un elemento de gran ayuda a la hora de profundizar en los temas que susciten más interés al lector. En el libro que comentamos, en cambio, su extensión es equilibrada y muy actual. Una rápida mirada indica que las citas con más de 10 años son raras y corresponden, en su mayoría, a trabajos "clásicos". Además, el formato elegido, con títulos completos, es el más informativo, aunque requiera más espacio.

Finalmente, quisiera resaltar que el libro consigue el objetivo general de mostrar que la microbiología no es una ciencia del pasado, sino una disciplina actual, en la que se generan y aplican muchas de las tecnologías más avanzadas, y que tiene una incidencia social y económica de primera magnitud. Como dice el editor en su excelente prólogo, el límite en el avance de la microbiología está en la imaginación de los investigadores. Los dos voluminosos tomos que ahora se presentan son un buen ejemplo de que no es imaginación lo que falta a muchos de nuestros investigadores. Creo que el Prof. Casadesús, los diversos autores de los capítulos, la Universidad de Huelva y la Sociedad Española de Microbiología pueden sentirse muy orgullosos del resultado.

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Diccionario terminológico de ciencias médicas, 13ª ed.

MASSON, S. A., Barcelona, 1995. 1648 pp. Precio: 11.100 PTA. ISBN 84-458-0095-7.

"Hechos, observaciones y experimentos son los sillares del edificio. A nosotros nos toca clasificarlos, adscribirlos al lugar que les corresponde." Con estas palabras, Lavoisier (*The Enlightenment of Matter*, 1766) dejaba constancia de la capacidad clasificadora inherente a los científicos. La base de toda clasificación es, sin duda, una nomenclatura coherente y correctamente establecida. En cualquier doctrina científica, el tema de la nomenclatura es uno de los que precisan mayor atención y exigen continua actualización. Paradójicamente, éste es un campo muchas veces olvidado y que da pie a confusiones indeseables, complicando así lo que tendría que ser una tendencia acrisoladora del lenguaje. En este sentido, el *Diccionario terminológico de ciencias médicas* de Masson adquiere un papel muy importante en el campo de la ciencia en general, y en el de la materia médica en particular. Desde su aparición, en 1918, este diccionario ha ido ampliando el número de definiciones, hasta contar con las casi 100.000 que constituyen esta última edición. La incorporación de muchas voces nuevas, acuñadas en los últimos años y pertenecientes a las diferentes especialidades médicas, hacen de este diccionario médico uno de los más completos y modernos de que se dispone.

Las definiciones anatómicas de este diccionario se adaptan a las utilizadas en la última *Nomina Anatomica*. Paralelamente al papel terminológico del libro, se incluye un valioso anexo que recoge diversas constantes biológicas, las cuales abarcan desde índices hematológicos hasta determinantes enzimáticas. De igual forma, y remarcando así el carácter preferentemente médico del diccionario, al final del libro se incluye un atlas en color con 48 láminas de ilustraciones anatómicas tomadas de F. H. Netter (Colección Ciba de Ilustraciones Médicas), y que destacan por su exactitud y la gran cantidad de información que muestran.

Podemos recordar aquí otras obras similares, todas las cuales han tenido una amplia difusión y han resultado de gran utilidad para la clase médica. Las dos principales en español son las traducciones de los famosos *Diccionario de ciencias médicas* de Stedman (25ª edición, Editorial Médica Panamericana, 1993) y el *Diccionario médico de bolsillo* de Dorland (Interamericana/McGraw-Hill, 1989). Está, además, el práctico y gráfico *Diccionario médico ilustrado* de Melloni (Editorial Reverté,1983), de gran utilidad para los estudiantes y personas que se inician en la investigación. Finalmente, no podemos olvidar una importante obra publicada en catalán (con sus versiones en forma de libro y de CD-ROM) y apropiada continuadora del famoso *"Diccionario Corachán"*. Esta obra, *Diccionari Enciclopèdic de Medicina*, realizada bajo la dirección de Oriol Casassas (Enciclopèdia Catalana, 1990), constituye uno de los mejores y más precisos trabajos sobre terminología médica existentes, en cualquier lengua, con amplias y certeras incursiones en diversos campos afines, como pueden ser la química orgánica, bioquímica, botánica y ecología.

Tal como indica el título, el diccionario de Masson se dirige, principalmente, á profesionales de la medicina. Pese a ello, ese título puede llevar a confusión, ya que el libro constituye una referencia excepcional para todas aquellas personas relacionadas con todas las ciencias de la vida. Los términos biológicos se definen con exactitud, incluyendo muchas veces vocablos que escapan de la práctica puramente médica. Esto resulta lógico, en tanto que el hilo argumental del diccionario hace referencia a con-

ceptos de carácter anatómico, fisiológico y sanitario. El libro abarca ampliamente materias biológicas que se podrían agrupar bajo el nombre de ciencias de la salud, como son la inmunología, microbiología, fisiología animal y vegetal, bioquímica y genética. Si bien no registra algunos conceptos especializados de estas materias, sí en cambio explica los términos más usuales y con implicación directa en el campo de la medicina.

Este diccionario constituye, por tanto, una herramienta valiosa en diversos campos de las ciencias de la vida. El amplio espectro terminológico que se sistematiza en el libro adquiere un papel excepcional en el establecimiento de un marco lingüístico, sin el cual es imposible tener acceso a ninguna ciencia. La capacidad de hablar con exactitud se dificulta muchas veces por la cada vez más frecuente incorporación de palabras adaptadas incorrectamente del inglés. En este sentido es importante remarcar que el diccionario consta de un glosario inglés–español, con unos 19.000 vocablos, y otro francés–español, con más de 18.000. No obstante, debemos precisar que estos "glosarios", como los denomina el libro, son en realidad "vocabularios", porque no explican el significado de los términos, sino que dan sólo la equivalencia entre las parejas de lenguas mencionadas. Aunque pueda parecer un detalle insignificante —y en el diccionario pueden encontrarse todas las definiciones requeridas—, nos permitimos sugerir a la editorial que, tratándose de una obra léxica, corrija esa pequeña imprecisión en futuras ediciones del libro.

La importancia del glosario inglés–español se hace evidente por la gran influencia del inglés en la terminología científica en lengua castellana. La débil barrera lingüística que tiende a separar los vocablos científicos se hace, a veces, demasiado permeable. Esto acaba por permitir el flujo, casi por ósmosis, de palabras inglesas mal adaptadas al acervo terminológico español. El uso continuado de este glosario puede dificultar este trasiego indiscriminado e indeseado de términos.

La delineación de un vocabulario científico no es un trabajo intuitivo, sino que requiere un estudio detallado. Libros como éste son lo que ayudarán a definir, cada vez más exactamente, los límites semánticos de la terminología de las ciencias de la vida. El carácter enciclopédico del libro también supone una ayuda excepcional, en tanto que define, con gran exactitud, muchos conceptos confusos. Esto hace del libro una herramienta muy útil para estudiantes que necesitan definiciones claras y breves, y para profesionales de las ciencias de la salud y, por extensión, de la vida.

La editorial Masson, responsable de la obra, ha informatizado en una base de datos todos los vocablos incluidos en el diccionario, lo cual facilitará en el futuro la expansión de la obra, su revisión y la continua actualización, tan necesaria para mantener al día el marco lingüístico de las ciencias de la vida. Es indudable, por tanto, el carácter de abierto de esta obra. Precisamente las 13 ediciones del libro remarcan el hecho de que haya sido objeto de una constante y extensa revisión y actualización, lo cual le confiere un amplio valor y actualidad.

Sin duda, el rápido incremento de los conocimientos en las doctrinas que componen las ciencias de la vida implica la exigencia, cada vez más patente, de una mayor exactitud en la terminología, tanto desde el punto de vista cualitativo como cuantitativo. Y es de ahí, precisamente, de donde surgirán las herramientas que permitirán una correcta comunicación oral y escrita, algo esencial para aquilatar el lenguaje científico.

Alfredo Mayor
Redacción de Microbiología SEM

Fundamental Virology, 3rd ed.

Bernard N. Fields, David M. Knipe, Peter M. Howley (editors-in-chief)

Lippincott-Raven Publishers, Philadelphia, EE.UU., 1996. 1340 pp. Precio: $ 97,75
ISBN 0-7817-0284-4.

Fields Virology, 3rd ed.

Bernard N. Fields, David M. Knipe, Peter M. Howley (editors-in-chief)

Lippincott-Raven Publishers, Philadelphia EE. UU., 1996. 2 volúmenes, 3216 pp. Precio: $ 339,50. ISBN 0-7817-0253-4.

Bernard N. Fields, eminente virólogo de Harvard, falleció el 31 de enero de 1995, víctima de un cáncer de páncreas. Desde que le fue diagnosticada la enfermedad en 1992, y conocedor del poco tiempo de que disponía para culminar sus proyectos, Fields estuvo sumergido por completo en la tarea de preparar las terceras ediciones de *Fields Virology* y de su versión resumida, *Fundamental Virology*. Los capítulos finales fueron entregados a Raven Press justo un mes antes del óbito. *Fundamental Virology* y *Fields Virology* constituyen, sin ninguna duda, las dos mejores obras disponibles actualmente para el estudio de la virología. Por ello, deben ser recomendadas abiertamente, tanto al investigador especializado como al estudiante de cualquier nivel de virología.

La formación científica de Bernard Fields se sitúa a caballo entre la medicina y el estudio de las enfermedades infecciosas, especialmente las de etiología vírica. En este último campo ha brillado particularmente gracias a sus contribuciones sobre la patología molecular de los reovirus, tema en el que se había especializado hacía años.

La concepción de estas obras, *Fundamental Virology* y *Fields Virology*, aúna las perspectivas de la virología molecular y de la virología médica. La virología es, en la actualidad, una de las ciencias que genera mayor cantidad de descubrimientos. Su trepidante dinámica de cambio transforma, con pasmosa celeridad, datos vigentes en anticuados. Es lógico, por tanto, que se aprecien notables cambios de una edición a otra de la obra. Respecto de la segunda edición, hay grandes variaciones referentes a los virus de la inmunodeficiencia humana, virus emergentes, priones, herpesvirus y hepatitis delta.

Fundamental Virology condensa en un solo volumen, de 1360 páginas, 37 capítulos extraídos del hermano mayor, *Fields Virology*, con el objetivo de proporcionar un texto orientado a estudiantes de segundo y tercer ciclo de ciencias biomédicas, y a investigadores con un interés específico sobre aspectos básicos de la virología. La obra está especialmente indicada para cursos de virología general o de virología molecular, y tiene un notable sentido didáctico. El libro contiene 482 ilustraciones, algunas de ellas a todo color y muy espectaculares. Como en las anteriores ediciones, la obra se divide en dos partes. La primera (capítulos 1 a 15) está dedicada a aspectos generales como, entre otros, historia de la virología, taxonomía, morfología, multiplicación y patogenia víricas, etc. Extraordinario es el capítulo sobre la estructura de los virus, con unas magníficas reconstrucciones tridimensionales de partículas víricas y de proteínas de diferentes virus. En esta primera parte se tratan también, en sendos capítulos, los virus de insectos, los virus de levaduras, de otros hongos y de microorganismos parásitos, los virus de plantas y los bacteriófagos. Precisamente, uno de los po-

cos defectos de esta obra, y también de *Fields Virology*, es que los bacteriófagos y los virus de vegetales están tratados muy someramente..

En la segunda parte de *Fundamental Virology* (capítulos 16 a 37) se describen la bioquímica, biología molecular y aspectos celulares de la multiplicación de los virus de diferentes grupos. Aunque se estudian los aspectos generales, el trabajo está urdido muy a fondo, sobre todo la estructura y el modelo replicativo de cada familia. *Fields Virology*, además, dedica a cada familia un segundo capítulo, en el cual se tratan aspectos de interés médico. En ambos libros, cada capítulo está elaborado por destacados especialistas en los temas tratados, lo cual garantiza la calidad del contenido de los mismos. En total, participan en esta tercera edición del *Fundamental Virology* 55 autores, que llegan a ser 118 en el caso del *Fields Virology*. No obstante, este hecho permite apreciar cierta falta de uniformidad, en algunos casos.

En cuanto a *Fields Virology*, esta edición, al igual que las anteriores, está estructurada en dos voluminosos tomos, con 3216 páginas en total, que abarcan setenta y dos capítulos. La obra, que está profusamente ilustrada (969 figuras y esquemas), contiene, aparte de las novedades ya reseñadas para *Fundamental Virology,* otros temas de reciente actualidad, como son los capítulos sobre los astrovirus, la familia *Flaviviridae*, el herpesvirus humano de tipo 7, el virus de la hepatitis E y el virus elevador de la lactato deshidrogenasa y afines. También hay material nuevo referente al virus del papiloma, el virus de la hepatitis C, los hantavirus y las vacunas víricas.

La última parte de *Fields Virology* trata de los agentes víricos que todavía no han recibido una adscripción definitiva dentro de las distintas familias de virus. En este caso se encuentran el virus de la hepatitis delta, el virus de la hepatitis E (que ya ha sido asignado a la familia *Caliciviridae*), y los agentes causales de las encefalitis espongiformes, tema de enorme actualidad, al cual se dedican tres capítulos. Resulta interesante que este tema sea discutido de forma exhaustiva por autores de tan reconocido prestigio y opiniones tan contrarias como Carleton Gajdusek y Stanley Prusiner.

En resumen, aunque no estemos delante de unas obras diseñadas con un objetivo prioritariamente didáctico, puede considerarse que *Fundamental Virology,* al estar editado en un solo volumen, es más aconsejable para estudiantes de primer y segundo ciclo, exceptuando a aquéllos especialmente interesados en temas médicos. Por otro lado, sí que puede considerarse esta tercera edición de *Fields Virology* un manual insustituible para investigadores interesados en profundizar en cualquier aspecto de la virología animal. En este tratado puede encontrarse, con gran profusión de detalle, la más completa información recogida en una sola obra sobre virus que infectan animales.

Finalmente, cabe mencionar que se han preparado dos colecciones de diapositivas extraídas de *Fields Virology*, de gran interés científico y didáctico: «Basic Virology Slide Set», y «Clinical Virology and Pathogenesis Slide Set». Estas diapositivas, que todavía no están a la venta, podrán adquirirse a través de la misma editorial. En un momento de gran desarrollo de las técnicas de la imagen, no se explica lo difícil que resulta acceder a este tipo de material. Por ello, la iniciativa es de agradecer, y más aún teniendo en cuenta la indiscutible calidad de las ilustraciones del libro en las que se basan.

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Science for the Earth

Can Science Make the World a Better Place?

Tom Wakeford, Martin Walters (eds.)

John Wiley & Sons, Chichester, West Sussex, Gran Bretaña, 1995. 370 pp. Precio: £ 7,99. ISBN 0-471-95284-2.

El libro *Science for the Earth* es un crítico recorrido por lo que se podría llamar la epistemología científica y su interrelación directa con la Tierra como un todo. La frase de Nietzsche ("La Tierra es un bello lugar, pero tiene una pústula llamada hombre") que Lynn Margulis presenta en su artículo, sirve como eje argumental unificador, del que derivan los diferentes capítulos del libro. Esta obra es una crítica del papel del científico en nuestra sociedad, y de cómo la ciencia actual se aleja cada vez más de lo que debería ser una ciencia global. En este sentido, en el libro se encuentran un valioso grupo de artículos, escritos desde diferentes disciplinas, que se enfrentan con este problema. Ya en el prefacio, Stephen Hawking define el argumento que da pie y sirve de conclusión del libro: la humanidad no puede dar la espalda a la ciencia, pero necesita aprender cómo usarla responsablemente. Con estas palabras se constata un razonamiento general del libro: si, hasta hacía poco, la ciencia era algo orientado a averiguar la verdad objetiva, sin otra implicación que este puro descubrir, el estado presente de las cosas no permite la disociación de la ciencia y la responsabilidad que conlleva. Frente a los muchos problemas actuales (hambre, cambios climáticos, contaminación, crisis urbana, etc.), la pregunta que el libro intenta sugerir queda clara desde el principio: ¿cómo puede hacerse una "ciencia para la Tierra"? Se trata, pues, de un libro vital, en tanto que supone un espejo crítico de la ciencia, cuyos reflejos —desde un marco científico, social, diplomático, ambiental, de derechos humanos— sirven para remarcar la estrecha interacción que existe entre ciencia, sociedad y ambiente. La profunda crítica que se respira desde las primeras páginas plantea cómo la actividad científica, en un principio completamente objetiva, ha acabado por modular el mundo. Si bien es constante la presencia de un juicio mordaz del papel del científico en el mundo, en ningún momento el libro emplea conceptos anticientíficos o en contra de la ciencia. Su intención va mucho más allá: propone un replànteamiento de los principios científicos que ahora empiezan a hacer temblar los pilares de esta casa común que es la Tierra.

El libro consta de dieciséis capítulos, cada uno elaborado por un escritor diferente, y agrupados en tres partes. En la primera se hace un repaso crítico de las prácticas científicas actuales. Los autores de esta sección se enfrentan a varias preguntas: ¿por qué los científicos no piensan filosóficamente?, ¿puede el científico ser independiente de la cultura?, ¿qué es progreso y dónde encajan los indígenas en este concepto?, ¿por qué existen tantas disciplinas en ciencia que parecen no comunicarse entre sí?... De este modo, David Suzuki, en el primer capítulo, saca a relucir una serie de "conceptos sagrados" de la sociedad industrial, cuyo abandono o replanteamiento podría conducir a un futuro sostenible. Lynn Margulis destaca la estrechez de mente que, cada vez más, oprime el trabajo científico, y afirma que la ciencia debería incluir en su argumentación una concepción filosófica y otras vías de conocimiento paralelas para solucionar los problemas a los que se enfrenta la sociedad actual. De igual modo, James Lovelock resalta el carácter corrupto, perezoso y lucrativo de la ciencia actual, y concluye afirmando la necesidad de unos científicos más generalistas —lo que él denomina

físicos planetarios— para así abarcar con éxito los problemas. Claude Alvares discute en el cuarto capítulo el papel que la ciencia ha jugado en el mundo y que ha acabado por aumentar el hambre y la pobreza, privando a la gente de su conocimiento del mundo natural y gestando sus propias supersticiones y costumbres violentas.

En la segunda parte del libro se abarca la interacción de ciencia, sociedad y ambiente en varios contextos particulares. Así, se plantea si los principios científicos pueden ayudar al desarrollo del mundo y si el progreso científico es realmente progreso. George Monbiot, en su artículo, hace una valiosa crítica a *The tragedy of the Commons*, de Garrett Hardin. John Whitelegg dirige una mirada crítica a la tecnología social como un mecanismo de ahorrar tiempo, constatando este papel como una causa importante de la contaminación. Crispin Tickell describe el impacto de los cambios climáticos en las ciudades. Tony Cooper y Aubrey Meyer, desde su enfoque economicista, plantean el estrecho enfoque actual de los problemas mundiales, mientras que Nigel Woodcock abarca el problema de la falta de comunicación entre los geólogos y la sociedad, y propone la necesidad urgente de una ciencia global. Richard Lindsay explica las implicaciones de la relación entre la humanidad y el medio ambiente. Darrell Posey critica la explotación de los indígenas y cómo se está llegando a una pérdida de su cultura y de su acervo cultural. Por último, Stephen Tomkins plantea el papel de la escuela en la inculcación de una ética ambiental.

En la tercera parte del libro se proponen preguntas sobre aspectos más generales de la ciencia y de nuestra relación con la Tierra: ¿es el reduccionismo la aproximación adecuada a la ciencia?, ¿existen expertos?, ¿es la ciencia un proyecto unificado o no es más que un mito particular de nuestro tiempo? Wangari Maathai describe su concepción del mundo como una pirámide de riquezas, en cuya base confluyen la carencia de recursos para satisfacer las necesidades básicas, y en cuyo pico descansa el exceso de apetitos innecesariamente satisfechos por los conocimientos científicos. Brian Moss desarrolla una ácida comparación de la ciencia con el emperador desnudo de Hans Christian Andersen. Harmke Kamminga resalta la contingencia de nuestros criterios para el conocimiento científico. Por último, Tom Wakeford concluye que los científicos necesitan desprenderse de su exclusividad para orientar la investigación hacia un camino abierto y más comunicativo.

El carácter multidisciplinario de este pequeño gran libro remarca la exigencia, cada vez más patente, de un abordaje global de los problemas científicos. El desgarre profundo que se pone de manifiesto entre ciencia y mundo sólo puede ser considerado desde un planteamiento más generalista y menos reduccionista. De igual modo, el libro critica la concepción, muchas veces demasiado estrecha, de la mentalidad científica, propugnando una visión menos arcaica del mundo y requiriendo, cada vez más, la inclusión de unas ideas filosóficas y otras vías de conocimiento. El libro supone una profunda crítica de las barreras culturales y sociales que impiden la dedicación de los científicos a los problemas más importantes de la humanidad. El nexo común de los artículos en este libro es la idea de que la ciencia debe reformarse, para que pueda contribuir a solucionar los problemas sociales y ambientales que empañan el mundo en el umbral del siglo XXI. El libro no es una denuncia de la incapacidad de la ciencia para abordar la problemática mundial; es una crítica constructiva que pretende revalorizar, o simplemente otorgar el beneficio de la duda, ese nuevo mito o superstición en que se está convirtiendo la ciencia. Lo que los artículos proponen es una nueva mentalidad, no sólo en el nivel científico, sino en todos los niveles de conocimiento. El mundo ha

dejado de ser un sistema cerrado y abarcable desde un solo ángulo, para convertirse en una casa global, cuya comprensión requiere una metamorfosis radical en los caminos del conocimiento.

Se trata de un libro que merece la pena leer, por cuanto no se limita a exponer una crítica de la ciencia, sino que presenta también un proceso de renovación en el que todos los científicos juegan un papel fundamental. Este libro puede servir para plantar la semilla de la duda, esa simiente madre de la ciencia. Pero ahora no se trata de dudar del mundo exterior, sino del propio cuerpo científico. En este sentido, se trata de un libro de múltiples preguntas y respuestas veladas. Los dieciséis capítulos dibujan un gran interrogante, cuya contestación surge paulatinamente en el lector.

El libro resume sólidos conceptos con un lenguaje divulgativo. Esto hace que esté dirigido tanto a la comunidad científica como a un público mucho más amplio. En el científico, el texto acabará por aguijonear muchos de los conceptos sobre los que, inamoviblemente, se basa su conocimiento. En el lector no especializado, despertarán el interés por el importante papel de la ciencia en el mundo actual. Cada capítulo desvela una aproximación a esa nueva manera de contemplar el mundo, estimulando la imaginación y convirtiéndose en un placer para los lectores.

En definitiva, el libro plantea la exigencia de un nuevo diálogo que permita unificar y no disgregar, y propone un cambio de mentalidad, una nueva visión del mundo y de la mente humana. Haciendo eco de las palabras de Eugene Cernan, la última persona en caminar sobre la Luna, "You wonder, if you could get everyone in the world up there, wouldn't they have a different feeling, a new perspective?"

Alfredo Mayor
Redacción de Microbiología SEM

DNA Repair and Mutagenesis

Errol C. Friedberg, Graham C. Walker, Wolfram Siede

American Society for Microbiology. Washington, D.C., EE. UU., 1995. 698 pp. Precio: $ 79. ISBN 1-55581-088-8C.

Este libro es la segunda edición ampliada de la obra *DNA Repair*, publicada en 1985 por Errol C. Friedberg (W. H. Freeman & Co., New York). La imagen de la cubierta reproduce la estructura de la glicolasa de dímeros de pirimidina del fago T4 (endonucleasa V). La historia de las observaciones sobre este enzima es uno de los ejemplos que contiene esta obra, acerca del progreso alcanzado en el campo de la reparación del DNA. Ya en 1947 Salvador Luria reconoció el fago T4 como extraordinariamente resistente a la radiación ultravioleta, a diferencia de los fagos T2, T5 y T6. La causa era la endonucleasa V, que muchos años después se purificó y que finalmente, en 1992, se determinó su estructura. La enzima cataliza la hidrólisis de sólo uno de los enlaces N-glicosil en un dímero de pirimidina; no libera bases al medio, sino que la escisión de parte del nucleótido ocurre durante la degradación postincisional del DNA.

De los catorce capítulos del libro, los dos primeros analizan los hechos que originan la reparación del DNA. Éstos son las lesiones del DNA y la mutagénesis. Por una parte, se estudian las lesiones espontáneas y las inducidas por agentes ambientales, así como su detección y medida. Se hace énfasis en la aplicación de las bases moleculares de la mutagénesis para el estudio de polimerasas purificadas. A continuación, en los seis capítulos siguientes, se exploran sistemáticamente los mecanismos conocidos de reparación del DNA, tanto en procariotas

como en eucariotas —con un énfasis especial en el caso de las células de mamíferos—. Por ejemplo, se tratan los modelos de reparación por reversión de la lesión (fotorreactivación), escisión de bases (glicosilasas de DNA), escisión de nucleótidos en procariotas, y eucariotas , las AP endonucleasas, y la importancia de la reparación por escisión de nucleótidos. El capítulo octavo incluye apartados especialmente interesantes, uno de los cuales está dedicado a la reparación por escisión del DNA mitocondrial. Otros, tratan los genes implicados en la reparación por escisión y la importancia de estos procesos en los sistemas libres de células. El noveno capítulo está dedicado íntegramente a la reparación de los errores de emparejamiento de bases ("mismatch repair") en eucariotas y procariotas. Trabajos recientes han clarificado que los mecanismos moleculares de este tipo de reparación son similares a los de escisión de bases y de nucleótidos. Además, la enzimología básica del proceso es casi idéntica entre procariotas y eucariotas. No obstante, la diferenciación entre la base correcta y la incorrecta en el desemparejamiento continúa siendo esencial y característica de este proceso. Los capítulos finales estudian la tolerancia a las lesiones del DNA en procariotas y eucariotas, así como la mutagénesis y los agentes que lesionan el DNA. El capítulo trece se centra en los procesos de regulación de la respuesta celular a las lesiones del DNA en eucariotas; destacan los apartados dedicados a la detención del ciclo celular en respuesta a las lesiones del DNA y a los procesos de muerte celular programada, o apoptosis. Un último capítulo –el catorce– expone las enfermedades y síndromes humanos asociados a defectos en la respuesta celular a las lesiones en el DNA (entre otros, el síndrome de Cockayne, el xeroderma pigmentoso, la anemia de Fanconi, el síndrome de Bloom y el de Louis-Bar, etc.).

La obra está especialmente indicada como libro de texto en cursos especializados de biología molecular, y como obra de referencia para investigadores y biólogos moleculares dedicados a la biología del cáncer, regulación, recombinación y transcripción genética y replicación del DNA. Presenta, de una manera muy clara, la perspectiva histórica de los conocimientos actuales sobre las respuestas celulares a las lesiones del DNA. Recopila de forma ordenada y completa toda la información existente acerca de los mecanismos moleculares –y las bases genéticas implicadas– que realizan la reparación del DNA, sea cual sea el tipo de lesión. Además, sus más de 4000 citas bibliográficas y 380 figuras en color son extremadamente útiles, tanto para el docente como para el investigador en esta materia.

La aparición de esta obra coincide con un desarrollo notable del conocimiento de los genomas de diferentes organismos. Los diversos proyectos que están en marcha en relación a este tema se fundamentan en las ventajas de índole diversa que puede suponer desvelar la información genética completa de un organismo. Todo ello puede resultar insuficiente sin una respuesta celular capaz de preservar dicha información genética frente a los ataques de los agentes ambientales y a las mutaciones espontáneas. El estudio de sistemas de reparación de las lesiones en el DNA permiten mantener nuestra salud, conocer los mecanismos de la evolución y contribuyen a planear una política científica frente a los riesgos de los contaminantes ambientales. Por todo ello, la prestigiosa revista *Science* distinguió de modo especial a la enzima de reparación del DNA, la endonucleasa V, como la Molécula del Año 1994.

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Intoxicaciones por plantas y hongos

Josep Piqueras

MASSON, S.A., Barcelona, 1996. 153 pp. Precio: 5.400 PTA. ISBN 84–458–0416–2

Este libro ha conseguido reunir, de forma muy concisa, clara y organizada, los datos básicos sobre plantas y hongos que producen intoxicaciones en la especie humana. Como médico, su autor lo ha orientado básicamente hacia la práctica médica, para facilitar a sus colegas el diagnóstico y el tratamiento de una serie de afecciones que pueden resultar peligrosas precisamente por su baja frecuencia, a causa de la cual la formación y la actualización de los facultativos se hace difícil o incompleta.

El autor ha reunido, durante muchos años de práctica hospitalaria, un acervo de experiencia directa, de casos citados y de datos antes muy dispersos en la bibliografía, que le ha permitido confeccionar un libro de una gran precisión de cara a la práctica. Él mismo advierte en el prefacio que no es su objetivo describir con detalle las plantas y hongos potenciales causantes de intoxicaciones, para lo cual existen textos botánicos y micobiológicos. También indica el autor que no es su deseo que el libro sea utilizado en la práctica hospitalaria para identificar las especies o restos de plantas o setas que los acompañantes de las personas intoxicadas aporten a los servicios de urgencias. Sin embargo, su formación biológica, y especialmente micobiológica —es miembro de la Societat Catalana de Micologia desde su fundación—, le ha permitido dar unas descripciones breves, pero útiles y a menudo ilustradas con fotografías en color o en blanco y negro, que pueden suministrar una buena orientación sobre las especies implicadas cuando no sea posible recabar el dictamen botánico de un especialista, o en tanto se obtiene el mismo.

El libro consta de 36 capítulos, distribuidos en cuatro partes. La parte I comprende cuatro capítulos introductorios, dedicados a precisar los diversos síntomas de intoxicaciones y los principios generales para el tratamiento. A continuación, en la parte II (capítulos 5 a 22), el autor aborda las intoxicaciones por plantas fanerógamas agrupándolas en capítulos, no por afinidades sistemáticas (aunque a menudo exista una correlación), sino por el tipo de substancias tóxicas, de síntomas y de manifestaciones clínicas, que son los factores más importantes para establecer un tratamiento adecuado, incluso en aquellos casos en que no pueda determinarse la especie causante de la intoxicación. Cada capítulo incluye una extensa bibliografía básica para profundizar en el tema.

Nos encontramos en una época en que muchas personas sin conocimientos botánicos o micológicos, ni siquiera rudimentarios, vuelven su atención hacia las plantas y setas silvestres como alimento, condimento o incluso medicina, en la creencia de que por tratarse de productos naturales no pueden ser perjudiciales. En las últimas décadas hay que añadir el consumo cada vez más frecuente de algunas plantas y setas como substancias alucinógenas. Se trate de vegetales o de hongos, no es raro que se produzcan confusiones entre especies de aspecto parecido, o se incurra en sobredosis. Aún son muchas las personas que confían en los "métodos" tradicionales —y erróneos— de determinación de la toxicidad de las setas, como hervir ajos o introducir una cucharilla de plata en el mismo recipiente donde se cocinan, confiando en que se ennegrezcan si los espécimenes son venenosos. En el caso de intoxicaciones por plantas, las ingestiones accidentales por niños

continúan siendo muy frecuentes. En todas estas situaciones, una duda del médico en el diagnóstico puede tener graves, incluso fatales, consecuencias; este libro contribuirá en buena medida a evitar las confusiones en el diagnóstico.

Las partes III, IV y V están dedicadas a los micetismos o intoxicaciones por setas, campo en el que el autor, buen micólogo, además de médico, ha podido acumular sus experiencias desde su trabajo hospitalario. Tras considerar los aspectos generales, clasificación de las intoxicaciones y diagnóstico sindrómico y botánico de las intoxicaciones por setas (parte III, capítulos 23 a 25), describe los dos grandes tipos de síndromes que se dan en los micetismos; los de período de latencia breve (parte IV, capítulos 26 a 31), en general benignos, y que sólo exigen una terapia de soporte (gastroenteritis agudas, intoxicación neurológica, alucinógena, muscarínica, cardiovascular, hemolítica), y los de período de latencia prolongado (parte V, capítulos 32 a 36), especialmente las intoxicaciones por setas nefrotóxicas y hepatotóxicas, que pueden tener consecuencias mortales y requieren una actuación rápida, incluso antes del establecimiento de un diagnóstico definitivo. Es en el último tema de los síndromes coleriformes y de agresión hepática donde el autor muestra su vasta experiencia, su dominio de la bibliografía y el resultado de sus propias investigaciones, que le han permitido limitar al 7% la mortalidad en el total de las 135 intoxicaciones tratadas por él, causadas por *Amanita phalloides*, *Lepiota* del grupo *helveola* y otros hongos ricos en amatoxinas, citados en los libros como mortales.

El autor termina el libro con unos interesantes y útiles algoritmos para facilitar la rápida toma de decisiones en el caso de intoxicaciones por setas. Creemos que estos gráficos hacen del libro un instrumento que debería estar disponible en los servicios de urgencia hospitalarios, con objeto de que pueda ser utilizado en cualquier eventualidad derivada del consumo o manejo de plantas o setas tóxicas.

Hemos detectado pequeñas imperfecciones nomenclaturales o de grafía terminológica. Estos errores están distribuidos sólo en algunas páginas, que quizás fueron omitidas en la revisión final. También nos sorprende la inclusión de la ortiga blanca (*Lamium album*), entre las plantas urticantes, quizás por una confusión debida a su nombre popular. En la figura 22.3 se alude a *Suillus granulatus* como especie alergénica, sin que se hable de ella en el texto. En cuanto a la terminología, en general muy correcta, en uno de los casos no nos parece lógica. Se trata de *orelanina* que, por la pronunciación latina de la doble ele, debiera escribirse así en castellano, y no con elle, como aparece en el texto comentado.

Pero estos comentarios detallistas sirven sólo para demostrar que el autor de esta revisión, aun no siendo médico, ha leído con gusto e interés la totalidad de este libro, que pese a ser lacónico, riguroso y pensado para los profesionales de la medicina, resulta de un gran atractivo y amenidad para cualquier naturalista y especialmente para los micólogos. Por todo ello, recomendamos también su lectura, y uso, a los diversos profesionales y estudiantes de las ciencias de la vida y de la salud.

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Fonaments de Bioquímica

Juli Peretó, Ramon Sendra,
Mercè Pamblanco, Carme Bañó

Universitat de València, 1996. 310 pp.
Precio: 2.400 PTA. ISBN: 84–370–2323–8.

Este texto general de introducción a la bioquímica incide en la descripción de los conceptos fundamentales, a partir de los cuales se explican los procesos bioquímicos de todo organismo. A diferencia de otros textos sobre el tema, no se centra exclusivamente en la bioquímica de vertebrados o en la humana. Cumple así el difícil compromiso de sintetizar los fundamentos básicos de la bioquímica sin perder la perspectiva de la gran diversidad metabólica de los organismos, consecuencia del proceso de adaptación necesario para la conquista de distintos nichos ecológicos durante la evolución. Sería de desear que este libro fuera traducido al castellano y al inglés, para que pudiera beneficiarse de este excelente texto toda la comunidad científica.

Es una obra de bioquímica general con enfoque docente, realizado por docentes, cuyo contenido se adapta perfectamente a los 7–7,5 créditos con que cuenta la asignatura en los nuevos planes de estudio. Tiene la extensión adecuada para servir al alumno en la preparación de los temas y, en este sentido, ayuda también al profesor. Substituye con ventaja a los "apuntes", como complemento de las lecciones explicadas en clase, aunque de ninguna manera ha de considerarse —ni es la pretensión de los autores— un sustituto de los manuales clásicos sobre la materia, cuya extensión y profundidad, tanto en el texto como en las ilustraciones, resultan imprescindibles. Es el caso de las obras de Stryer, Voet & Voet, Devlin, Rawn, Lehninger,... que están disponibles en inglés y en español.

El libro se divide en seis partes:

(i) *Introducción.* Comprende una atractiva introducción a la bioquímica, en la que cabe résaltar la perspectiva histórica y el resumen de sus principales objetivos, además de una magnífica síntesis sobre la problemática del origen de la vida. En la introducción se aprecia lo que serán dos de las características distintivas del libro: la perspectiva evolutiva en la que se presentan algunos temas y la inclusión de ejemplos que abarcan desde la bioquímica humana a la de microorganismos.

(ii) *Estructura y función de proteínas.* El primer capítulo se dedica a la descripción de los distintos niveles de estructura de las proteínas, presentando de manera clara y concisa no sólo el nivel actual de conocimiento en este tema, sino también las incógnitas que todavía existen por descifrar sobre el proceso de plegamiento de las proteínas in vivo. En el segundo y último capítulo de este bloque se explican los conceptos de cooperatividad y alosterismo, ilustrándolos con el clásico ejemplo de la interacción de la hemoglobina con el oxígeno.

(iii) *Enzimología.* En dos capítulos se resume la naturaleza química de las enzimas, la cinética enzimática monosubstrato y los distintos mecanismos moleculares que permiten regular la actividad de algunas enzimas en la célula. Es sorprendente ver cómo un libro de texto de estas características consigue, por un lado, sintetizar los conceptos básicos y, por otro, hacer hincapié en los aspectos que todavía deben ser ampliamente investigados. Así, por ejemplo, se hace referencia al escaso conocimiento actual sobre las ribozimas y se comenta cómo mediante la mutagénesis dirigida y los anticuerpos monoclonales se están esclareciendo los mecanismos de la catálisis enzimática.

(iv) *Estructura y función de los ácidos nucleicos.* Consta de tres capítulos. El primero se dedica a la estructura y organización de los ácidos

nucleicos; el segundo a la replicación, reparación y transcripción del DNA; el último al proceso de traducción o biosíntesis de proteínas.

Es de destacar la actualidad con la que se trata el tema, la inclusión de aspectos evolutivos y el perfecto compromiso entre la exposición concisa y clara de los conceptos generales, como la universalidad del código genético, sin olvidar la biodiversidad. Son muy ilustrativos los ejemplos particulares del distinto significado de algunos tripletes en bacterias.

(v) *Bioenergética.* Comprende dos capítulos, el primero dedicado al flujo de la energía y el segundo a las cadenas de transporte electrónico y síntesis de ATP. Esta parte representa una novedad respecto a la organización habitual que encontramos en otros manuales de bioquímica. En este libro, en lugar de presentar la cadena respiratoria después de la glicolisis y del ciclo de Krebs, se presentan todos los aspectos bioenergéticos antes del metabolismo. Se destaca así en esta parte que el destino de cualquier oxidación es la cadena respiratoria, y se presentan conjuntamente la cadena de transporte electrónico mitocondrial y la fotosintética del cloroplasto, resaltando su punto en común, que es la hipótesis quimiosmótica.

(vi) *Metabolismo intermediario.* El primer capítulo en el que se presenta el panorama general del metabolismo incluye una novedad importante respecto a la mayoría de manuales de bioquímica: contiene una sección dedicada a las herramientas teóricas desarrolladas en los últimos años para cuantificar la distribución del control entre las distintas enzimas de una vía metabólica. A continuación se describen las rutas metabólicas en cuatro bloques: acetil-CoA y ciclo del ácido cítrico, metabolismo de glúcidos, metabolismo de lípidos y metabolismo de compuestos nitrogenados. La obra termina con dos capítulos dedicados a la comunicación intracelular y a la integración del metabolismo. Cabe señalar que en este último capítulo se presentan ejemplos muy diversos de adaptaciones metabólicas, tales como la adaptación al ayuno, la sincronización de ritmos estacionales en plantas, o la adaptación de *Escherichia coli* a ambientes anaeróbicos. Una vez más se alcanza con éxito el compromiso entre presentar la universalidad del metabolismo intermediario y la diversidad de combinaciones posibles entre las distintas reacciones metabólicas, que configuran el soporte metabólico de la biodiversidad.

Como resumen, puede decirse que este libro es un excelente manual adaptado a un curso de introducción de bioquímica, recomendable a cualquier estudiante que desee introducirse en esta disciplina. La bibliografía seleccionada en cada capítulo está actualizada y se aprecia un esfuerzo por incluir una buena parte de textos en español, a fin de facilitar al alumno la ampliación de sus conocimientos. Los aspectos evolutivos están muy bien tratados, permitiendo comprender como se ha llegado a la complejidad actual que presentan los seres vivos.También se incluye en varios capítulos bibliografía actualizada sobre los aspectos evolutivos del tema en particular. La estructuración, el rigor y la claridad con los que está escrito facilitan la comprensión de su contenido. Faltaría, sin embargo, un índice general alfabético que permitiese acceder rápidamente a la información deseada. Es de esperar que en la próxima edición de este libro se incluya un índice al final. Tenemos que añadir que la calidad y claridad de las numerosas figuras, así como el precio moderado del libro, hacen que esta obra sea altamente recomendable a los estudiantes, no sólo de bioquímica, sino también a los de biología celular o microbiología, ya que es un magnífico complemento de diversos temas de estas asignaturas.

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Automated Microbial Identification and Quantitation.

Technologies for the 2000s.

Wayne P. Olson (ed.)

Interpharm Press, Inc. Buffalo Grove, IL, EE. UU., 1996. 397 pp. Precio: $ 189. ISBN: 0–935184–82–1

Desde que el estudio de los microorganismos tiene nombre propio, los avances científicos y técnicos, lentos en sus primeras etapas, vertiginosos en nuestra época, han ido proporcionando cada vez mayor conocimiento y comprensión de los múltiples procesos que tienen lugar con o gracias a la intervención de microorganismos. La tecnología, a la que tanto debe el desarrollo científico, ha sido igualmente un potente motor de desarrollo para la industria, llegando a producir, hasta nuestros días,un flujo unidireccional de información.

Wayne P. Olson presenta en este libro las técnicas más innovadoras para la identificación de los microorganismos, aplicables a los laboratorios de investigación más modernos. *Automated Microbial Identification and Quantitation* comprende 15 capítulos, que pueden distribuirse en cuatro grupos temáticos, a cargo de diferentes autores.

En el primero de ellos (capítulos 1, 2 y 3), dedicado a la identificación general de diversos microorganismos, se describen desde la identificación por cromatografía de gases (considerando sus limitaciones y errores de aplicación), hasta los "Vitek", sistema de 30 tarjetas de identificación con substratos bioquímicos deshidratados de uso específico, o el sistema "Biolog Microstation", microplacas que en su interior presentan un centenar de tests de identificación y clasificación.

El segundo grupo temático (capítulos 4, 5 y 6) trata los sistemas de identificación de grupos concretos de microorganismos: (i) anaerobios, con dos tipos de "kits", los microbioquímicos, que dependen del crecimiento, y los enzimáticos rápidos, no dependientes de crecimiento, sino de la unión a su cromóforo; (ii) microorganismos Gram-positivos, con sistemas muy similares, test automatizado para bacteriología (ATB) y sistema automicrobiano (AMS), ambos basados en "kits" de pruebas bioquímicas; (iii) levaduras, mediante quimiotaxonomía, realizada por análisis químico de los componentes microbianos (lípidos, polisacáridos, proteínas y ácidos nucleicos), muy útil, pero que no puede aplicarse de manera exclusiva en la identificación de gran número de grupos de origen filogenético diverso.

El tercer grupo (capítulos 7, 8 y 9) describe métodos de identificación poco específicos, a excepción de las técnicas para el género *Mycoplasma*. En " Tecnología de sondas y automatización" se muestra una metodología de amplio espectro, que permite identificar, enumerar y detectar microorganismos celulares o virus, mostrando a la vez gran especificidad, por su unión restringida a su objetivo molecular (DNA, RNA, proteínas, etc.). La descripción se centra básicamente en las sondas de DNA y RNA, por su posible combinación con la tecnología de la reacción en cadena de la polimerasa. No es de extrañar que el libro dedique un capítulo entero a describir las dos técnicas principales para la identificación de *Mycoplasma* (la metodología de cultivo y el método de tinción por fluorescencia, que puede ser aplicado directamente al cultivo celular). Desde que, en 1956, se detectó como contaminante de un cultivo celular, se ha visto que la presencia de micoplasmas en cultivos utilizados para obtener productos farmacéuticos es bastante frecuente. El capítulo "Análisis de los ácidos grasos celulares para la clasi-

ficación e identificación de bacterias" es una descripción detallada de una técnica quimiotaxonómica utilizada para identificar microorganismos de importancia biológica y médica.

El último grupo temático (capítulos 10 a 15) trata de la cuantificación y caracterización de poblaciones microbianas, y describe seis tipos de métodos. El capítulo "Enumeración y caracterización de las poblaciones microbianas por medio de contadores de partículas ópticos o sensibles a campos eléctricos" describe la tecnología disponible para medir el tamaño de bacterias o células aisladas y hacer recuentos de poblaciones, así como las aplicaciones de esta metodología a las poblaciones microbianas que se encuentran en suspensión en el aire o en medio acuoso. Son técnicas auxiliares muy útiles para conocer el tamaño de las células, los cambios cuantitativos en una población, cambios físicos tales como alteraciones del morfotipo o presencia de esporas bacterianas; también para controlar variaciones temporales en las relaciones intercelulares, como la fusión celular, gemación, adherencia o fagocitosis.

Se describen detalladamente los principios en los que se basan el contador de Coulter, el contador por extinción de luz, la citometría de flujo y la espectroscopia de correlación de fotones, entre otras técnicas, con indicaciones para elegir la metodología adecuada. El capítulo sobre el "Análisis cuantitativo de ATP", describe una técnica muy sensible, específica y rápida para calcular el número de células viables por medio de bioluminiscencia. Se basa en la presencia universal de ATP en las células vivas, en cantidad relativamente constante dentro de un mismo tipo, y en el hecho de que este nucleótido desaparece rápidamente en las células muertas. El siguiente capítulo está dedicado a las "Pruebas de lisado de amebocitos de *Limulus* (LAL)", que se utilizan para la detección de contaminación por endotoxinas. El nombre se tomó a partir de la prueba descrita por Levin and Bang, en 1968, que utilizaba como reactivo una suspensión de células sanguíneas, o amebocitos, del artrópodo marino *Limulus polyphemus* (cacerola de las Molucas) para detectar la presencia de endotoxinas (pirógenos) en sangre.

Además de la naturaleza de las pruebas, metodología y aplicaciones, el capítulo describe las regulaciones y estándares en diferentes países. Los capítulos "Recuento microbiano por cromatografía de gases" y "Análisis cuantitativo del DNA en productos proteicos y en medios de fermentación" se centran en productos obtenidos a partir de microorganismos. La primera de estas técnicas permite detectar la existencia de contaminantes microbianos, y se basa en el conocimiento de la cantidad aproximada de células en el cultivo y de la producción final de la fermentación, mediante la utilización de la cromatografía de gases (GC). La segunda es utilizada para la detección de residuos celulares o de DNA microbiano contaminante, en la gran cantidad de proteínas recombinantes obtenidas para uso farmacéutico. El último capítulo, "Recuento semiautomático de microorganismos en el aire", se centra en la importancia de la cuantificación de los microorganismos en el aire, por los aspectos económicos y de salud pública.

Como resumen, podemos decir que se trata de una obra de consulta casi obligada para las personas que trabajan en microbiología clínica, en la industria farmacéutica o de producción de alimentos y bebidas, así como en cualquier campo que precise de técnicas rápidas para la identificación y recuento de microorganismos.

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Bacteriological Analytical Manual, 8th ed.

AOAC International, Gaithersburg, MD, EE. UU., 1995. Precio: $145 (en Europa). ISBN 0–935584–59–5.

Publicado por la AOAC International y bajo la dirección de la US Food and Drug Administration (FDA), esta octava edición del *Bacteriological Analytical Manual* (BAM) describe en sus 28 capítulos los métodos seleccionados por la FDA, bajo diferentes criterios, para el análisis microbiológico de alimentos, bebidas y cosméticos. Se citan, aunque no se explican, otros métodos de detección rápida de microorganismos en un apéndice al final del manual, donde se insiste en los constantes cambios que se producen en este tipo de pruebas.

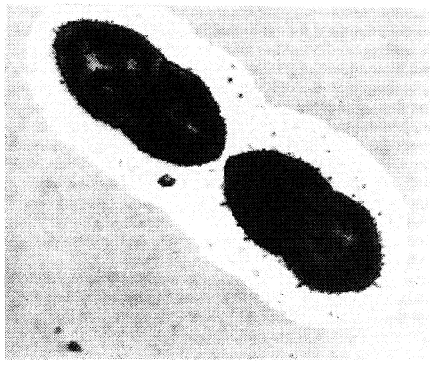
La actualización obligada de un manual de estas características, en este caso con respecto a la edición anterior, de 1992, se ha llevado a cabo revisando y modificando la mayor parte de capítulos y añadiendo otros, concretamente los que tratan de la utilización de la PCR para la detección y cuantificación del virus de la hepatitis A en bivalvos (capítulo 26), y para la detección de *Vibrio cholerae* en alimentos (capítulo 28), así como los métodos de detección de la fosfatasa alcalina para determinar si los productos lácteos han sido elaborados con leche pasteurizada (capítulo 27).

Cada capítulo consta de una breve explicación acerca de los fundamentos científicos de cada método. Sigue una descripción detallada de los aparatos, medios y reactivos y, a continuación, la parte descriptiva de la técnica que, gracias a una buena distribución, facilita la consulta rápida. La bibliografía del final de cada capítulo combina los dos fundamentos de cada método, el aspecto teórico básico (con textos clásicos) y el tecnológico, con las publicaciones más recientes que permiten al usuario obtener información adicional.

Los tres primeros capítulos incluyen procedimientos generales y recomedaciones para llevar a cabo las distintas pruebas. Los capítulos 3 al 17 tratan de los microorganismos patógenos que, utilizando los alimentos como vehículo de transporte y entrada, son causantes de distintas infecciones humanas. Se incluyen desde bacterias (*Escherichia coli*, *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, *Vibrio*, *Listeria*, *Staphylococcus aureus*, *Bacillus*, *Clostridium*), hasta levaduras y hongos, y parásitos de animales. El resto de capítulos, del 18 al 28, no pueden englobarse bajo un denominador común, debido a que en todos ellos se tratan temas distintos. Sirvan de ejemplo los tres capítulos dedicados al estudio microbiológico de la leche, que describe la detección de substancias inibitorias en ella, la determinación de sulfametazina en leche mediante HPLC y la determinación de la fosfatasa alcalina en productos lácteos. La detección mediante el uso de sondas de microrganismos patógenos que se transmiten vía alimentos, y la detección y cuantificación del virus de la hepatitis A en bivalvos mediante PCR, son el tema de otros tantos capítulos. Merece la pena destacar la existencia de un capítulo sobre las técnicas microbiológicas utilizadas en la industria de la cosmética.

Estamos, pues, ante una obra de manifiesta utilidad, de carácter eminentemente práctico que, gracias a su constante actualización y fácil consulta, permite resolver de manera eficaz los problemas de tipo metodológico que se pueden encontrar en el análisis de aguas, alimentos y cosméticos.

David Ràfols
Universidad de Barcelona



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Doc. 6. The Concept of a Virus
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Continued from p. 340

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Regulation of virulence gene expression in pathogenic *Listeria*

Klaus Brehm,[1,2] Jürgen Kreft,[2] María-Teresa Ripio,[1]
José A. Vázquez-Boland[1]*

[1] *Unidad de Microbiología e Inmunología, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, España*
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On p. 225, Table 1, in the last epigraph, "Growth within macrophages", the text "Selective induction of 32 proteins (including HSP and oxidative stress proteins)" in the "Effect" column should read:"Selective induction of 32 proteins (excluding HSP and oxidative stress proteins)". For more details, see p. 226, 2nd paragraph, on the left column.

On p. 233, left column, line 18, should read "...as the many open questions..." instead of "as there are many open questions..."

The authors apologize for these errors.

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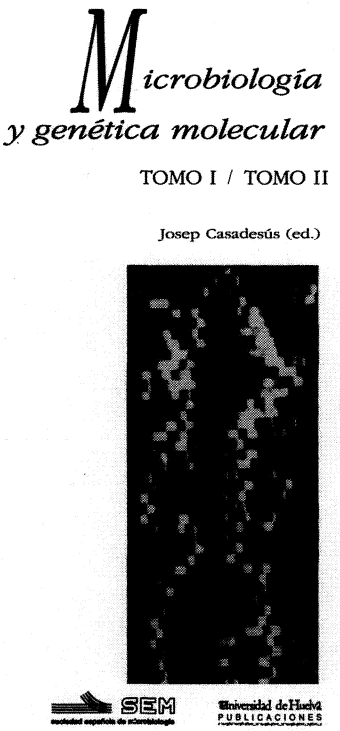
Josep Casadesús (ed.)

Coedición de la Sociedad Española de Microbiología y la Universidad de Huelva



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