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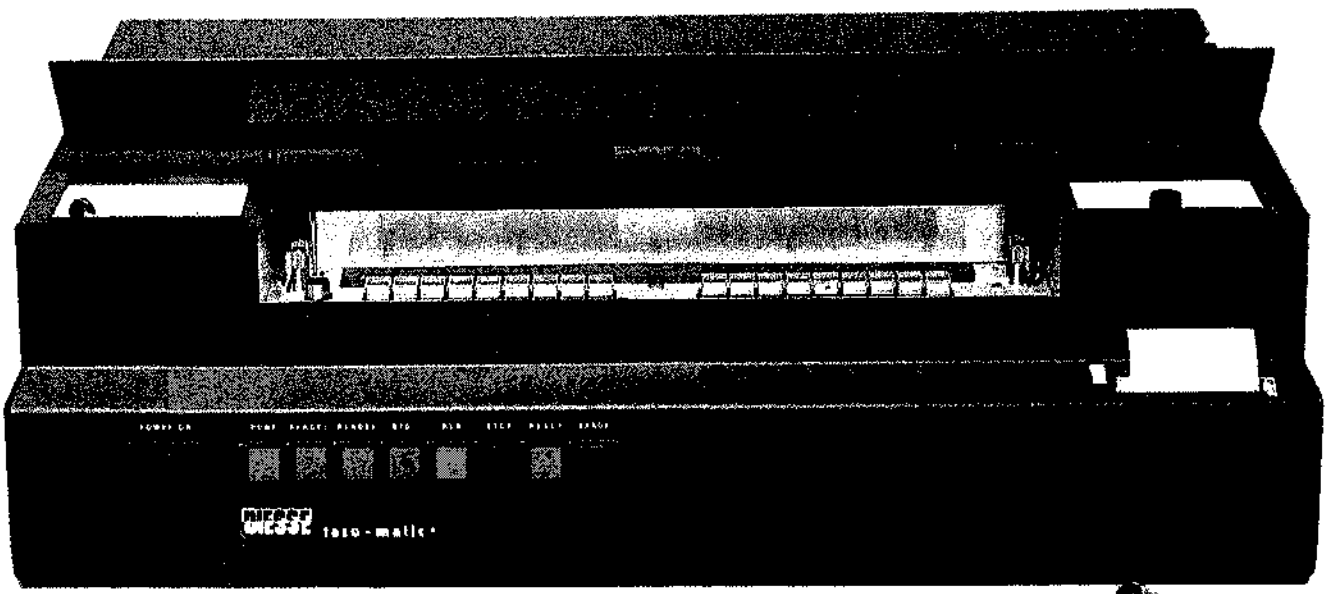

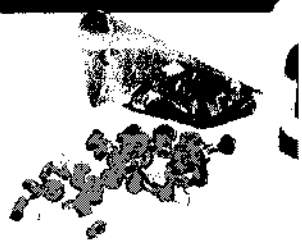

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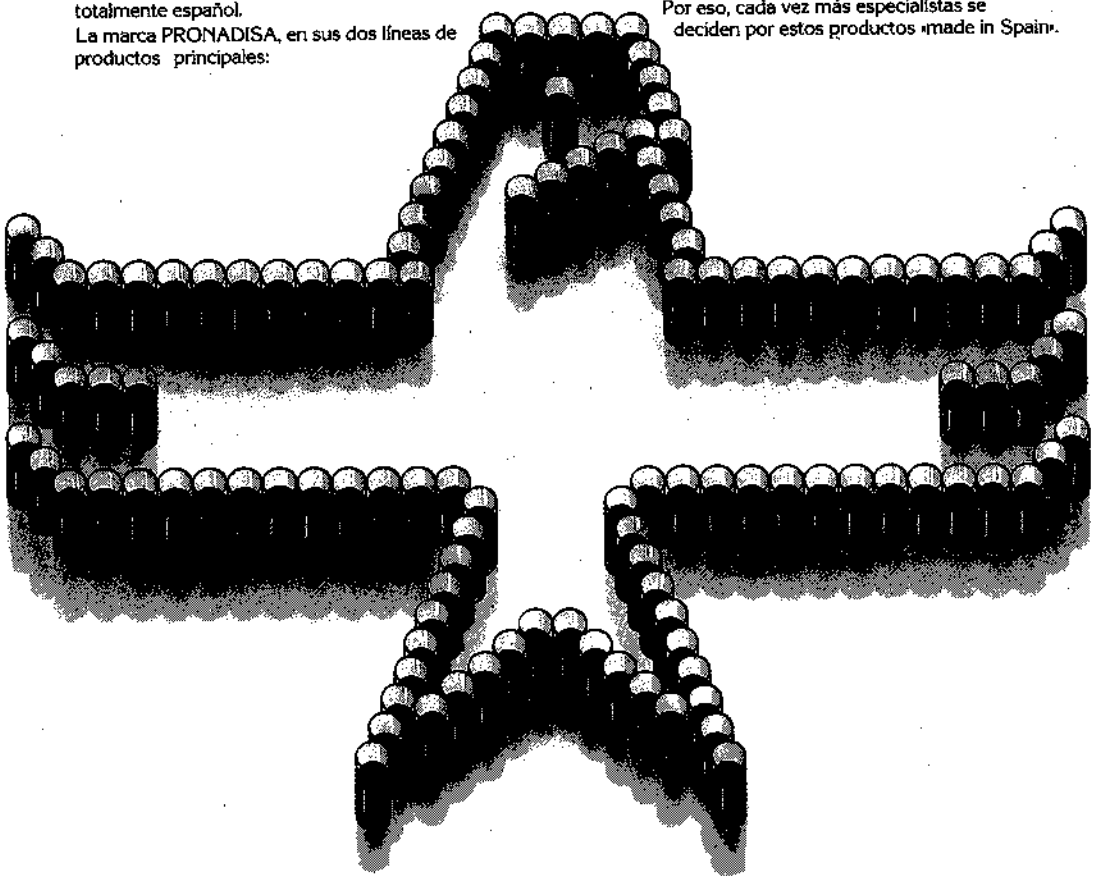

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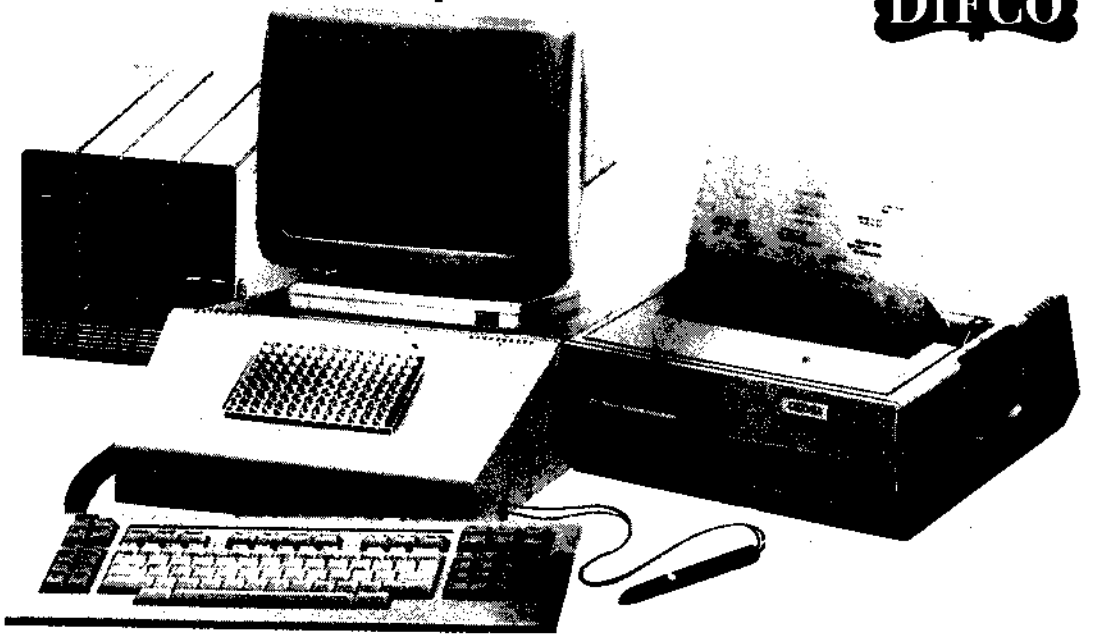

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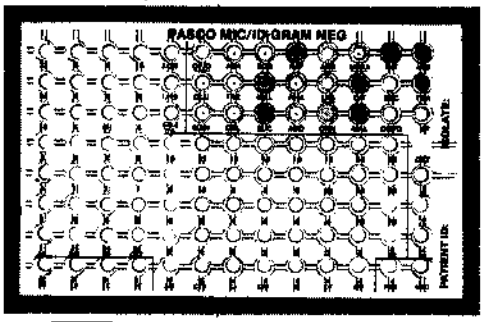

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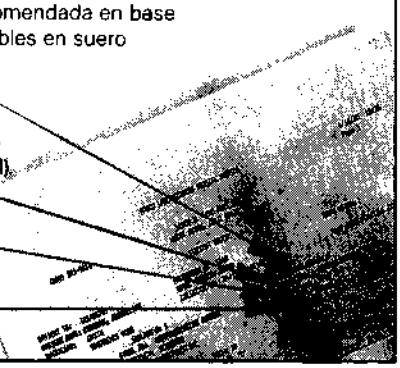

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MICROBIOLOGIA SEM 4 (1988), 5-27 **MINIREVIEW**

Archaebacteria: Their phylogenetic relationship with the eubacterial and eukaryotic kingdoms

José Luis Sanz and Ricardo Amils (*)

Centro de Biología Molecular, CSIC - UAM, Canioblanco. 28049 Madrid. Spain.

(Received January 15, 1988)

Summary

In microbiology the discovery of archaebacteria ten years ago has wrought a profound change in the concepts of physiology, taxonomy, ecology, biochemistry, molecular biology, genetics and phylogeny. This review offers a concise summary of the state of the art in this field with special reference to taxonomy and ecology as well as to the different methodologies used to study the phylogeny of this unusual group of microorganisms that question many well established biologycal concepts.

Key words: Archaebacteria, phylogeny, rRNA, taxonomy, primary kingdoms.

Resumen

El descubrimiento de las arqueobacterias hace diez años ha supuesto en microbiología un profundo cambio en los conceptos de fisiología, taxonomía, ecología, bioquímica, biología molecular, genética, y filogenia. Esta revisión ofrece un sumario conciso de la situación en la que se encuentra este campo, con especial referencia a la taxonomía, ecología, así como a las distintas metodologías utilizadas para el estudio de la filogenia de este grupo inusual de microorganismos, que cuestionan muchos de los conceptos biológicos establecidos.

Introduction

The concept of archaebacteria was first proposed by Woese and Fox in 1977 to describe the phylogenetic differences between methanogenic bacteria and the many prokaryotic bacteria.

These authors proposed the existence of three primary kingdoms, putting archaebacteria on the same taxonomic level as eubacteria and eukaryotes. The name referred to the apparent antiquity of the methanogenic phenotype which fit the atmosphere that a primitive Earth was supposed to have had, rich in CO_2 with some H_2 and virtually no O_2. Their metabolism, especially adapted to the conditions that presumably existed at the beginning of life on Earth, and the detailed analysis of certain molecular characteristics led Woese to propose that these archaebacteria were ancestors of both eubacteria and eukaryotes (108), thus implying that this new group of organisms might in fact be the oldest.

Several factors such as the absence of murein in cell walls (43, 44), membranes made up of isopranyl glycerol ether lipids (53, 54, 55), the structure of the RNA polymerase (114, 117, 119, 122) and most significantly, a series of characteristics related to the translational apparatus consisting of the structure and function of the elongation factors (26, 45, 63), the sequence and structure of their

(*) Corresponding author.

rRNAs (23, 24, 25, 30, 38) and proteins (68, 104), their sensitivity to protein synthesis inhibitors (2, 3, 7, 11, 37, 63, 74, 81), and the genetic organization of the rRNA operons and regulation signals (13, 16, 18, 58, 60, 62), all seem to offer ample justification for the consideration of archaebacteria as a kingdom separate from the rest of the prokaryotes (eubacteria) (Table 1).

Archaebacteria live under extreme conditions. The least extreme of all are the methanogen's ecological niches, which are extremely anaerobic but not unusual. The high saline concentration of the habitats of the extreme halophiles makes it impossible for other organisms to colonize them. The sulfur metabolizing thermophiles have been isolated in every kind of hot springs (extremely acid, anaerobic, deep ocean, etc.).

Archaebacteria constitute a coherent phylogenetic unit with a «status» similar to that of eubacteria and eukaryots. This unit has two principal branches with methanogens and extreme halophiles on one and sulfur metabolizing thermophiles on the other. *Termoplasma acidophilum* is not clearly related to either, but appears to have some periferal relation to the former, in spite of the acidothermophilic nature of its habitat (109).

TABLE 1
DIFFERENTIAL PROPERTIES BETWEEN THE THREE PRIMARY KINGDOMS. Woese and Olsen (110)

	Archaebacteria	Eubacteria	Eukaryotes
Size (μm)	~1	~1	~10
Organelles	–	–	+
Nuclear membrane	–	–	–
Cell walls	Extremely diverse	Murein	Lack, in some cases cellulosa chitin or saccha.
Membrane lipids			
Chain type	Phytanil & byphytanil	Faty acids	Faty acids
Glycerol linkage	Ether	Ester	Ester
ATPases	DCCD insensitive	DCCD sensitive	DCCD sensitive
RNA polymerase	Diverse	Eubacterial	Eukaryotic
Splicing	+	–	+
Histone-like proteins	+	–	+
Ribosomes			
Subunits size	30S, 50S	30S, 50S	40S, 60S
Shine & Dalgarno sequence	+	+	–
Initiator aatRNA	Methionine	Formylmethionine	Methionine
Antibiotic sensitivity	Extremely variable	Eubacterial type	Eukaryotic type
5' terminal of 5S rRNA triphosphorilated	–a	–	+
Modified nucleotides in the 16/18S rRNA	10[a]	10	25
Dihydrouracil in tRNA	–b	+	+
Protein/rRNA relation	variable[c]	low	high
Order of transcription of rRNA genes: 5' (16s–(tRNA)–23S–5S) 3'	+d	+	–
mRNA with poly-A	–a	+	+

a: except thermophilic sulfodependent. b: except Methanomicrobiales. low: Mehtanobacteriales, Methanomicrobiales and c: Halophiles. High: All the others. d: except *Thermoplasma* and the thermophilic sulfodependent.

According to their genotype (the distances between their rRNA sequences appear to be greater than those between eubacteria) and their extremely variable phenotype, archaebacteria appear to be the most diverse of the three kingdoms. The sulfur metabolizing thermophiles seem so different from the methanogens and halophiles that some authors have suggested they be divided into two different kingdoms (12, 50, 51, 52). The reasons for this wide genotypic and phenotypic diversity are presently unknown. They could reflect a rapid evolutionary rate or simply be due to the fact that they are much more ancient than the other kingdoms, or possibly that the common ancestor was a simple entity, more primitive and less evolved than the ancestors of eubacteria and eukaryotes.

This review consists of a concise description of each of the three main archaebacterial groups, focusing on their ecology, taxonomy, energetic metabolism and phylogenetic relationships measured by different techniques in an attempt to summarize their most notable characteristics.

Methanogenic archaebacteria

The biological production of methane is a process that takes place every time organic material decomposes under anaerobic conditions. It is the result of the metabolic activity of a small highly specialized group of microorganisms that perform the final stage of the trophic chain in these environments. They convert the products of fermentation, especially CO_2, H_2 and formiate, produced by other anaerobes, into methane. This property led H. A. Baker, in 1956, to group a series of morphologically diverse but physiologically similar bacteria into one family known as *Methanobacteriaceae* (6).

Methanogenic archaebacteria, or more simply, methanobacteria, obtain their energy by forming methane from the reduction of CO_2, using electrons generated by the oxidation of H_2. Some species belonging to the *Methanomicrobiales* order are able to ferment compounds such as formiate, acetate, methanol and methilamine. Many can reduce molecular sulfur to H_2S with greater or lesser loss of their methanogenic efficiency (91). Recently, the ability of different methanobacteria to reduce CO_2 to CH_4 using elemental Fe as the only source of electrons, albeit with substantially less efficiency in their production of methane than when H_2 is used directly, has been described (14). To date, no secondary or alternative sources of energy for the production of methane have been found. A broad review of the sources of carbon and of essential, as well as growth stimulating vitamins, was carried out by Whitman in 1985 (103).

Methanogenic bacteria are the only archaebacteria that are not restricted to extreme ecological niches. In fact, methanobacteria are found in a wide variety of anaerobic habitats including acuatic sediments (swamps, ponds, lakes and oceans), insect and mammal intestinal tracts, sewage processors, trees and hot springs (4). They are common in anoxigenic environements in which organic material is in anaerobic decomposition. There, they occupy the last link in the transfer of electrons generated by this decomposition, especially at redox potentials of less than 200 mV. In their natural habitats the fermentation of organic material is initiated by non methanogenic heterotrophic eubacteria producing H_2, CO_2 and volatile fatty acids, all direct substrates for the production of methane.

In the 8th edition of Bergey's Manual methanobacteria are grouped for the first time in one family: *Methanobacteriaceae,* made of three genus, *Methanobacterium, Methanosarcina,* and *Methanococcus.* In 1979, the taxonomy of methanogenic bacteria was reorganized to reflect the phylogenetic relationships determined by the analysis of the sequences of 165 rRNAs (4). The results obtained by comparing the oligonucleotide catalogs of sixteen species led to a new schematic outline describing the relationships among the methanobacteria. The degree of difference between some of its members is as great as that of Gram + and Gram – eubacteria, justifying its division into three orders, four families and seven genus. The criteria for establishing such taxonomic levels is found in

the range of values of their association coefficients which are similar, in each case, to those found in eubacteria when the same taxonomic levels are established.

Currently, three orders (*Methanobacteriales, Methanococcales* and *Methanomicrobiales)* have been described and include five families *(Methanobacteriaceae, Mehtanothermaceae, Methanococcaceae, Methanomicrobiaceae* and *Methanosarcinae),* fourteen genus and more than thirty described species (Fig. 1). The comparison of the 16S rRNA sequences (60, 109) indicates a profound difference between *Methanobacteriales* and *Methanomicrobiales* on one hand and *Methanococcales,* on the other, which is confirmed by buoyant density measurements (12). Buoyant density is higher for the *Methanococcales* than for the other two methanogenic orders and related archaebacteria like Halobacteria.

Evidence to support this new taxonomy can be found in the structure and composition of the cell wall, the distribution of lipids, intermediate metabolism, nucleic acid composition, etc... A detailed description of the orders, families, genus and species was carried out by Balch (4) and Whitman (103). New species with intermediate properties have been recently incorporated to the methanogenic order: extreme thermophiles (59), halophiles (126) and new metabolic properties (39, 71).

Extreme halophilic archaebacteria

The extreme halophilic archaebacteria, also known as extreme halophilic bacteria, red halophilic bacteria or simply Halobacteria were identified long ago because they give a reddish tint to any medium they grow in, which can result in significant economic losses in the salt fish, bacon, preserved meat and tanning industries.

Halobacteria can be found anywhere, in extremely saline natural lakes and ponds, or in marine salt flats where salt is concentrated by evaporating sea water in the sun. They appear quickly in any aquatic habitat where the saline conditions can support them, turning the water red. They probably travel in salt crystals, are blown in the wind or on the legs and feathers of birds that inhabit these areas.

The halophilic archaebacteria most frequently studied come from salt lakes such as The Dead Sea (Israel), The Great Salt Lake (USA) and The Wadi Natrum Lakes (Egypt). They are defined as terminal desert lakes with no natural efluents, or as marine salt flats. The ionic composition of these habitats differ widely, from 250 to 400 gr/l, although the most important differences are qualitative. In The Great Salt Lake, Na^+ is the main cation, while in the Dead Sea it is Mg^{++}. In the Wadi Natrum and Magady (Kenya) the concentrations of HCO_3^-/CO_3^- are very high leading to pH values greater than eleven and the complete elimination of soluble Mg^{++}.

Halobacteria are chemorganotrophic and need organic material in order to grow, preferably proteins and aminoacids rather than carbohydrates. The former are supplied by algae and halotolerant cyanobacteria that eventually burst as the osmotic pressure increases, and by primary producers including cyanobacteria, sulforeducers and photosynthetic eubacteria of the genus *Ectothiorhodospira.* Halobacteria are the last link in the trophic chain and act as mineralizing agents. Most are strict aerobes, although some strains develop anaerobic respiration using nitrates as electron acceptors.

The taxonomy of halobacteria is rather confused and is currently being revised. This is due to several factors: 1) the existance of «strains» defined as different species that appear to constitute only one, e.g. *H. salinarium* CCM 2148, *H. halobium* CCM 2090 and *H. cutirubrum* CCM 2088, which should probably be considered *H. salinarium;* 2) the existance of strains in different collections, e.g. *H. halobium* NCIB 8720, CCM 2090, NCMB 736, NCMB 777, *H. cutirubrum* NRC 34001, CCM 2088, NCMB 763, etc..., which makes comparisons of results from different laboratories extremely difficult because in some cases the analysis of their nucleic acids (79) and polar lipids substantiate that they are different species; 3) the appearance of new isolates that are not readily attributed to an

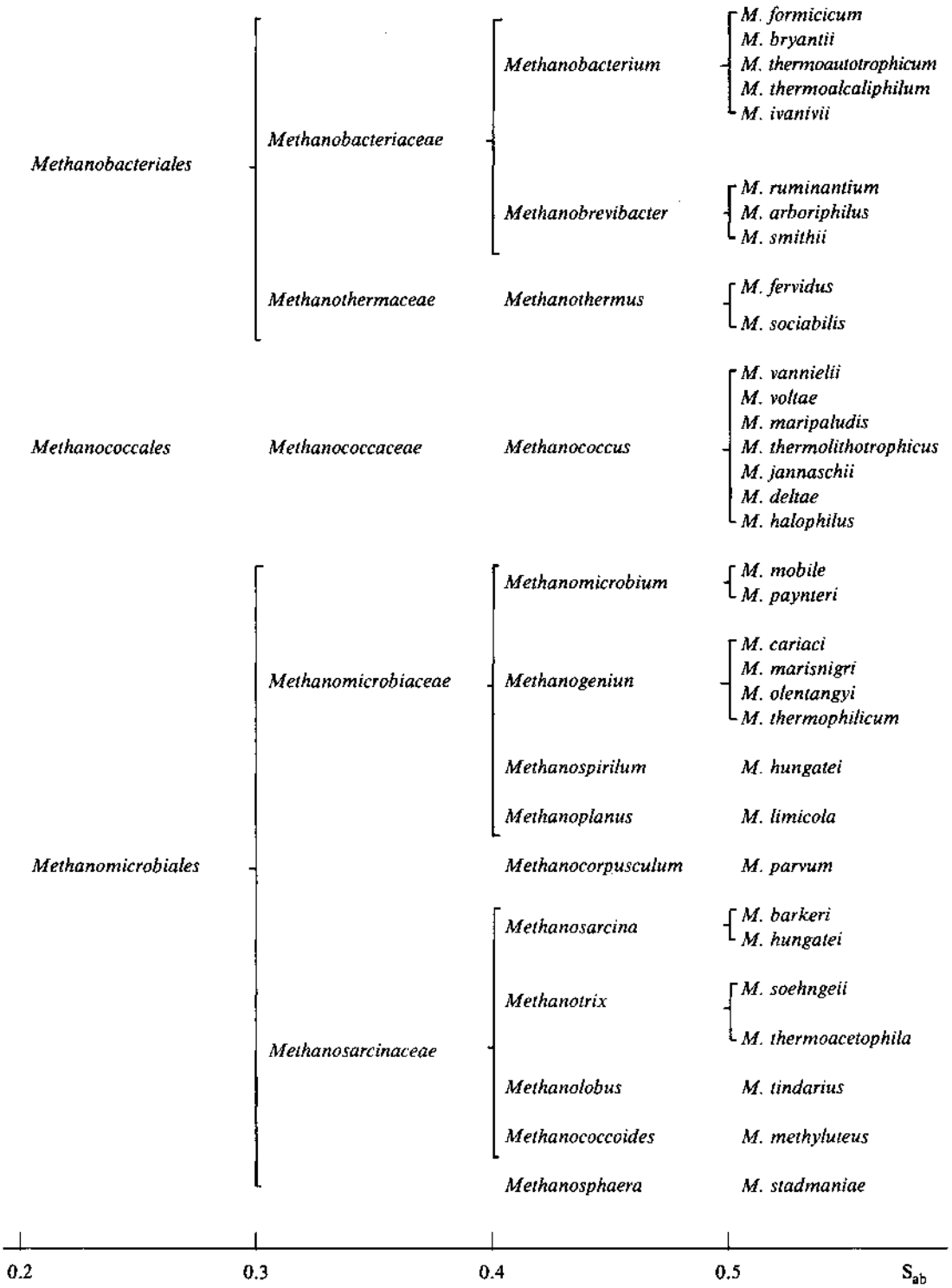

Fig. 1 Association coefficients between the different methanogenic archaebacteria (after 103).

established species and are described in awkward terms, e.g. the square or box-shaped species isolated by Walsby in 1980 and the genus *Haloarcula* (98); 4) or even the loss of species, e.g. the first isolate of *H. maris-mortii* carried out by Elazani-Volcani in 1940 in The Dead Sea, the type strain of *H. halobium* NCIB 8720 or *H. trapanicum* NRC 34021.

The 8th edition of Bergey's Manual recognizes two genus: *Halobacterium* (Elazani-Volcani 1957) and *Halococcus* (Shoop 1935). These contain the six recognized species *(H. salinarium* NRC 34002 - ATCC 33171, *H. volcanii* NCMB 20212, *H. saccharovorum* ATCC 29252, *H. vallismortis* ATCC 29715, *H. pharaonis* DSM 2160, *Hc. morrhuae* ATCC 17082; and the «incertae sedis» *H. trapanicum* and *H. vallismortis* NRC 34021). Also in 1984, Tindall and coworkers described two new genus of alcalophilic halobacteria *Natronobacterium* and *Natronococcus* (97).

Ross and Grant proposed in 1985 the existance of nine different groups of extreme halophiles based on DNA/rRNA hybridization data (79). Polar lipid patterns and oligonucleotide catalogues supported their hybridization data. The representative organism of each taxa is given by type culture collection species (CCM 2084 —*Hb. salinarium,* NCMB 777-*Hb. halobium,* NCMB 2012-*Hb. volcanii,* NCMB 2192-*Nc. occultus,* NCMB 787-*Hc. morrhuae,* ATCC 29715-*Hb. vallismortis,* NRC 34021-*Hb. trapanicum* and NCMB 763-*Hb. cutirubrum).* This proposal has been quoted in a recent report from Tindall and Trüper (98).

Rodríguez-Valera and collaborators, after analyzing phenotypic characteristics by numerical taxonomy and taking into consideration the composition of the polar lipids of the membranes, have proposed that extreme halophilic rod shaped and non alcalophilic archaebacteria be classified in three genus: *Halobacterium, Haloferax* and *Haloarcula* (99). The main distinguishing characteristics of the six genus, which according to the authors constitute the family *Halobacteriaceae* are included in Table 2.

Several authors have revised this area extensively in recent years (42, 49, 57, 72). They have proposed the reclassification of old species, the establishment of type strains, and the definition of new genus. In addition genomic organization studies do not agree with the extremely conserved sequences of 16S rRNA results (Amils unpublished results). In any case, it seams reasonable to suggest that the taxonomy of this important group of archaebacteria needs some further clarification.

The phylogenetic status of halobacteria in relation to the rest of the archaebacterial kingdom seems clear to most authors. Oligonucleotide catalogs (23, 24), rRNA/DNA hybridization (100, 123), 5S rRNA sequences (24), total 16S rRNA sequences (109, 110), and antibiotic sensitivity (74), all indicate that halobacteria are located in the methanogenic branch of the archaebacteria, generally near the *Methanomicrobiales.* Only Lake (51) differs by pointing out the tridimensional structure of the ribosomal subunits, indicating that eubacteria and halobacteria evolved together. His proposal is not supported by other techniques, and has been discussed critically by several authors (109, 110).

Sulfur-metabolizing thermophilic archaebacteria

The name, sulfur dependent thermophilic archaebacteria, was proposed by Zillig, Stetter and coworkers (22, 121) to substitute the earlier name, thermoacidophilic archaebacteria, because some of the newly discovered species tolerate or prefer near neutral pHs and all of them can obtain energy by metabolizing sulfur. Their ecological niches are the most restricted and limited of all, although they can be found anywhere on the planet.

Currently, three main groups are recognized; *Sulfolobales, Thermoproteales* and the organisms from submarine volcanic areas. Lately a new order has been proposed *Thermococcales,* as a fourth branch of the sulfur dependent archaebacteria. *Thermoplasma* although it is not sulfur dependent is thermoacidophilic and is included in this section for historical reasons.

Prior to any detailed description of this group, it is important to note the diversity of the organ-

TABLE 2
DIFFERENTIAL FEATURES BETWEEN THE DESCRIBED GENERA OF HALOPHILIC ARCHAEBACTERIA (adapted from 42)

	Halobacterium	*Haloarcula*	*Haloferax*	*Halococcus*	*Natronobacterium*	*Natronococcus*
Cell morphology	Long rods	Short pleomorphic rods	Short pleomorphic rods	Cocci	Long rods	Cocci
G+C	66-71	62-78	59.5-64	64.6	62	64
Gram	–		–	+	–	+/–
Origin	In the most concentrated pons of solar salterns.	Solar salterns and salt lakes of very diverse features.	Intermediate salt concentrations. Dead Sea.	Salt fish	Saline soda lakes	Saline soda lakes
Optimum pH for growth	6-5-7.5	7.0	7.0	7.2	7.7-9.5	9.5
Aminoacid requirement	+	–	–	+	ND	ND
Mg^{++} requirement for growth (mM)	5	5	10-40	ND	10	ND
Minimal total salts to grow at 38° C	20 %	15 %	10 %	15 %	12 %	8 %
Polar lipids						
PGP and PG	+	+	+	+	+	+
PGS	+	+	–	–	–	–
S-DGD	–	–	+	–	–	–
DGD-1	–	–	+	?	–	–

N.D.: Not determined. PGP: Phosphatidilglycerophosphate. PGS: Phospatidylglycerosulfate. PG: Phosphatidylglycerol. S-DGD: Sulfated diglycosyl diether. DGD-1: Diglycosyl diether.

isms that make it up: 1) the association coefficient of the oligonucleotide catalogs of *Thermoplasma* and *Sulfolobus* is only 0.17 (the lowest found between two members of the same kingdom); 2) the sulfur metabolisms of *Sulfolobus* and the *Thermoproteales* appear to be opposites (although this is not absolutely correct as will be discussed further on) and furthermore there is no crossreaction of their RNA polymerases with immunodiffusion techniques which indicates at least interfamily distances; 3) to date the phylogenetic relation of marine isolates with the rest of the families of the group is still unknown and lastly; 4) the continuous appearance of new species with concomitant changes in taxonomic status of species and genus.

Knowing full well that new discoveries will shed more light on this situation we will, for the sake of clarity, briefly describe each of the four orders separately.

Thermoplasma

Thermoplasma acidophilum, the only species in this genus, was isolated in a coal refuse pile in the Friar Tuck mines in Indiana by Darland and coworkers in 1970 (15). Brock in 1978 (10), Langworthy and coworkers in 1984 (56). In 1985 Stetter and coworkers revised its properties (93). *Thermoplasma* lacks a cell wall, which made some consider it a thermophilic mycoplasm, but the nature of its membrane lipids (55), its 16S rRNA sequences (23, 110) and its RNA polymerase (112, 114, 107, 119) put it undoubtedly among the archaebacteria.

Their presence in their natural habitat, burning coal refuse piles at temperatures of 55-60 °C and pH of 2, only two years after the pile was formed supports the idea that the organism grows and multiplies easily under adequate conditions. This does not necessarily eliminate the possibility that *Thermoplasma* was already in the coal. An organism similar to *T. acidophilum* was isolated in hot springs in Japan and although little or nothing has been published about them since then, the references available (93) seem to indicate that it is, in fact, a *Thermoplasma,* thus greatly widening the ecological niche of this archaebacteria. The presence in *Thermoplasma* of histone and actin-like proteins, citocrome b, as well as the nature of its superoxide dismutase and its RNA polymerase, have led to the idea that it may be specifically related to the eukaryotes. *Thermoplasma* might be an ancestor of the urkaryote (84).

Within the archaebacterial kingdom, the comparison of oligonucleotide catalogs (106) and studies of DNA/RNA hybridization (100) and 5S rRNA sequences (24, 64) all seem to place *Thermoplasma* between methanobacteria and sulfodependent thermophiles, closer to the former.

Recent studies using measurements of DNA/rRNA crosshybridization velocities (125) and 16S rRNA sequence comparison (109, 110) clearly place *Thermoplasma* on the Methanobacteria-Halobacteria branch of the archaebacteria.

TABLE 3
PROPERTIES OF THE THERMOPHILIC SULFOMETHABOLIZING ARCHAEBACTERIA

Species	Temperature	pH	Strict Aerobiosis	Autotrophy (CO_2 utilization)	Energy Source
Thermoplasma	45-62	0.96-3.5	+	–	Organic compounds
S. acidocaldarius	55-85	2-3	+	+	S^0, organic compounds
S. solfataricus	50-90	3	+	+	S^0, Fe^{++}?, organic comp.
S. ambivalens	94	2.3	–	+	S^0
A. brierleyi	45-75	1.5-2	–	+	S^0, Fe^{++}
A. infernus	65-95	1.5-4.5	–	+	S^0, organic compounds

Sulfolobales

Sulfolobales have been isolated in acidothermal springs all over the wororld, including the USA, Italy, New Zealand, Japan and Iceland. *S. solfataricus* and *S. acidocaldarius* have been found in solfataras in North America, Italy and Japan, indicating that there are no geographic barriers to their propagation. The temperature in the springs ranges between 60°-100° C with a pH between 1-5. Most of the isolates were found in 80-90° C waters with a pH between 2-3 containing elemental sulfur and generally low ionic content (Table 3).

Their metabolism is varied. Many strains are facultative heterotrophs (using yeast extract and sugars as carbon sources) or chemolithoautotrophs (using CO_2 as the only carbon source) obtaining energy by oxidizing sulfur into H_2SO_4 or, in some species, by reducing it to H_2S. The oxidation of sulfur or in some cases of Fe^{++} takes place through aerobic respiration using O_2, or MoO_4^- as electron acceptors. Other species can only grow heterotrophically and the strain B 6/2, isolated in Japan, is a strict chemolithoautotroph.

J. A. Brierly isolated and preliminarily described the first example of the *Sulfolobales* order in an acidothermal spring in Yellowstone Park in 1966. The first complete description was not carried out until 1980 when Zillig and Stetter (114) named it *Sulfolobus brierleyi* after its discoverer. In the

same paper they renamed another organism that had been isolated and named *Caldariella acidophila* by Rosa and coworkers in 1975. Zillig and Stetter called it *Sulfolobus solfataricus*. As a result, in the 8th edition of Bergey's Manual only one species is described as belonging to the genus *Sulfolobus: S. acidocaldarius* (8), which was isolated by Brock in 1970 at Roaring Mountain in Yellowstone Park. In 1985 Stetter's and Zillig's groups, working separately, described two microorganisms that were isolated in Pisciarelli (Pozzuoli, Italy) and Leirhnukur (Iceland), and included initially in the genus *Sulfolobus* because in addition to sharing the well known sulfo oxidant nature of this genus, they were able to grow in strict anaerobic conditions, using CO_2 as the sole source of carbon and reducing elemental sulfur in order to obtain energy. These microorganisms, called SO-4 (85) and *S. ambivalens* (122), respectively, are the first known living organisms which, depending on the redox potential are able to grow both aerobically and anaerobically, using the same energy source, elemental sulfur, and the same metabolic routes in either direction, producing H_2SO_4 or H_2S. Studies of the known members of this group revealed that *S. brierleyi* was also able to grow anaerobically reducing sulfur autotrophically. In 1986 Stetter and coworkers (86) proposed the creation of a new genus: *Acidianus* which would include two species: *A. Infernus* (previously known as *SO-4*) and *A. brierleyi*. The addition of *S. ambivalens* to this new genus has not, as yet, been carried out.

Brock in 1978 and Stetter and collaborators in 1985 and 1986 reviewed the genus *Sulfolobus* (9, 93, 94). Table 3 is a summary of some of its properties. The pattern of the RNA polymerase components (113, 123), the sequence of the ribosomal «A» protein (69), the tridimensional structure of the ribosome (50) as well as numerous characteristics related to the translation system (see Table 1) indicate that *Sulfolobales* and *Thermoproteales* seem to be close to the eukaryotic cytoplasm.

Thermoproteales

The order of *Thermoproteales* (115, 116) constitute the second great branch of the sulfur dependent thermophiles. It differs from *Sulfolobales* in being strictly anaerobic. Classically it was made up of two families: *Thermoprotaceae* and *Desulfurococcaceae* and four genus: *Thermoproteus* (116), *Desulfurococcus*, (118), *Thermofilum* (120) and *Thermococcus* (121). Members of this order have been isolated in Iceland *(Thermoproteus, Thermofilum* and *Desulfurococcus)*, California *(Thermoproteus)* and Italy *(Thermococcus)* in hot springs, marine water holes and solfataras fields, at pHs of 1.7-6.8 and temperatures of 90-100° C.

All of the *Thermoproteales* are able to grow by sulfur respiration of organic matter (yeast extract, peptides or proteins) except for *Thermoproteus neutrofilus* V 24, which is a strict chemolithotroph. *Thermoproteus* can grow chemolithotrophically and *Desulfurococcus* and *Thermococcus* can do so in the absence of sulfur by fermenting organic matter in an undetermined and inefficient system. *Thermofilum pendens* requires a fraction of the polar lipids synthesized by *Thermoproteus* in order to grow. Stetter and collaborators have reviewed the morphological characteristics, chemical composition and molecular biology of this group (93, 94). From a phylogenetic point of view the *Thermoproteales* are closely linked to the *Sulfolobales*. Their RNA polymerases, DNA/RNA hybridization, pattern of sensitivity to translation inhibitors, 16S rRNA sequences, etc., confirm the proximity of the two orders. *Thermococcus* is the furthest from the group since it is located midway between the sulfodependent and the methanogens-halophiles (109), making it likely to be the archaebacterial line with the slowest evolution and placing it nearer the root of the archaebacterial tree.

Recently, different authors (21, 125) have described a new genus: *Pyrococcus*, with two species: *P. furiosus* and *P. woesei*, strict anaerobic organisms that are heterotrophic by sulfur respiration and whose optimal growth temperature is around 100° C. These organisms have all the typical archaebacterial characteristics and the pattern of their RNA polymerase components is similar to that of *Thermoproteales* and *Sulfolobales*. Immunodiffusion crossreaction among RNA polymerases and

DNA/RNA hybridization places them close to *Thermococcus,* leading Zillig and coworkers to propose the creation of a new order: *Thermococcales,* with only one family, *Thermococcaceae* and two genus: *Thermococcus* and *Pyrococcus.*

Thermoproteales seems to be a very ancient «philum» from which *Sulfolobales* emerged when O_2 appeared in the biosphere enabling it to oxidize sulfur. This parallels the relationship of methanogens to halophiles. The similarity of the characteristics of both groups: cell envelope, membrane, ribosomes, RNA polymerase, synthesis of glucogen, etc..., all support this point of view. In addition, their method of obtaining energy appears to be adapted to the conditions of primitive Earth, and the absence of binary cell division and the low efficiency of its glucosyl transferases also strongly support this theory (93).

Organisms from submarine volcanic areas

In 1982 Stetter reported the existance of microorganisms isolated in a field of submarine solfataras located in Porto Levante Bay on Vulcano Island (Italy). The pH was 6 and the temperature of the sediments was 103° C. Two types of organisms with different morphology and physiological properties were isolated and classified as two new genus: *Thermodiscus (T. maritimus)* and *Pyrodictium (P. occultum* and *P. brockii)* (92).

The presence of lipids with ether bonds, the sensitivity of their ribosomes to diphteria toxin, the existence of a cell envelope protein instead of a murein sacculus and the composition of their 16S rRNA, indicate that these new submarine isolates are, in fact, archaebacteria. Analysis of their nucleotide catalogues shows a relation with sulfur metabolizing thermophiles which is confirmed by comparing their 16S rRNA sequences (110).

Recently, in the same area, Stetter and coworkers (20) have isolated a strictly anaerobic, S° dependent microorganism whose optimal pH is 6.5 and temperature is 92° C, for which they have proposed a new genus: *Staphylothermus marinus.* Its relation to the other sulfurdependent thermophiles has not yet been established.

Archaeglobus; a new archaebacterial phenotype?

Stetter and coworkers (1985) have recently described an organism tentatively called *Archaeglobus fulgidus,* whose characteristics do not seem to fit any of the three classical archeobacterial phenotypes. *Archaeglobus* is able to reduce sulfates (it is the only archaebacteria known to do so) and produced small quantities of CH_4 (quantities of 0.1 % or lower than those produced by normal methanogenesis) although it lacks some of the cofactors usually related to methanogenesis.

Its thermophilic habitat, isolated in marine hydrothermal systems, and its means of obtaining energy through anaerobic respiration of highly oxidized sulfur compounds, seems to indicate that *Archaeoglobus* is a sulfodependent thermophile, whose ability to produce methane places it closer to Methanobacteria. Phylogenetically it could be the transition between the two archaebacterial phenotypes, on one hand, the sulfodependent, whose metabolism appears to be more primitive (109, 110) and ou the other Methanogeus. The 16S rRNA sequence appears to confirm this hypothesis, placing *Archaeoglobus* between *Thermococcus* and *Methanococcus* (1).

Phylogenetic relationship between archaebacteria and the Eubacterial and Eukaryotic kingdoms

One of the most exciting questions posed by the discovery of archaebacteria regards their evolutionary relationship with the other two well established kingdoms: Eubacteria and Eukaryotes. As

Woese has stated, one of the biggest problems in the study of evolutionary relationships is a direct result of the negative definition of the prokariotic kingdom in relation to the eukaryotic kingdom, and the subsequent difficulties in sorting out evolutionary information from this prokaryotic-eukaryotic dichotomy (105, 107). The introduction of a new kingdom whose outstanding property is that it shares partial homologies with the reference systems: eubacteria and eukaryotes, while having at the same time other unique properties, demonstrates unequivocally that archaebacteria are taxonomic cluster entities as different from the reference kingdoms as eubacteria and eukaryotes are from each other.

This part of the review is not intended to be an extensive survey of the current studies of phylogenetic relationships between archaebacteria, eubacteria and eukaryotes. There are several recent reviews which cover this in detail (109, 110). We propose to survey the different methodologies used to approach this important field, in order to discuss the state of the art of phylogenetic studies in archaebacterial research.

Any attempt to study phylogenetic relationships between different cell lines is based on the existence of reference elements. These reference elements fall into two classes: semantophoretic and syntactic moleculas (5, 127). The semantophoretic molecules (DNA, RNA, proteins) have primary sequences that are accurate copies of genetic information. Their comparison permits the establishment of relationships with direct phylogenetic significance. Syntactic molecules are the substrates or products of functions performed by semantophoretic molecules and make up most of the phenotypic cellular properties (metabolic routes, composition of the cell wall, coenzymes, etc.). These structures are useful in establishing taxonomic relationships, but they are considered of little phylogenetic value.

Taxonomic studies predate studies of cell line evolutionary relationships and their goal was to classify organisms using syntactic elements. Although all biological classifications are subject to the controversy over which methodologies are most appropriate (70), fast sequencing techniques have revolutionized the study of evolution. While the phenotype, at least in its classical conception, is currently considered too complex to be used in phylogenetic studies, especially in microbiology, Zuckerland and Pauling state that the direct comparison of the genotype should, in principle, allow the measurement of phylogenetic relationships.

Different genotypes exist for any given phenotypic property. This means that most of the changes fixed in the genome are selectively neutral, conferring a chronometric quality on them. A wealth of different genomic sequences that codify for the same function, i.e. rRNA, have been well documented. Genetic variability of this sort, unrelated to important phenotypic changes, is not submitted to selective pressure, making mutational changes detected on the primary sequence level serve as a chronometer not only of evolutionary relationships but also of relative times of divergence. In principle, they can allow the molecular characteristics of the common ancestor to be inferred.

The possibility of ascertaining phylogenetic relationships raises challenging questions. The most important is to find an appropriate molecular chronometer, which is not as easy as it may appear. Some molecules such as cytochromes might be appropriate for eukaryotes but not for eubacteria. A good molecular chronometer must have the following properties: 1) universal distribution; 2) slow and gradual rate of change, so early relationships can be detected; 3) constant function and 4) simple purification process.

Several molecules belonging to the translational apparatus fulfill all the above requirements. They are the rRNA, which have a constant function, an extremely conserved sequence, an easy purification process and universal distribution. Most phylogenetic studies have been focused on the protein synthesis machinery, some using rRNA sequences (5S rRNA, 16S rRNA, oligonucleotide catalogs, DNA/rRNA hybridization) and others, ribosomal protein sequences, structural ribosomal features, ribosomal function, etc. Still others circumvent the problem using different semantophoretic molecules like RNA polymerase, or more classical phenotypic approaches based on the comparison

of the cell wall structures, lipid content of the membranes and metabolic properties. The majority of the systems, however, are based on the comparison of primary sequences from the translational apparatus.

rRNA based methodologies

As stated previously, few cell components meet all the requirements of a good evolutionary chronometer. Of the translation apparatus components none are adequate, except for rRNA.

5S rRNA

5S rRNA has been sequenced and catalogued many times since 1967 when the first sequence for *E. coli* 5S rRNA was published (19), Kimura and Ohta first used these rRNA sequences in 1973 to study the differences between procariotes and eukariotes (46). The first phylogenetic trees were based on comparisons of 5S rRNA sequences by Hori and collaborators (32, 33, 34).

In 1978 Nazar and coworkers published the first 5S rRNA sequence for an archaebacteria, *H. cutirubrum* (73), followed in 1981 by *T. acidophilum* (64) and *S. acidocaldarius* (89). For the first time, it was possible to compare the three primary kingdoms and draw phylogenetic trees based on their sequence similarity (23, 35, 48) and secondary structure (24). While eubacterial 5S rRNA adopts a secondary structure that implies the formation of four helices the eukaryotic 5S rRNA follows a five helix model. The fact that both types of RNAs are different makes them excellent markers. Archaebacteria have eubacterial and eukaryotic characteristics as well as their own, and their 5S rRNAs can be placed on a gradient that range from *Methanobrevibacter smithii*, whose 5S rRNA follows a typically eubacterial model, to *Metanospirillum hungatii*, which is throroughly eukaryotic. Thermoacidophilic archaebacteria have pronounced eukaryotic characteristics (24). Based on this data the following evolutionary outlines have been proposed:

1. Eubacteria → Archaebacteria (type I) → Archaebacteria (type II) → Eukaryotes.
2. Protoarchaebacteria ↗ archaeb. (type I) → Eubacteria
   archaeb. (type II) → Eukaryotes

It would seem that the second scheme is more reasonable and would agree with the fact that type I archaebacteria have pseudomurein, although some inconsistencies do exist, like, for instance, the homology corresponding to ribosomal protein «A» from some members of the group is greater in eukaryotic type proteins. The existance of lateral genetic transfer should not be discounted, because, although it is greatly restricted in modern organisms, it could have had an important role in early evolution during the age of the progenotes (24).

Oligonucleotide catalogues

The obtention of oligonucleotide catalogues of 16S rRNA from different organisms and organelles was a fundamental step in the development of the concept of archaebacteria and the establishment of its kingdom status. Whereas 5S rRNA, with only 120 nucleotides, is too small for detailed phylogenetic analysis, the oligonucleotide catalogues allow much larger rRNA molecules to be compared, thus minimizing errors. In 1980, Fox and collaborators, using this technique, presented the phylogeny of the prokaryotes, suggesting a taxonomy based on genotypic rather than phenotypic data (23). The classification of archaebacteria and eubacteria, as well as the description of the phylo-

genetic relationships between the main phyla of this new kingdom, constitute the keystone on which all future studies were based (88). Since then oligonucleotide catalogues have been used for everything from the phylogenetic analysis of mycoplasma and their subsequent inclusion in the Gram + phylum (106) to the study of the origin and evolution of mitochondria and chloroplasts (47, 48), as well as to assign recently isolated organisms to an order, family or genus (87).

Total sequences of large rRNAs

The rRNA from the small ribosomal subunit (16/18S) is particularly useful as a molecular chronometer, since it is smaller than the rRNA from the large subunit (23/28S) but sufficiently bigger than the 5S rRNA to insure that any phylogenetic results obtained by comparison of their homo-

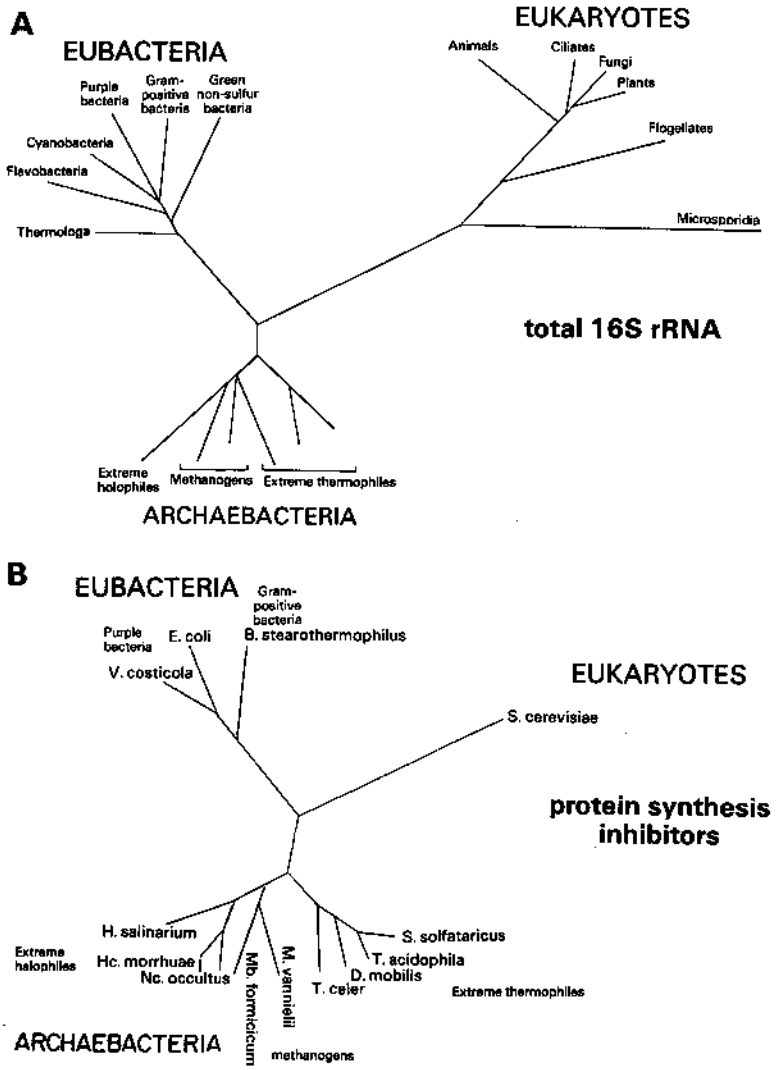

Fig. 2. Unrooted phylogenetic tree for the three urkingdoms. A) using total 16S rRNA sequences after Woese and Olsen (109), B) using antibiotic sensitivity (Amils et all, unpublished).

logy are not affected by small non chronometric changes (107). Its sequence contains regions of different degress of conservation which permits, not just the detection of close relations but also very distant ones, making the study of a wide range of phylogenetic relations possible. The comparison of 16S rRNA sequences allows much more statistically precise homology values than other techniques such as oligonucleotide catalogues or DNA/rRNA hybridization. Sequence variations in their primary structure can be correlated with secondary structure features or functional tertiary structures.

Close to sixty total sequences of the rRNA of the small ribosomal subunit and thirty from the big subunit from the three kingdoms and different organelles have been published to date (27, 38). In archaebacteria, the sequences of the 16S rRNA from four halobacteria have been published: *H. volcanii* (29), *H. morrhuae,* (61), *H. cutirrubrum* (36) and *H. halobium* (65). The 23S rRNA of the last is also known (66). In Methanobacteria, the 16S and the 23S rRNA of *M. vannielii* (40, 41) as well as the 16S rRNA of *M. formicicum* (60) and *M. hungatei* (111) have been studied. The 16S rRNA of *T. tenax* (85) and *S. solfataricus* (75) have been sequenced from the sulfodependents.

Woese and collaborators using these sequences and other unpublished ones, (110) have divided the archaebacteria into two main groups, thermophilic sulfur dependent being the first, and the methanogens and relatives the second. The degree of relatedness of 16S rRNA sequences both between kingdoms and within each, is shown in Figure 2. The three kingdoms are well defined with the archaebacteria grouping together in a coherent fashion, and the eubacterial and eukaryotic systems arising away from the archaebacterial taxon. The position of *Thermoplasma* and *Thermococcus* in the archaebacteria is somewhat intermediate, closer to the methanogenic branch than the sulfo dependent one (109, 110). The three kingdoms can also be easily defined when specific positions on the rRNA molecule, that distinguish one group from another (sequence signature), are analyzed. This type of analysis does not allow the position of the common ancestor of the three lines of descent to be located (110).

DNA/rRNA hybridization

Using the de Ley and de Smedt methods (17), Tu and coworkers (100) constructed phylogenetic trees for several archaebacterial species. Only comparison between related species can be made with this technique, relationships between different kingdoms can not be studied. But in spite of this limitation, Ross and Grant have used this method to establish relations among the halobacterias (79).

Other methodologies based on the characteristics of the translation apparatus

Ribosomal proteins

Very few archaebacterial ribosomal proteins have been sequenced to date. The only complete sequences we are aware of is the Hcu-L12 (also known as HL20) from *H. cutirubrum* and the Hma-S3, Hma-13b and Hma-S12 from *H. marismortui.* The partial sequences of several *H. cutirubrum, H. marismortui, M. vannielii* and *S. acidocaldarius* are also known (for a more thorough review see 104). Two domains, the 5S rRNA complex and the ribosomal «A» protein complex have been studied in some detail. The eubacterial and archaebacterial 5S rRNA complex is made up of two or three proteins bound to the RNA, whereas in eukaryotes it consists of one very large protein. When these riboproteins are compared strong homologies among the three kingdoms appears (104).

The ribosomal «A» protein domain in *E. coli* consists of four copies of the L7/L12 protein and

one copy of L10 protein. The equivalent domain in *H. cutirubrum* is made up of four copies of the HL20 ribosomal protein and one copy of HL11. Each of the phylogenetic groups have one class of «A» proteins with their own structural characteristics. In spite of this, we can deduce from sequence data that the similarities between archaebacterial and eukaryotic «A» proteins are greater than those between either of them and eubacteria. Other archaebacterial ribosomal proteins appear to be more closely related to eubacterial ones (104, 68, 69, 86). The paucity of known sequences must be born in mind before any firm conclusions are drawn.

Another unusual characteristic of archaebacterial ribosomal proteins is their extreme acidity, much higher than the values measured in eubacterial and eukaryotic systems. It seems that a correlation exists between the acidity of the proteins, the cytoplasmatic ionic concentration and the phylogenetic relationships between the archaebacteria (67).

Ribosomal size and shape

Although archaebacterial ribosomes have a sedimentation coefficient analogous to that of the eubacterial ribosome, 70S, they present a series of characteristics, like number of proteins, RNA/protein relation, buoyant density and shape which are unhomogeneus when compared to the reference systems. In eubacteria the ribosomal mass and the RNA/protein relation remain constant from the cyanobacteria to the extreme thermophiles, indicatyng that structural complexity has been / uomaintained constant throughout the adaptation to different conditions. In archaebacteria this uniformity does not exist. The ribosomes from *Methanobacteriales* have a isodensity sedimentation value similar to the eubacterial ribosomes and the *Methanococcales* show a lower value which is close to the sulfodependent thermophilic type. This indicates that their ribo somes are richer in proteins, like the eukaryotic ones, whose density is maintained constant throughout the kingdom (12).

Another difference to consider is the number of ribosomal proteins, which range from 52-55 in eubacteria to 70-84 in eukaryotes. Again archaebacterial ribosomes present enormous variability. While *M. bryantii* and *M. thermoautotrophicum* have 55 and 54 proteins respectively. *M. vannielii* have 58-60, *S. solfataricus* 62, *S. acidocaldarius* 61-64 and for *H. cutirubrum* there are discrepancies in the literature, from 54 to 60-65 (12, 67, 82, 83). These data seem, again, to place the *Methanococcales* closer to the thermophiles, although, other techniques, like the RNA polymerase, tend to group all the methanogens with the halophiles.

It seems clear that these ribosomal differences have a phylogenetic, rather than a simple adaptative origin. A reasonable suggestion would be that the two types of ribosomes found in archaebacteria represent different evolutionary stages of the translational apparatus. Cammarano and coworkers interpreted that the older ribosomes are bigger, and structurally closer to the particle which could have existed when the cell line diverged from the common ancestor. Due to the fact that during the logarithmic phase of growth around 30 % of the celular proteins are ribosomal, there is an obvious disadvantage in maintaining particles with a higher number of proteins than those strictly needed. If this is correct, the ribosomal miniaturization in the halophiles and most of the methanogens represents an evolution of their ribosomes similar to that of eubacterial.

Another aspect related to the use of ribosomes to establish phylogenetic relationships between the different kingdoms is their morphology (50, 51, 52). Using different morphological structures that appear when the ribosomes are analyzed by electron microscopy Lake has proposed four kingdoms, different from the ones proposed by Woese, which have been questioned extensively (96, 109, 124) due to the fact that they are based on variable, unquantified properties, which are not present in all of the population and which need external properties of dubious semantophoretic value to support them.

Ribosomal sensitivity to protein synthesis inhibitors

Antibiotics, when they act in a specific mode, are very useful tools to study physiology, genetics, molecular biology and evolution. Classically they have been used, from a phylogenetic point of view, to differenciate eubacterial and eukaryotic protein synthesis. They were an important molecular marker in ascertaining the eubacterial origin of mithocondria and chloroplasts. The more useful antibiotics for this type of work have been the specific protein synthesis inhibitors, whose specificity is not only related to the type of ribosome but to the different steps of the translation system (101).

In the case of archaebacteria many different specific inhibitors have been tested to determine the eubacterial or eukaryotic membrane, the RNA polymerase, the DNA replication and in great detail, the protein synthesis process (3, 7, 74). The pioneer studies using protein synthesis inhibitors in archaebacteria were done *in vivo* (31, 77, 82, 102), later followed by studies done using cell-free systems, whose interpretation is straightforward because possible complications related to transport and/or inactivation of the inhibitor are eliminated. Our group in collaboration with Dr. Böck's group in Munich and Dr. Cammarano's group in Rome, have developed a ribosomal program for the screening of archaebacterial ribosomal sensitivity to inhibitors with different structures and different structural and functional specificity (2, 3, 11, 63, 74, 81, unpublished results).

The comparative functional study was carried out using several statistical methods of analysis (74). The results obtained allow us to conclude that this functional analysis is of phylogenetic value because it shows a phylogenetic relationship between the different types of archaebacteria and the other two kingdoms that is similar to the one obtained using rRNA sequences (109, 110). The comparative results obtained using signature sequences of 16S rRNA from different organisms and protein synthesis inhibitors are displayed in Fig. 2.

Other techniques

RNA polymerase

Zillig, Stetter and collaborators have made a detailed study of the DNA dependent RNA polymerases from several archaebacteria and compared them with the structure and function of eubacterial and eukaryotic transcription systems (113, 117, 123, 125). The archaebacterial RNA polymerase type differs from the eubacterial one in structural complexity (nine to eleven components versus four to seven in eubacteria). They do, however, resemble the eukaryotic systems, especially yeast type I, in the following characteristics: 1) complexity, estequiometry, and molecular weight of the components, 2) insensitivity to rifampicin and streptolydigin and stimulation by silibine and 3) immunological cross reaction of specific antibodies rised against the heavy components. Within the archaebacterial RNA polymerases, two groups can be clearly distinguished: the thermophilic sulfometabolizing group that is closer to the eukaryotic transcription systems and the methanogenic halophilic group that is structurally less similar to them.

Cell wall

Classically, bacteria have been clasified into two groups according to the composition and structure of the cell wall, Gram – having a monolayer and Gram + a multilayer of murein. It seems reasonable to consider the origin of the eubacterial cell wall as monophyletic, with the development of

murein occuring after the separation of the eubacterial line from the progenote and a posterior divergence with two different cell wall structures.

The eukaryotic cell wall may have had a polyphyletic origin. While animals lack any envelope, plants, fungi, and algae have a rigid cell wall composed of cellulose, chitin, and a great variety of heteropolysaccharides respectively. Archaebacteria are very unusual in this respect. Their lack of murein and the wide variety of structures and polymers that constitute their envelopes and cell walls are characteristic of this kingdom. Table 4 shows a survey of the cell wall and cell envelope structures found in archaebacteria. Using these data and the association coefficient values of Fox and coworkers (23, 44) Kandler has constructed a phylogenetic tree that shows the evolution of the three kingdoms from a common ancestor which basically accords with the others, previously mentioned, obtained by genotypic or functional analysis. As opposed to eubacteria, the other two kingdoms did not develop a rigid sacculus or specific cell wall structure before the diversification to different cell lines. Even today animals and *Thermoplasma* lack cell walls, and the heteropolysaccharides of the cell wall of *Halococcus* and *Methanosarcina*, the protein of the envelope of *Methanospirilum* and the pseudomurein of the *Methanobacteriales* seem to be of recent development. The most frequent structures in archaebacterias are formed by subunits, suggesting that these types of envelopes were present in primitive archaebacteria. More information is needed on their chemical structure before concluding that glycoproteins and proteins have derived from the same gene.

The enormous discrepancy that exists between the cell walls of the two prokaryotic kingdoms, with one being uniform and the other multiform, might be explained by supposing that the development of the murein sacculus in eubacteria made it easier for them to adapt to a wide variety of biotopes, especially those with variable, low osmotic pressures. The first archaebacteria, on the other hand, lacking any wall, were forced to remain in their much more limited original habitats. These habitats are considered extreme now, but may very well have been «normal» when life on Earth began. Over time, most of the archaebacteria have developed a cell covering that is as effective, or almost, as murein, but its physiological specialization did not allow them to colonize new ecological niches, since these were already inhabited by eubacteria thanks to their early adaptation. Archaebacteria were limited to a species restricted diversification.

TABLE 4
ARCHAEBACTERIAL CELL WALL AND CELL ENVELOPE STRUCTURES.
After O. Kandler (44)

Organism	Rigid sacculus	Protein envelope	Polymer
Methanobacteriaceae	+	–	Pseudomurein
Methanosarcina	+	–	Heteropolysaccharide
Methanococcus	–	+	Protein subunits
Methanospirillum	–	+	Fibrillary protein sheath
Halobacterium	–	+	Glycoprotein subunits
Halococcus	+	–	Sulfated heteropolisaccharide
Sulfolobus	–	+	Glycoprotein subunits
Thermoproteus	–	+	Glycoprotein subunits
Desulfurococcus	–	+	Glycoprotein subunits
Thermoplasma	–	–	None

Lipids composition

All the techniques described so far are undoubtedly of value in establishing taxonomic and phylogenetic relationships between different organisms, but they are time consuming and inappropriate

for the rapid identification of new isolates. Within the methanogens and halobacteria the utilization of phenotypic characteristics for identification of new organisms is limited by their uniformity. The value of their morphology is very relative due to the pleomorphism of many species. Chemotaxonomic procedures like cell wall composition, polyamine distribution, etc. are not being used due to the fact that they only allow the family, or in the best of the cases, the genus of the new isolate to be assigned.

Grant and coworkers (28, 78, 80) proposed that the chromatographic analysis of polar lipids by TLC is a simple, fast and reliable technique capable of differenciating at the species level by providing extraordinarily complex patterns. As an example we can mention the utilization of this technique by Rodríguez-Valera and coworkers to establish a classification of the halophilic archaebacteria (42).

New techniques

Recently new methodologies or adaptations of old ones have been incorporated into the analysis of taxonomical and phylogenetic relationships. For instance, the comparison of energy obtainment systems, the structure and regulation of key metabolic enzymes, polyamine composition, etc., and even complex molecular biology techniques like heterologous reconstitution of ribosomes allow the degree of structural and functional homology of ribosomal components to be tested, leading to significant advances in the understanding, not only of phylogenetic relationships between different organisms but the deciphering of patterns of evolution. Quick karyotyping of different microorganisms and physical genetic maps of organisms with difficult or non existent genetic data can be carried out with fast sequencing techniques combined with electrophoretic systems with macrorestriction resolution capability (up to 10^7 base pairs).

Final considerations

In the midst of the avalanche of new discoveries unleashed during the ten short years of archaebiology's existance, it is difficult to summarize everything that has been learned. Every day something exciting appears that contradicts well establish dogmas. Probably the most important aspect of archaebacteria is the opening of new aspects of life that could not have been considered before because they were one order of magnitude away from normal biological behavior. It is obvious that the measurement of only one characteristic, even when it is considered a good chronometer, will not give the correct answer. Many different complementary techniques must basically agree. If all were to give the same type of results then it would be reasonable to infer that archaebacteria is not only a different primary kingdom but represents a primitive phenotype. In that case the studies on evolution will have a very important back up. Probably one of the most important findings in this area is the possibility of rescuing halobacterial cells trapped in salt sediment for one billion years, as recently reported by Grant and collaborators (personal communication). If this is so, microbiologists will have a precise chronometric ruler that can enable them to measure the rate of evolution in this group of archaebacteria by comparing it with extant related microorganisms. This could then be used to accurately date their phylogenetic trees.

Finally, the biotechnological potential of this group of microorganisms should be mentioned, which is mainly due to their outstanding properties which could be of interest in technological processes, from enzymes with unusual resistance to temperature, pH, and salt; to metal resistant microorganisms; to open air fermentation processes in extreme conditions; to unusual sources of biological products (membranes, antibiotics, polymers, etc.). There is no doubt that a few years from now

we will have a better understanding of microbiology thanks to the discovery and exploration of this unusual kingdom.

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Degradación de pelo humano «in vitro» por *Trichophyton mentagrophytes*

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Summary

The degradation «in vitro» of human hair, invaded by the dermatophyte *Trichophyton mentagrophytes* was studied by means of scanning and transmission electron microscopy. The digestion of the hair started as well in the cuticle as in the cortex, in the regions with low content of keratin, especially in the intercellular spaces. The exocuticle and epicuticle were the most resistent parts of the cuticle in fungal degradation, due to its high content in cystine. In the cortex the macrofibrils were first separated from each other and later attacked on the surface and the interior, arriving to be almost completely degraded. At the end only pigmented granules and rests of membranes could be observed. After a month the hair was almost entirely destroyed probably due to an enzymatic process although some mechanical alteration could also be observed.

Key words: Trichophyton mentagrophytes, *hair degradation, queratinolysis, dermatophytes.*

Resumen

Se han estudiado los mecanismos de degradación de pelo humano «in vitro», por el hongo dermatofito *Trichophyton mentagrophytes*, utilizando microscopía electrónica de transmisión y de barrido. La digestión del pelo se inició, tanto en la cutícula como en la corteza, en aquellas regiones con un menor contenido de queratina, especialmente en los espacios intercelulares. La exocutícula y la epicutícula eran, debido a su alto contenido en cistina, las partes más resistentes al ataque del hongo. Las macrofibrillas de la corteza fueron, en un principio, separadas unas de otras para ser posteriormente atacadas, tanto en su superficie como en su interior, llegando a ser totalmente degradadas. En las fases finales únicamente se podían observar gránulos de pigmento y restos membranosos. Al cabo de un mes el pelo había sido ya totalmente destruido, debido probablemente a un proceso enzimático, aunque también fueron observadas algunas alteraciones de origen mecánico.

Introducción

La queratina es una proteína fibrosa que forma parte de la piel de los vertebrados y sus anejos, se caracteriza por su alto contenido en cistina. Este aminoácido, con sus enlaces disulfuro, es el responsable de la estabilidad de la molécula de queratina haciéndola más resistente a la digestión enzimática. El mecanismo por el cual los diferentes dermatofitos atacan y digieren a la misma ha sido estudiado por diferentes autores, publicando en algunos casos resultados contradictorios en cuanto al mecanismo de acción de dichos hongos, ya que según algunos investigadores se trata básicamente de una acción enzimática, mientras que según otros el mecanismo es casi exclusivamente mecánico (1,

3, 4, 5, 6, 7, 13, 16). En los referidos trabajos se han investigado tanto hongos patógenos como saprofitos aislados del suelo.

En el presente trabajo nos hemos propuesto, utilizando técnicas de microscopía electrónica, aportar nueva información sobre la acción queratinolítica de los dermatofitos a nivel ultraestructural, utilizando *Trichophyton mentagrophytes* creciendo sobre pelo humano «in vitro» y comprobar lo apuntado por otros autores referente a la acción preponderantemente enzimática del hongo.

Material y métodos

Hongo estudiado. Fue utilizada la cepa FMR 1512 de *T. mentagrophytes* aislada de una «tinea capitis» humana. La suspensión de conidios fue preparada a partir de un cultivo de PDA (patata dextrosa agar) de 15 días.

Preparación de pelo. Pelo humano prepuberal, lavado con agua destilada y detergente, fue cortado en fragmentos que se agruparon en haces de unos 1-1,5 mm de diámetro, mediante hilo de nylon y se sometieron a 110° C durante 10 minutos. Fueron colocados en el interior de una placa de Petri estéril a la que se añadieron 25 ml de agua destilada también estéril y 1 ml de extracto de levadura al 10 %. Las placas fueron sembradas con una suspensión de conidios del hongo a investigar e incubadas a 25° C durante un mes. A la semana y al mes fueron recogidas muestras para su observación al microscopio electrónico.

Preparación de la muestra para microscopía electrónica. Se siguió la metodología publicada por nosotros en un anterior trabajo (2). Las observaciones de SEM y TEM se llevaron a cabo con microscopios «Jeol JSM 840» y «Zeeis EM 10C», respectivamente.

Resultados

En la muestra control la estructura del pelo coincide plenamente con la descrita por los ya clásicos trabajos de Mercer (9) y Roth (14) sin signos evidentes de alteración de la misma (Fig. 1-1). Las primeras hifas invasivas se observaron en las zonas de menor resistencia, como son los espacios intercapilares. En las muestras de 7 días de incubación aparecieron ya evidentes signos de degradación, en todas las secciones realizadas, tanto verticales como horizontales, incluso en aquellas sin la presencia de elementos fúngicos (Fig. 1-2).

La degradación del pelo «in vitro» comienza con un ataque de la cutícula, que se inicia en las células más externas y va progresando hacia las más próximas a la corteza. Los primeros signos de invasión se manifestaron en el complejo de membrana que separa las células cuticulares (Fig. 1-3) que acaba digiriendo tanto la totalidad de la sustancia cementante como las propias membranas citoplasmáticas que son disueltas probablemente por enzimas fúngicos (Fig. 1-5). En ocasiones la separación entre las células cuticulares es facilitada por la acción mecánica de las hifas introducidas al incrementar su diámetro (Fig. 1-4).

Otra parte que pronto se puede observar invadida por el hongo es la endocutícula, sólo parcialmente al principio, pero pronto desaparece completamente, apreciándose perfectamente la exocutícula con un claro borde irregular que la delimita (Fig. 1-5). Con la desaparición de la endocutícula se pone de manifiesto la presencia de una delgada capa densa a los electrones que en un principio está íntimamente unida a la membrana citoplasmática interna y aparentemente intacta aún después de la disolución de las estructuras adyacentes (Figs. 1-4, 1-6). En las células cuticulares no alteradas es difícil poder apreciar la existencia de la misma. Otra capa altamente resistente a la degradación es la epicutícula, que rodea externamente a la célula (Fig. 1-6), y que puede observarse incluso en los más avanzados estados de degradación junto a restos de exocutícula (Fig. 2-1). Esta también resulta

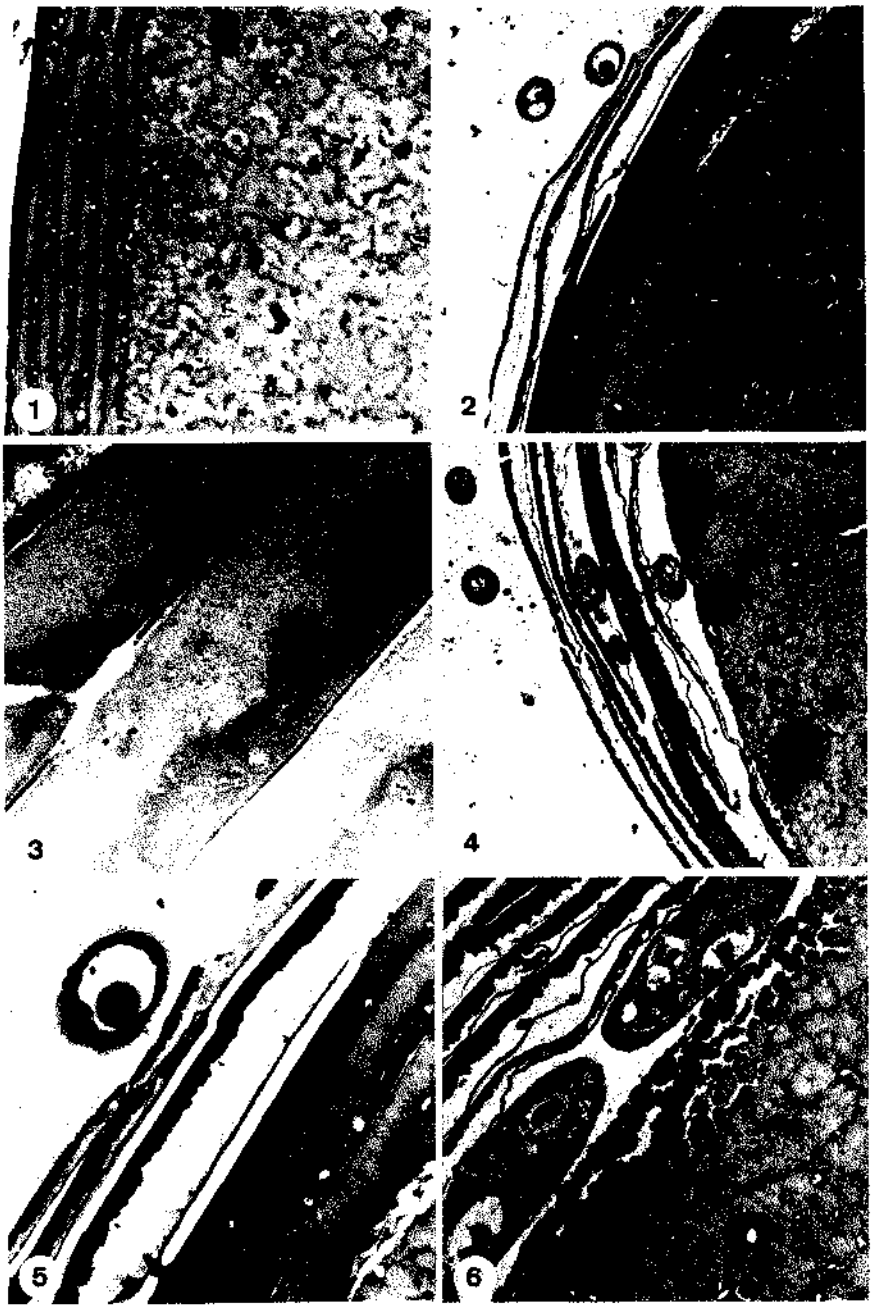


Fig. 1. Cortes transversales de pelo humano bajo la acción de *Trichophyton mentagrophytes*. Observaciones mediante microscopía electrónica de transmisión.
Fig. 1-1. Pelo intacto, x 1280.
Fig. 1-2. Capas externas de la cutícula separadas de las restantes por la acción enzimática del hongo, x 1.300.
Fig. 1-3. Detalle de la cutícula en la que puede observarse la degradación intracelular, x 32.980.
Fig. 1-4. Avanzada degradación de la cutícula del pelo, con hifas intercaladas, x 1.300.
Fig. 1-5. Detalle de la Fig. 2, en la que pueden observarse las células cuticulares externas desprovistas de endocutícula, x 11.000.
Fig. 1-6. Inicio de la degradación de la corteza, x 6.460.

atacada prontamente pero su digestión es muy lenta y difícilmente llega a desaparecer totalmente, presentando diferentes grados de descomposición según el tiempo de actuación del hongo. En preparaciones más viejas (un mes) la estructura fúngica de las hifas situadas a nivel de la cutícula fue menos evidente, sus paredes se habían desintegrado parcialmente y la estructura celular interna quedaba reducida a unos pocos restos de membrana (Figs. 2-2, 2-3).

En la zona cortical, a la semana, pueden observarse áreas en las que la parte adyacente a la cutícula evidencia ya claros signos de digestión (Fig. 1-6) mientras que en otras ha progresado más y aparece una gran zona desprovista de material apareciendo únicamente restos de queratina. Este espacio vacío continúa con el dejado por la desaparición de la endocutícula de la célula cuticular más interna (Fig. 2-1). Las células de las hifas que se insertan en la corteza siguen conservando su estructura interna. Aquí la queratinolisis empieza también probablemente en los espacios intercelulares. La sustancia cementante es también disuelta al igual que los restos citoplasmáticos y la digestión se desarrolla intracelularmente (Fig. 2-4); siendo las macrofibrillas separadas unas de otras (Fig. 2-3, 2-5) y volviéndose más osmiofílicas. Luego son degradadas tanto en su superficie externa como en su interior hasta llegar a desaparecer totalmente (Figs. 2-5, 2-6).

En la corteza pueden observarse hifas que ocupan los agujeros formados inicialmente por disolución de la queratina, adoptando la forma de los mismos (Fig. 3-1), mientras que en otras ocasiones (en estados más avanzados) presentan grandes zonas de lisis a su alrededor, en las que pueden observarse únicamente restos membranosos (Fig. 3-5). Tales hifas pueden ser solitarias o presentarse agrupadas con un frente de penetración que se ramifica arborescentemente. En sección pueden observarse varias de estas últimas dispuestas paralelamente e íntimamente asociadas (Figs. 3-2, 3-3, 3-4).

Quizá las estructuras más resistentes a la acción del hongo y que permanecen sin digerir a lo largo de todo el tiempo de ataque son una gran cantidad de gránulos de gran densidad electrónica que ya se observaban en el pelo intacto (Fig. 1-1) y que incluso llegan a aumentar su cantidad a lo largo del proceso (Figs. 1-2, 1-4, 2-2, 3-2, 3-4, 3-6).

En los últimos estadios del ataque fúngico (al mes) con microscopía electrónica de barrido únicamente se observan las hifas en disposición longitudinal, paralelas al eje del pelo y con muchas ramificaciones e interconexiones laterales, habiendo desaparecido casi la totalidad del pelo en su interior (Figs. 4-1, 4-2).

Discusión

Los mecanismos de degradación del pelo, observados en nuestro caso, se parecen mucho a los descritos por otros autores utilizando la misma especie fúngica (1, 10) u otros dermatofitos como *T. ajelloi* y *T. rubrum* (1), *Microsporum gypseum* (7) o incluso especies no patógenas como *T. terrestre* (3). De nuestras observaciones se desprende que *T. mentagrophytes* en su estado saprófito es capaz de degradar intensamente la queratina. Tal digestión fue observada tanto en la queratina amorfa de la cutícula como en la fibrosa de la corteza, siendo el mecanismo predominante de tipo enzimático, aunque alguna acción mecánica pudo evidenciarse también, especialmente en la cutícula, por engrosamiento de las hifas que crecen entre las células causando alteraciones de las mismas. En primer lugar son degradadas las estructuras con un menor contenido de queratina como son el complejo de membrana celular y la endocutícula.

En la cutícula, la pronta invasión fúngica origina una separación clara entre ésta y la corteza. La degradación más lenta y tardía de la exocutícula se debe precisamente a su composición química casi exclusivamente de queratina amorfa. La disolución desigual de esta capa sugiere una cierta heterogeneidad en su estructura y puede reflejar su construcción a base de acúmulos de bloques de queratina depositados en el curso de la queratinización (7, 10). La epicutícula es la parte con un mayor contenido en cistina (7, 15) y, por tanto, es la que mayor resistencia presenta a la degradación fúngi-

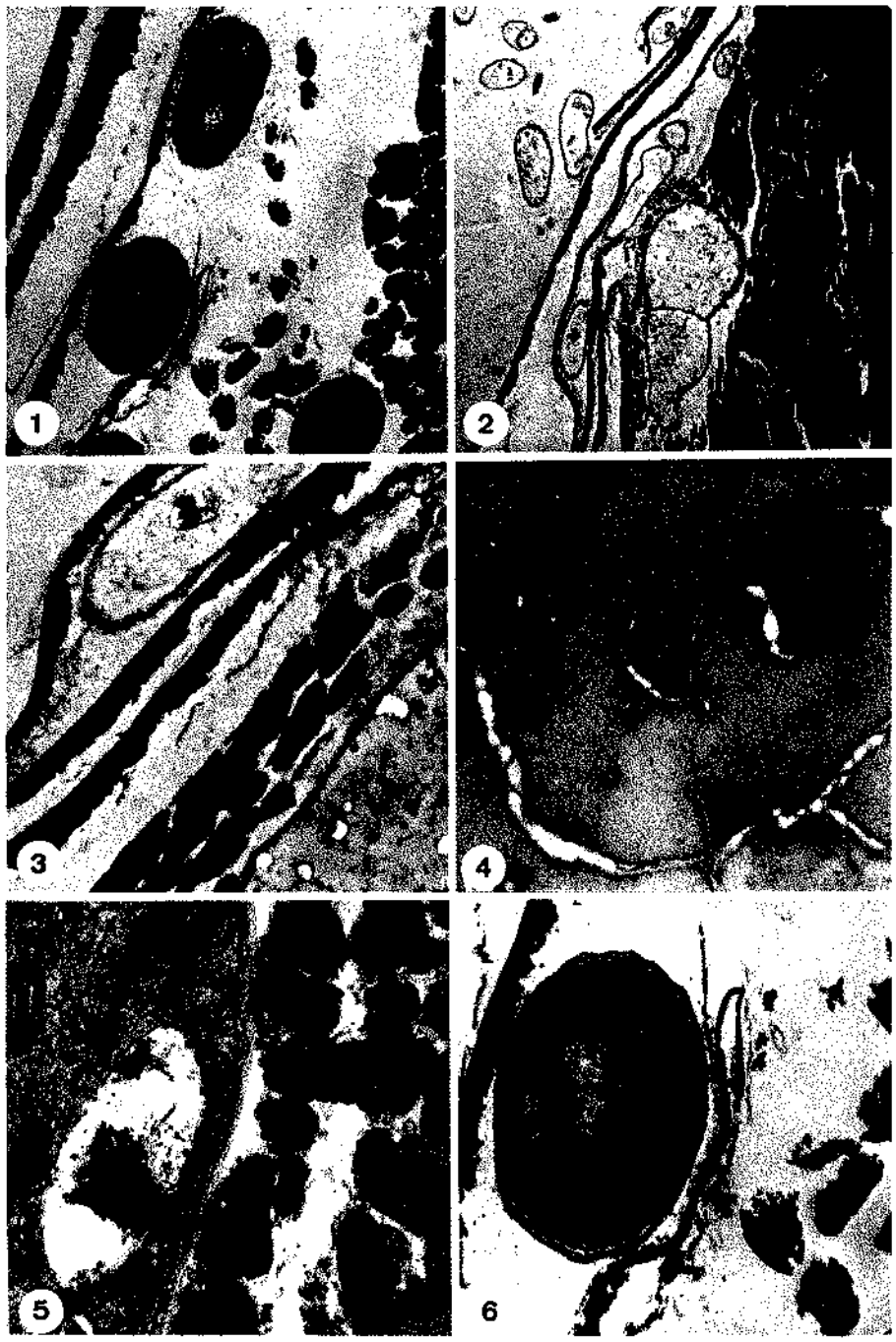


Fig. 2. Cortes transversales de pelo humano bajo la acción de *Trichophyton mentapophytes*. Observaciones mediante microscopia electrónica de transmisión.
Fig. 2-1. Estado avanzado de la degradación de la corteza, las células fúngicas todavía conservan su organización interna típica, x 11.050.
Fig. 2-2. Hifas fúngicas que han perdido su organización celular interna típica, x 1.700.
Fig. 2-3. Cutícula altamente degrada y corteza con gránulos densos de melanina, x 9.160.
Fig. 2-4. Inicio de degradación de la corteza, x 32.980.
Fig. 2-5. Macrofibrillas separadas, pudiéndose observar las microfibrillas que las integran, x 25.300.
Fig. 2-6. Detalle de la Fig. 9, x 24.300.

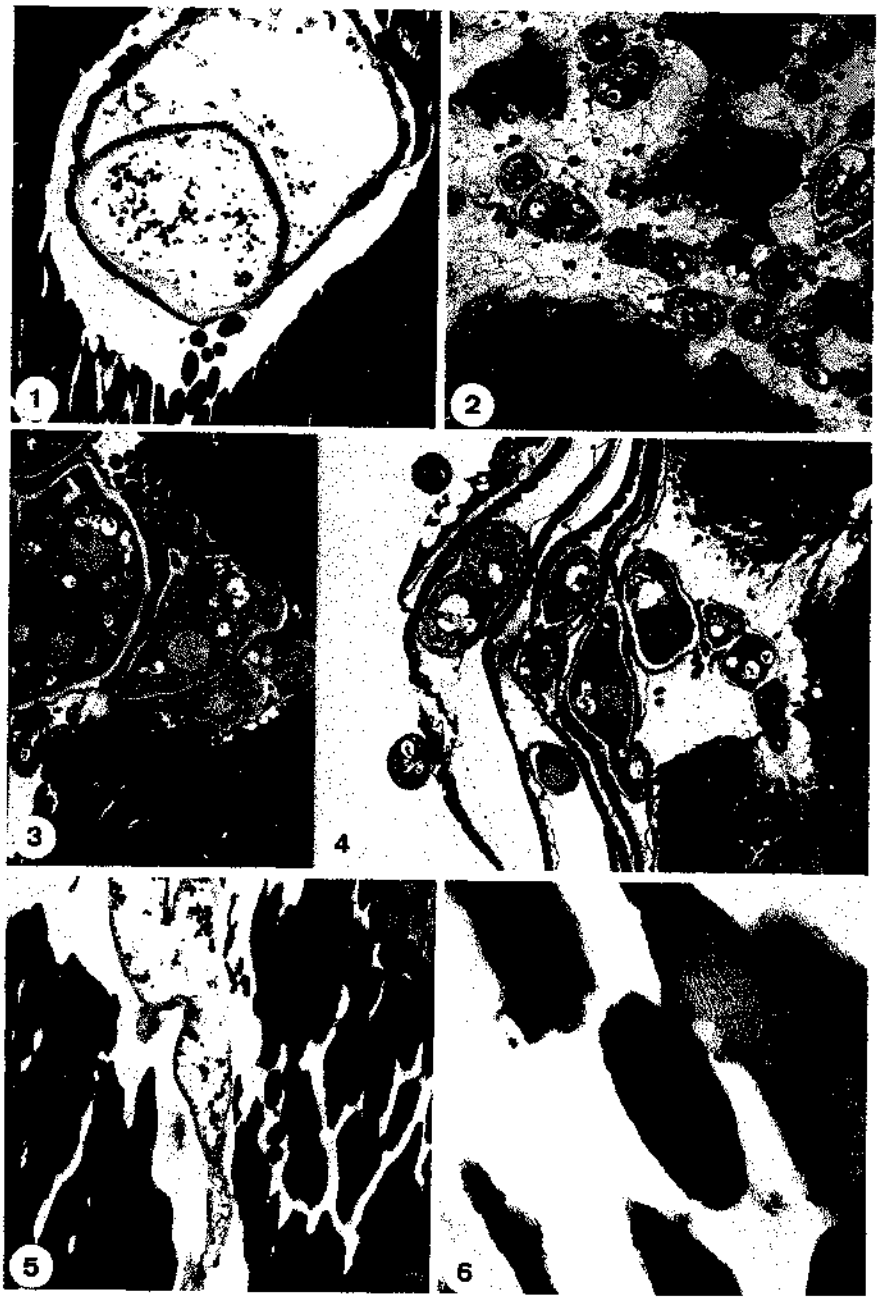
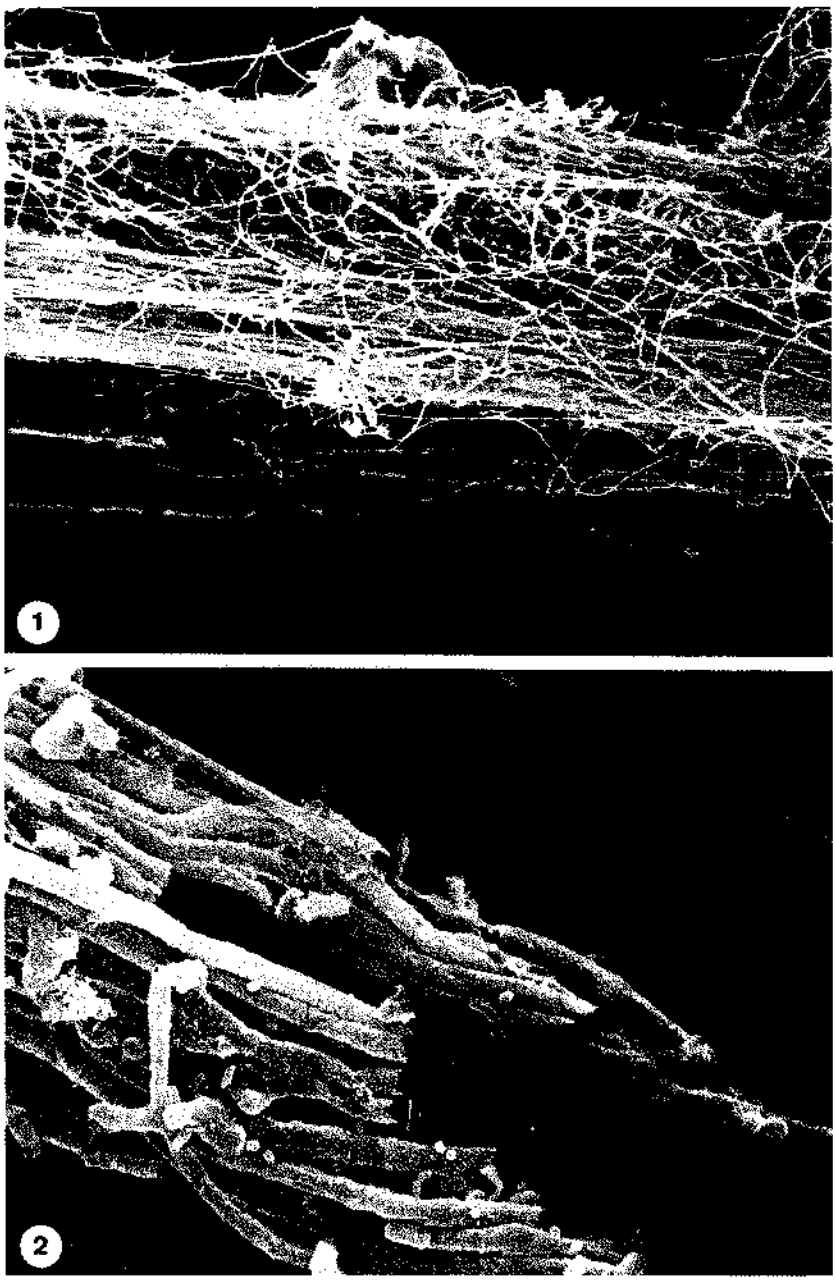


Fig. 3. Cortes transversales de pelo humano bajo la acción de *Trichophyton mentapophytes*. Observaciones mediante microscopía electrónica de transmisión.
Fig. 3-1. Corte de una hifa fúngica desprovista de contenido interno que rellena el espacio digerido previamente en la corteza, x 24.990.
Fig. 3-2. Amplias zonas líticas en el interior de la corteza, nótense los restos membranosos, x 4.930.
Fig. 3-3. Corte del frente de avance fúngico «fronds» con pocas áreas líticas a su alrededor, x 1.360.
Fig. 3-4. Corte de un frente de avence fúngico «fronds» que abarca la cutícula y la corteza con grandes zonas de degradación, x 840.
Fig. 3-5. Fase más avanzada que la anterior, en la que pueden observarse roturas en las paredes celulares fúngicas, x 15.200.
Fig. 3-6. Gota de melanina entre los restos de macrofibrillas de la corteza, x 43.860.



Figs. 4-1 y 4-2. Imágenes al microscopio electrónico de barrido del pelo humano invadido por *Trichophyton mentagrophytes* al mes de iniciado el ataque. Fig. 4-1, x 114. Fig. 4-2, x 1.140.

ca. Algo parecido ocurre con la capa altamente queratinizada que acompaña a la membrana citoplasmática interna.

En la corteza también los elementos con menos contenido o carentes de queratina fueron los más rápidamente atacados, así, por ejemplo, el complejo de membrana celular y la sustancia inter-

macrofibrilar. En la corteza muy degradada se observan únicamente remanentes de una capa queratinizada de origen desconocido parecida a la capa densa a los electrones que acompaña a la membrana citoplasmática interna y gránulos de pigmento correspondientes probablemente a eumelanina (12). Otros autores también han indicado la extremada resistencia de tales gránulos a la degradación (3, 7, 9).

Los típicos órganos de invasión «Fronds», sistema de hifas densamente ramificadas y creciendo paralelamente, que algunos autores describen ocupando el espacio entre la cutícula y la corteza (7) y creciendo paralelamente a aquélla, en nuestro caso los hemos evidenciado creciendo perpendicularmente a la cutícula y abarcando a ésta y parte de la corteza. Kanbe et al. (5) han demostrado que la hifas que se desarrollan en la corteza muestran una diferenciación estructural parecida a la de las hifas vegetativas, aunque en las de la corteza no se observan vesículas apicales debido probablemente al hecho de que en este caso el crecimiento no está polarizado en un lugar determinado sino que se ramifica apicalmente con varias protusiones en forma de dedo.

La degradación del pelo «in vitro» por *M. gypseum* (7), como ya hemos indicado, es muy parecida a la señalada por nosotros y por Mercer & Verna (10) con *T. mentagrophytes*. Por el contrario *T. ajelloi* mostró una menor capacidad queratinolítica, siendo quizá la degradación más mecánica que enzimática, como podrían demostrar la mayor preponderancia de órganos perforantes (1). *T. rubrum* también mostró poca acción enzimática y limitada a la zona cuticular (1).

En las macrofibrillas de la corteza la digestión se produce de forma desigual debido a una composición heterogénea en su parte central y periférica, hecho también demostrado por Orfanos & Ruska (11) y Kunert & Krajci (7). En las secciones en las que se observa una mayor degradación de la corteza también se aprecia un incremento en la densidad a los electrones y aumento del contraste. Kunert & Krajci (7) atribuyen este hecho a la pérdida de la estructura y a la formación de grupos sulfidrilos por sulfitolisis. Dentro de las macrofibrillas la matriz que ocupa los espacios intermicrofibrilares está formada por cortas cadenas polipeptídicas (gamma-queratinas) ricas en puentes disulfuro (1). La presencia de tales puentes explicaría la fijación preferencial del tetraóxido de osmio sobre la matriz que se traduciría por un incremento del contraste de las microfotografías (13). Las estructuras laminares más densas que se pueden observar en la corteza de los pelos más degradados podrían corresponder a esta matriz formada por proteínas de bajo peso molecular ricas en cistina y, por tanto, más resistentes.

Creemos que los detalles ultraestructurales sobre la queratinolisis del pelo por *T. mentagrophytes* aportados en el presente trabajo ayudarán a un mejor conocimiento del proceso infectivo de los hongos productores de tiñas, siendo precisamente la citada especie una de las más frecuentes en nuestro país (8).

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Extracellular galactosaminogalactan from *Penicillium frequentans*

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Summary

Penicillium frequentans secreted an exopolysaccharide composed mainly of N-acetyl-galactosamine (48.4 %) and galactose, mannose and glucose in the ratio 4:1:1, that were α - (1 → 4) and α - (1 → 3) linked predominantly. Greater polysaccharide production was achieved in old cultures grown statically with glucose as carbon source.

Key words: Penicillium frequentans, *exopolysaccharide production, galactosaminogalactan.*

Resumen

Penicillium frequentans produce un polisacárido extracelular compuesto principalmente de N-acetilgalactosamina (48,4 %) y galactosa, manosa y glucosa en la proporción 4:1:1, siendo sus enlaces predominantes del tipo α - (1 → 4) y α - (1 → 3). La mayor producción de polisacárido se alcanzó en cultivos viejos, incubados estáticamente con glucosa como fuente de carbono.

Introduction

Exopolymers with different composition and structure have been described in the genus *Penicillium*. An extracellular peptidophosphogalactomannan was isolated from *P. charlesii* culture fluids (12). In the culture filtrates of *P. chrysogenum*, Sakaguchi, Yakota and Suzuki (34) found a galactomannan with galactofuranosyl residues and phosphorus. In other species of this genus, exopolysaccharides composed of glucose and malonic acid (3, 9, 10, 22, 30, 31) and of galactose with malonic acid (11, 17) have been reported. Exopolysaccharides containing N-acetylgalactosamine and galactose were isolated from culture fluids of *Aspergillus* species (13, 15, 21, 32).

We report on the isolation and partial characterisation of an exopolysaccharide from *Penicillium frequentans* and the effect of culture conditions and carbon source in its production.

Materials and methods

Culture media and micro-organism

Penicillium frequentans Westling, strain CBS 345.51 was maintained on slants of Bacto-potato-dextrose-agar (Difco). Suspensions of conidia (10^6/ml of sterile medium) from 10-day-old agar slants were used as inocula. The basal medium for mycelial production (20) was distributed in 1 l portions into 2 l Erlenmeyer flasks. The medium was adjusted to pH 6.5 before autoclaving at 120° C for 15

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min. The flasks were inoculated with 1 ml of the conidial suspension and incubated for 4 days at 25 ± 1° C and 150 rpm in a Gallenkamp IH-465 orbital incubator.

After harvesting, the mycelium was collected on a fine mesh cloth. The culture fluids were treated with a volume of ethanol, and the polysaccharide separated as a stringy precipitate that wound around the stirrer. This material was washed twice with ethanol and then with acetone, dried at 60° C and ground. The precipitate from old cultures did not wind around the stirrer and had to be collected by centrifugation.

Chemical and structural analysis

Total hexose was determined by the anthrone procedure (23) with glucose as a standard. Nitrogen was measured by elemental analysis. Hexosamines were calculated from total nitrogen after subtracting the part corresponding to protein, and identified in 6N-HCl hydrolysates on a Biotroncik LC 7000 aminoacid analyser. Total protein was measured by the method of Lowry et al. (24) using bovine serum albumin as a standard. Phosphate was determined in the ashed polysaccharide by the phosphate-ascorbic acid method with $N_aH_2PO_4 \cdot H_2O$ as a standard (28). The acetate ion was determined according to Ludowieg and Dorfman (25) with methyl acetate as a standard. Volatile matter was determined by heating 150 mg of the polysaccharide at 100° C to constant weight.

For identification of monosaccharides the sample was hydrolysed with 1N H_2SO_4 at 100° C for 16h and the neutral sugars released were converted into their corresponding alditol acetates (18), which were identified and quantified by gas-liquid chromatography (17).

Infrared spectra were obtained by the KBr technique on a Perkin-Elmer 457 i.r. spectrophotometer. Optical rotation of the exopolysaccharide was determined with a Hartnack HA-4001 polarimeter.

Periodate oxidation was performed according to Aspinall and Ferrier (2). The formic acid produced was determined by the method of Kabat and Mayer (16). Ammonia was measured in the oxidation mixture according to Leal, Gallegly and Lilly (19). The oxidized polysaccharide was subjected to a modified Smith degradation (31) and the resulting products were converted into their corresponding alditol acetates and identified by gas-liquid chromatography. Elemental analysis was performed to quantify the periodate resistant hexosamines.

Results

Polysaccharide formation was first observed in 2 l Erlenmeyer flasks containing 1 l of medium, incubated at 25° C and 150 rpm. The exopolysaccharide obtained from 4-day-old cultures amounted to aproximately 5 mg/100 ml. This product was used to study its chemical composition and structure.

Effect of culture conditions

To study the effect of different culture conditions on polysaccharide production, the microorganism was incubated statically (250 ml Erlenmeyer flasks with 25 ml of basal medium at 25° C) and shaking at 100 rpm (1 l Erlenmeyer flasks with 500 ml of basal medium at the same temperature).

In static cultures (Fig. 1) it was observed that the polysaccharide yield increased with the incubation time, reaching a maximum (29.5 mg/100 ml) at the 15th day of incubation. From the 7th day

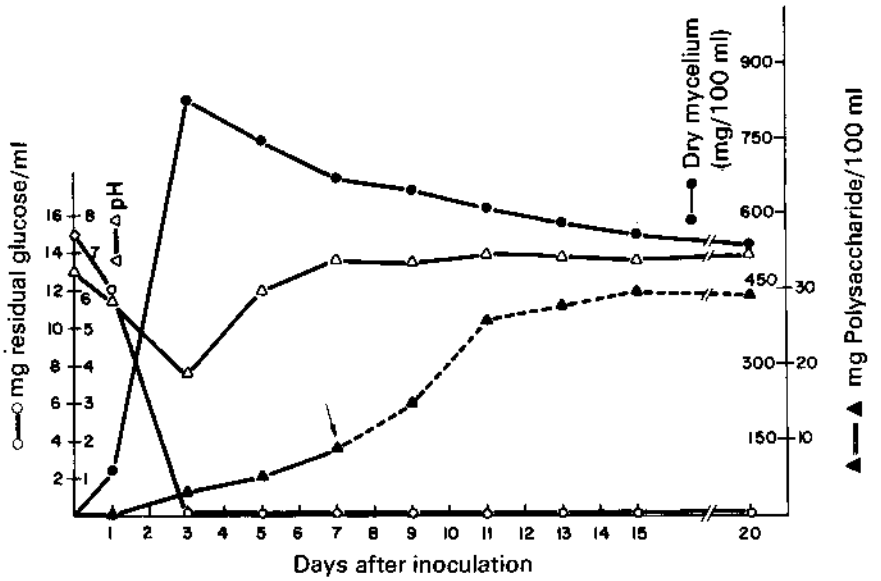

Fig. 1. Growth, changes in the pH of the medium, residual glucose and polysaccharide production when *P. frequentans* is cultured in 250 ml flasks containing 25 ml of the medium.

it had to be collected by centrifugation (broken line). Maximum mycelial growth (757.8 mg/100 ml) occurred at the 3rd day.

In submerged cultures (Fig. 2) the maximum polysaccharide yield was only 2.5 mg/100 ml at the 5th day of incubation. The mycelial growth was slower than in the static cultures. The maximum growth was reached at the 13th day of incubation (781.4 mg/100 ml). The polysaccharide had to be collected by centrifugation from the 11th day.

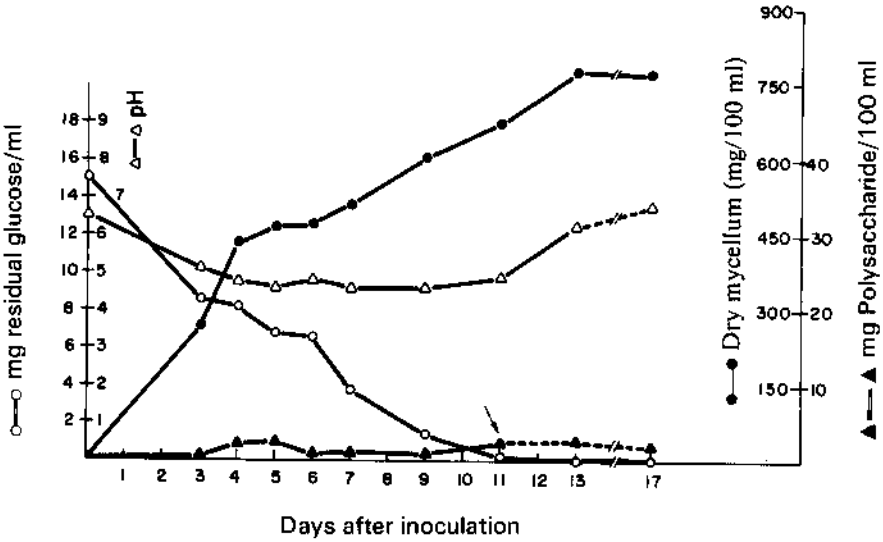

Fig. 2. Growth, changes in the pH of the medium, residual glucose and polysaccharide production when *P. frequentans* is cultured in 1 l flasks containing 500 ml of the medium at 100 rpm.

Effect of different carbon sources

To investigate the effect of different sugars on exopolymer production, glucose was replaced by 15 g/l of the sugar under test in the basal medium. Cultures in 250 ml Erlenmeyer flasks containing 25 ml of media were incubated statically at 25° for 3, 6 and 9 days. The greatest amount of polysaccharide was collected, by centrifugation, in the medium containing glucose at the 9th day of incubation (Fig. 3). In the mannose, fructose and sucrose media, the production amounted to 10 mg/100 ml at the end of the incubation period. In the medium with maltose the greatest yield was achieved the 6th day. There was no production of polysaccharide in the medium containing galactose.

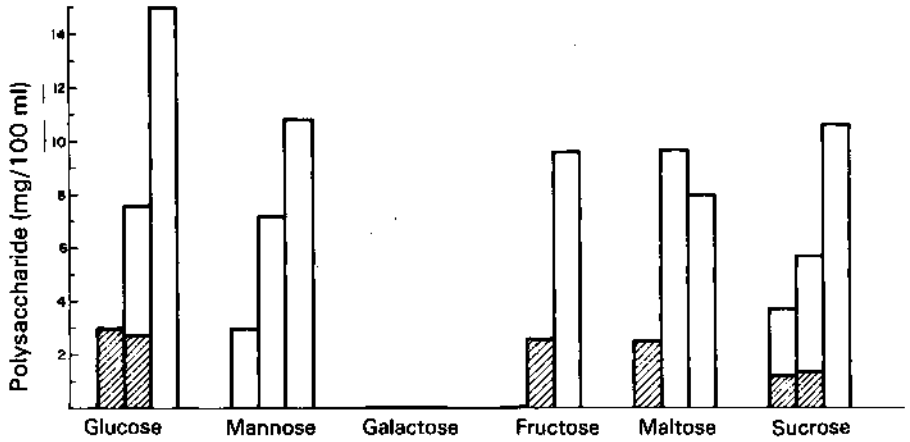

Fig. 3. Extracellular polysaccharide production with different carbon sources. From left to right 3, 6 and 9 days after inoculation. □ Collected with the stirrer. ▨ Collected by centrifugation.

Characterisation of the exopolysaccharide

The exopolysaccharide from *P. frequentans* was composed of N-acetylhexosamine and neutral sugars predominantly (Table 1). Neutral sugars were identified by G.l.c. as D-galactopyranose, D-mannopyranose and D-glucopyranose, D-galactose being the most abundant. Hexosamines were identified as D-galactosamine which was the main component of the polysaccharide, 40.7 % (Table 1). The acetate ion (8.4 %) indicated that 96.1 % of the galactosamine was N-acetylated; it was confirmed by the bands at 1650 and 1560 cm^{-1} of the i.r. spectrum of the polysaccharide (Fig. 4 A). The bands at 820 and 850 cm^{-1} are characteristic of polysaccharides having the α-configuration (5). In addition we have found a high positive optical rotation: $(\alpha) = +215.84$ (C = 0.19 in N-NaOH), that also indicated the presence of α-bonds.

TABLE 1
CHEMICAL COMPOSITION OF THE EXOPOLYSACCHARIDE (%)

Neutral hexose	37.7
Hexosamine	40.7
Protein	0.5
Phosphate ion	0.5
Acetate ion	8.4
Volatile matter	6.1
Ash	2.0

When the exopolysaccharide was extracted with deionized water at room temperature, 68 % of it was solubilized. The neutral sugars composition and i.r. spectra (Fig. 4 B, 4 C) of both the water-soluble and insoluble fractions were very similar to that of the native polysaccharide.

During the periodate oxidation of the polysaccharide the $NaIO_4$ consumed was 0.64 μmol/μmol hexose residue. The formic acid produced was 0.076 μmol/μmol hexose residue and there was no

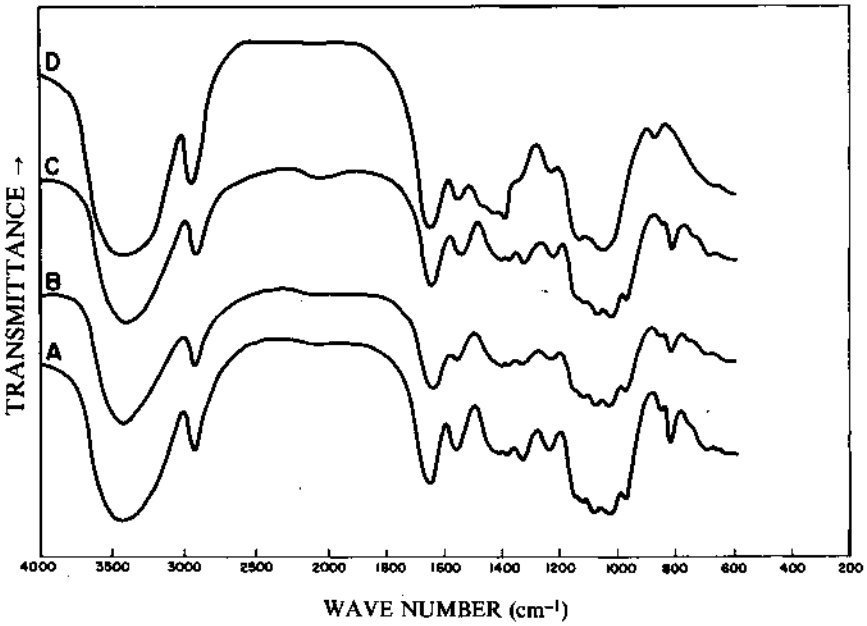

Fig. 4. Infrared spectra of the extracellular polysaccharide of *P. frequentans*. A: Native polysaccharide; B: Water-soluble polysaccharide; C: Water-insoluble polysaccharide. D: Oxidized and reduced polysaccharide.

ammonia production. The linkage types calculated from these results, according to Rankin & Jeanes (29), are 1 → 6: 7.6 %; 1 → 4: 48.8 % and 1 → 3: 43.6 %. The G.l.c. of the oxidized and reduced polysaccharide hydrolysed with 1N H_2SO_4 for 16 h at 100 °C revealed the presence of threitol as the main neutral sugar, and lesser amounts of glycerol, erythritol, arabinose, mannose, galactose and glucose. Most of the N-acetyl-galactosamine (80 %) was resistant to periodate oxidation (Table 3). The threitol proceeds from 1 → 4 galactopyranose residues. The absence of ammonia in the oxidation mixture, the amount of galactosamine recovered after degradation (Table 2) and the presence of the bands at 1650 cm^{-1} in the i.r. spectrum (Fig. 4 D) indicated that the galactosamine was N-acetylated.

Since polysaccharides containing N-acetyl-aminosugars are resistant to periodate oxidation although they were linked by 1 → 4 (4), we cannot say whether they are 1 → 3 or 1 → 4 linked.

Discussion

The extracellular polysaccharide isolated from culture fluids of *P. frequentans* is composed of N-acetylgalactosamine (48.4 %) and galactose, mannose and glucose (37.7 %) in the ratio 4:1:1.

The water soluble (68 %) and insoluble (32 %) fractions have the same composition. It can be explained considering that during the drying process some chains or chain segments could establish strong secondary bonds, losing their solubility.

TABLE 2
MOLAR RATIOS OF THE SUGARS DETERMINED IN THE NATIVE AND PERIODATE-OXIDIZED POLYSACCHARIDE OF *P. FREQUENTANS* (%)

	Native Polysaccharide	Periodate-oxidized Polysaccharide
Glycerol	0.0	6.0
Erythritol	0.0	0.3
Threitol	0.0	42.3
Arabitol	0.0	1.3
Mannose	6.8	0.5
Galactose	26.5	0.8
Glucose	6.5	0.8
N-Acetyl-Galactosamine	60.0	47.6

Neutral sugars determined as alditol acetates by G.l.c. in hydrolisates with 1N H_2SO_4 for 16h at 100° C. N-Acetyl-Galactosamine was determined from the elemental analysis.

The exopolysaccharide yield is different in static or submerged cultures. In the first case the production increased with the incubation period reaching the maximum at the 15th day (20.5 mg/100 ml). On the other hand, the submerged cultures yielded little amounts during all the experiment (2.5 mg/100 ml).

We also tried to increase the exopolymer production changing the carbon source in the basal medium, but after the experiment we verified that glucose was the best. There was no exopolymer production in the medium containing galactose as carbon source.

The composition resembles polysaccharides isolated from culture fluids of several species of *Aspergillus* (1, 4, 8, 15, 21, 32). Periodate oxidation followed by complete Smith degradation revealed that galactopyranose was 1 → 4-linked and galactosamine was completely N-acetylated and was resistant to periodate oxidation (1 → 3 or 1 → 4 linked). These results indicated differences between this polysaccharide and those produced by species of *Aspergillus*, which contain galatosamine partially N-acetylated and linked 1 → 6 predominantly (13, 32).

The presence of N-acetylgalactosamine has not been described up to now in polysaccharides isolated from the culture fluids of *Penicillum*.

This polysaccharide has not been found in the cell wall of *P. frequentans* (unpublished results). From the cell wall of species of *Talaromyces* and *Eupenicillium* a fraction amounting to 5 % of the cell wall has been isolated which contains galactosamine, glucose, galactose, mannose and xylose (33; 14). The structure of this fraction has not been investigated but its chemical composition is different from that of the N-acetyl-galactosamine galactan from *P. frequentans*. Gómez-Miranda and Leal (13) found that *Aspergillus alliaceus* released an α-galactosamino-galactan partially N-acetylated, while the cell wall of the same fungus contained an α-(1 → 3) glucan and a β-glucan-chitin complex, having only traces of galactosamine.

The highest production of exopolysaccharide was achieved at the end of the growth period. Some researchers (6, 7, 26, 35) suggested a relationship between extracellular polysaccharides and cell wall structure of the microorganism. Despite this, we consider that the extracellular polymer is not a result of the cell wall autolysis, since from the 3rd day of incubation we can isolate it from the culture medium.

There is probably a system of priorities within the cell, promoting first the synthesis of the cell wall and then that of extracellular polysaccharides (36).

Pitt's classification (27) includes *P. frequentans* in the serie Glabra of the subgenus Aspergil-

loides according to its morphological similarities with the genus *Aspergillus*. We have found a chemical resemblance between *P. frequentans* and *Aspergillus* sp. exopolysaccharides which is in good agreement with Pitt's classification.

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MICROBIOLOGIA SEM 4 (1988), 47-53

The effect of rifampicin on the development of the *Streptomyces* bacteriophage ØC31

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Summary

The production of ØC31 progeny virus was inhibited by rifampicin when it was added at any time before 20 minutes after induction of the thermoinducible lysogen *Streptomyces coelicolor* 01. The inhibition was gradually lost as the antibiotic was being added later on until the end of the latent period, which lasts about 45 minutes. This effect was not due to resistance of transcription to rifampicin but to accumulation of intracellular virions from around 20 minutes postinduction. When a rifampicin-resistant lysogen was induced in the presence of the antibiotic, no inhibition of RNA synthesis was detected, although a smaller population of progeny than in control cultures without rifampicin was obtained. Two possible explanations of this fact are discussed.

Key words: Bacteriophage, ØC31, rifampicin, Streptomyces.

Resumen

La producción de progenie del virus ØC31 es inhibida por el antibiótico rifampicina cuando éste se añade durante los primeros 20 minutos después de la inducción del lisógeno termoinducible *Streptomyces coelicolor* 01. Si el antibiótico se añade a tiempos posteriores el grado de inhibición disminuye de forma progresiva hasta el final del período de latencia, que dura aproximadamente 45 minutos. Este efecto se debe a la acumulación de viriones intracelulares a partir del minuto 20 del ciclo lítico, no tratándose de un fenómeno de resistencia de la transcripción al antibiótico. Cuando un lisógeno resistente a la rifampicina es inducido en presencia del antibiótico no se detecta inhibición de la síntesis de RNA, aunque la progenie viral obtenida es menor que la detectada en cultivos controles en ausencia de antibióticos. Se discuten dos posibles causas de este hecho.

Introduction

ØC31 is a broad host range temperate phage infecting *Streptomyces*. It is a naked virus with an icosahedral head and a long non contractile tail, with a basal plate (Family Styloviridae); up to 17 polypeptides have been detected in the viral particle (9). Heat treatment of germinated thermoinducible lysogens gives synchronous ØC31 induction with a 45 min latent period. Productive induction is only achieved in spores with germ tubes. The phage development is accompanied by a change in the rate of biosynthesis of macromolecules. Overall protein biosynthesis is reduced by a factor of 1.5 in

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induced cultures. Viral DNA biosynthesis starts at about 10 min postinduction and proceeds together with cellular DNA formation until the end of the latent period. RNA biosynthesis is lowered at least fourfold. In spite of it, the proportion of phage-specific RNA increases about 45 fold after induction of lysogenic cultures; on the other hand, stable RNA is not made by induced cultures although it is more than 60 % of the total amount of RNA synthesized by controls. Cellular RNA synthesis is not completely abolished during the lytic cycle (8).

In this paper we report that, after 20 min postinduction (less than half of the latent period), the production of the progeny virus becomes resistant to rifampicin and how this is accomplished.

Materials and methods

Bacterial and phage strains

Streptomyces coelicolor 01 (8), a thermoinducible lysogen of ØC31 cts 1 (7) was used. The quantitation of plaque-forming units (pfu) was done using *Streptomyces lividans* 66 as phage-host.

Growth conditions

S. coelicolor 01 and *S. lividans* 66 were grown to sporulation in Petri dishes on solid R2YE medium (4) at 28° C and 35 °C respectively. Obtention of spores, germination conditions, culture induction and quantitation of the progeny were carried out as previously described (8).

Determination of ØC31 period of eclipse

At intervals after induction, two aliquots (1 ml) of germinated cultures were taken. One of the samples was immediately centrifuged and the pfu of the suprenatant determined. The other aliquot was placed in a tube containing 3 g of glass beads (100 μm diameter), and 10 μl of chloroform and vortexed for 1 min to break the germinated spores. After appropriate dilution and centrifugation the pfu of the supernatant were determined (it was found that chloroform alone did not effectively lyse *Streptomyces).*

Isolation of a rifampicin-resistant mutant of S. coelicolor 01

Aliquots of 10^9 spores of *S. coelicolor* 01 were treated with EDTA according to Leive (6), just before plating on R2YE medium containing 200 μg/ml of rifampicin, and incubated at 28 °C. The colonies growing on the medium were allowed to sporulate and subsequently streaked onto plates with the same medium. Isolated colonies were tested for their thermoinducible lysogenicity and used as inocula to obtain fresh spore preparations which were tested for phage production in liquid medium in the presence or in the absence of rifampicin.

Determination of RNA synthesis

The RNA biosynthesis of cultures of *S. coelicolor* 01 was measured by continuous incorporation of [5-^{3}H] uridine (2 μCi/ml, 27 Ci/mmol) into the trichloroacetic acid (TCA)-insoluble material.

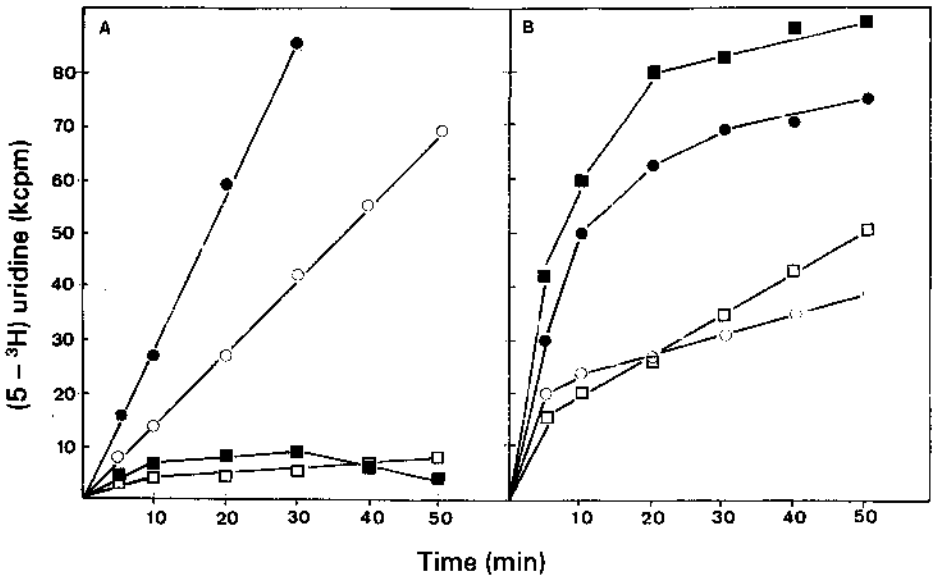

Fig. 1. RNA biosynthesis in the presence or absence of rifampicin of *S. coelicolor* 01 rif^S (A) and rif^r (B) cultures. The antibiotic (200 μg/ml) and ^{3}H-uridine (2 μCi/ml) were simultaneously added to 7 hour germinated cultures, immediately after the induction treatment. (●) RNA biosynthesis in uninduced cultures. (○) RNA biosynthesis in induced cultures. (■) RNA biosynthesis in uninduced cultures in the presence of rifampicin. (□) RNA biosynthesis in induced cultures in the presence of rifampicin. The radioactivity in TCA-insoluble material was determined in triplicate.

The effect of rifampicin on RNA synthesis was followed by measuring the radioactivity remaining in aliquots taken and placed on TCA at intervals, after pulse labelling (3 min) and addition of the antibiotic (200 μg/ml) to the cultures. The processing of radioactive samples has been previously described (5).

Results and discussion

Effect of rifampicin on the production of new virions

Rifampicin inhibits *S. coelicolor* RNA polymerase, since uninduced cultures of *S. coelicolor* 01 did not incorporate ^{3}H-uridine in its presence, whereas they did so in its absence (Fig. 1A). This sensitivity has been previously reported (3).

The production of new phages, expressed as pfu, by *S. coelicolor* 01 cultures, after the addition of rifampicin at different postinduction times and subsequent incubation for 90 min, is shown in Table 1. The antibiotic completely inhibited phage production when added before 20 min postinduction. After this time the yield increased until 40 min, when the normal burst size was reached. The latent period of the phage lasts about 45 min, so this premature insensitivity to rifampicin could be due to the production of a new rifampicin-resistant RNA polymerase, to an «eclipse» period of 20 min or to a complete set of viral macromolecules made by this time, which could assemble to give phage particles irrespective of the presence of the antibiotic.

The eclipse period of ØC31 development was about 20 min as shown in Fig. 2. RNA biosynthesis remained rifampicin-sensitive throughout the latent period (Fig. 3), even after the appearance of the insensitivity of phage production to the antibiotic (Fig. 3B). Both results taken together indicate

TABLE 1
EFFECT OF RIFAMPICIN ON ØC31 DEVELOPMENT

Time of antibiotic addition after induction (min)	PFU 90 min after addition of the antibiotic	Yield *
0	1.0×10^7	1
10	1.9×10^7	1.9
20	1.8×10^8	18.2
30	8.3×10^8	83
40	1.1×10^9	113
50	1.2×10^9	120
60	1.0×10^9	100
70	1.1×10^9	110
80	1.0×10^9	100
90	1.2×10^9	120

* Ratio of PFU/ml detected in induced *S. coelicolor* 01 90 min after antibiotic addition to PFU/ml detected in uninduced cultures.

that no rifampicin-resistant RNA polymerase was made as a consequence of ØC31 induction and that the progressive lack of effect of the antibiotic was due to a progressive increase in the number of virions harbored by the cells.

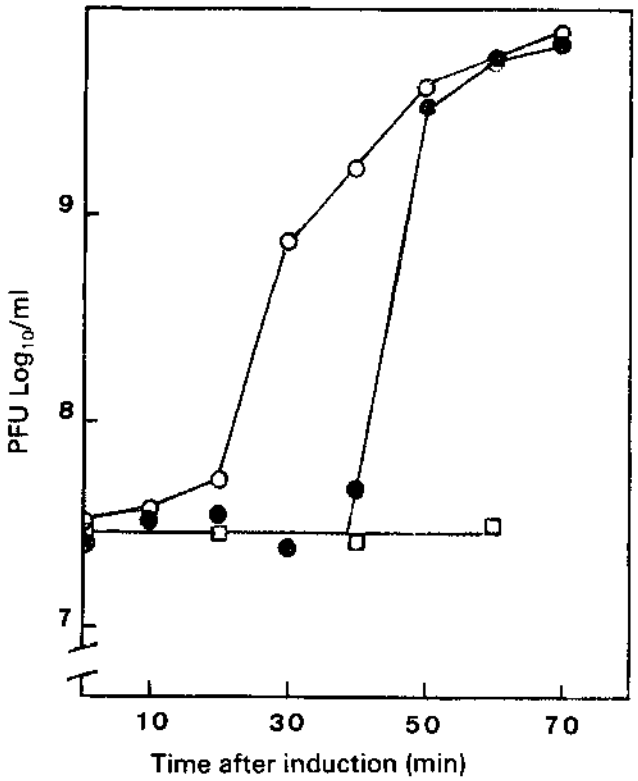

Fig. 2. Kinetics of appearance of intracellular ØC31 virions in induced *S. coelicolor* 01 cultures. The cultures were germinated for 7 h and then thermally induced. The pfu of supernatants (●) or of cultures after glass bead plus chloroform breakage (○) were determined in triplicate. (□) uninduced cultures.

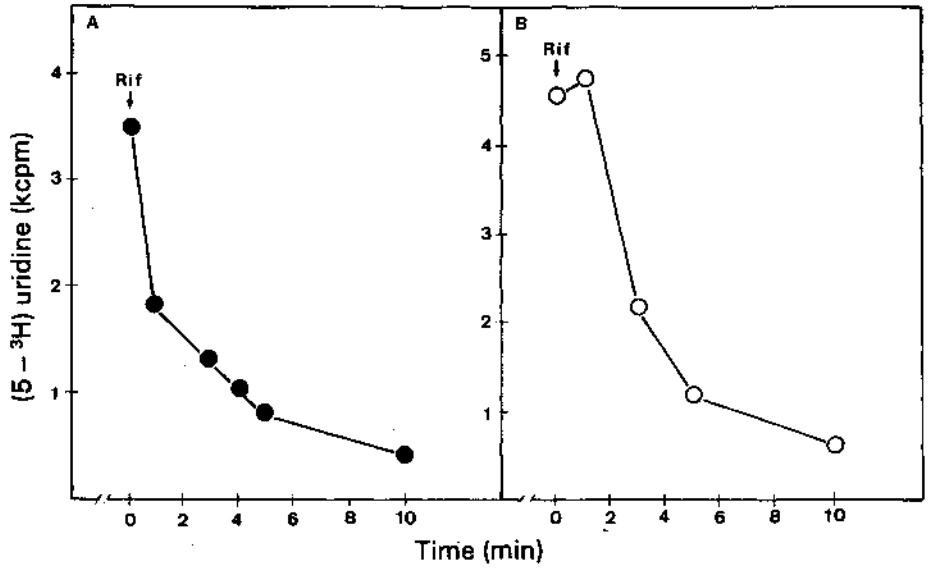

Fig. 3. Effect of rifampicin on RNA biosynthesis in induced *S. coelicolor* 01. ^{3}H-uridine (2 μCi/ml) was added to germinated cultures (7 h) at 10 min (A) and 30 min (B) postinduction. Three minutes later, rifampicin (200 μg/ml) was added. The radioactivity in TCA-insoluble material was determined in triplicate.

Isolation and induction of a rifampicin-resistant mutant

To further investigate the characteristics of RNA biosynthesis during the lytic development of ØC31, a rifampicin-resistant mutant of *S. coelicolor* 01 was selected under conditions which did not allow expression of vegetative phage functions. The mutant incorporated ^{3}H-uridine in the presence of rifampicin to approximately the same level than in its absence, both when the cultures were induced or uninduced (Fig. 1B). These data contrast with those shown in Fig. 1A in which a high inhibitory effect of rifampicin on *S. coelicolor* 01 RNA biosynthesis is represented. The mutant was a thermoinducible lysogen because it produced phages when placed on a plate of R2YE seeded with *S. lividans* 66, and no growth was observed at 37° C.

The increase of pfu in the supernatant of induced cultures with respect to uninduced controls was only of about 25 fold in the mutant (Table 2), whereas it was over 100 fold in induced cultures of *S. coelicolor* 01 *rif*^{R} (Table 1). This finding suggested that the mutation of the cellular RNA polymerase affected somehow phage production and consequently that it was involved in the transcription of phage DNA. On the other hand, the concentration of phages detected in supernatants from induced mutant cultures compared with uninduced ones was 3- fold lower in the presence of the drug than in its absence. Therefore, the production of phages by resistant cultures in the presence of the antibiotic confirms the effect of cellular RNA polymerase in the development of the virus; but the lower yield obtained with respect to cultures in the absence of the drug seems to indicate either that the RNA polymerase of the resistant culture is not completely insensitive to the drug or the presence of a new rifampicin sensitive RNA-polymerase, possibly encoded by phage DNA or even by the *Streptomyces*, that would participate in the lytic cycle through a preferential recognition of ØC31 DNA.

The first interpretation has to be conciliated with the finding that no RNA synthesis inhibition, as measured by continuous ^{3}H-uridine incorporation, was seen in the presence of the antibiotic (Fig. 1B); the paradox could be due to an only marginal sensitivity of the host polymerase which could thus not to be well determined by radioactive precursor incorporation, although it could result

TABLE 2
PHAGE PRODUCTION IN *Streptomyces coelicolor* 01 RIF^r CULTURES

Experiment	Yield *		A/B
	No rifampicin (A)	Rifampicin (B)	
1	27.0	9.0	3.0
2	18.0	6.1	2.9
3	31.0	9.3	3.3

Yield*: Ratio of concentrations of PFU detected in induced and uninduced cultures at 90 min postinduction of the Rif^r mutant. Rifampicin was added inmediately after induction of the cultures.

in a minor size of the phage progeny, because the formation of the virions is a process in which it is necessary the transcription of at least 16 molecules of phage RNA (J. E. Suárez, unpublished data), and the slight inhibition of the synthesis of each of them would result in a sinergistic inhibitory effect on the final product of the biosynthetic pathway. The second explanation is based in the fact that phages whose development is known to be exclusively dependent on host RNA-polymerases do not show differences in phage production between rifampicin-treated or untreated cultures, when the phage infects a rifampicin-resistant host (1, 10).

Indirectly, this result confirmed that the mutant was resistant to rifampicin by RNA polymerase modification rather than by impermeability to the drug because if the latter were the case, no effect on phage formation would have been detected.

The finding that ØC31 might encode an RNA polymerase or induce a host enzyme with preferential recognition of ØC31 promoters could partly explain why there is a preferential transcription of phage DNA in induced cultures of *S. coelicolor* 01. However, this is not the complete explanation; replication of phage DNA, which starts 10 min postinduction, should be also important because this increases the number of phage templates and the probability of an interaction between phage DNA and RNA polymerases (8).

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MICROBIOLOGIA SEM 4 (1988), 55-59 NOTES

Characterization of an hospital disseminated plasmid encoding resistance to gentamicin and other antimicrobial agents

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Summary

A preliminary report has shown the existence of an endemic R plasmid in the University Hospital of Zaragoza (9). The results presented in this paper demonstrate the dissemination of a new 73 kilobases plasmid into multiple strains and species of gram-negative bacilli. This transferable plasmid belongs to Incompatibility group P and mediates resistance to ampicillin, tetracycline, gentamicin, kanamycin, streptomycin, chloramphenicol and sulfamethoxazole, synthesizing the aminoglycoside-modifying enzymes 3-acetyltransferase, 3'phosphotransferase, and 3"nucleotidyltransferase, and a TEM-1 beta-lactamase. These results and the previous findings show that a family of gentamicin-resistance plasmids exists among the gram-negative bacteria in the University Hospital. Resistance to gentamicin in all these plasmids is associated with the formation of 3-N-acetyltransferases.

Resumen

En un trabajo previo se mostró la existencia de un plásmido R endémico en el Hospital Clínico Universitario de Zaragoza. Los resultados presentados en esta publicación demuestran la diseminación de un nuevo plásmido de 73 kilobases entre numerosas cepas y especies de bacilos gram-negativos. Este plásmido transferible pertenece al grupo de Incompatibilidad P y es responsable de resistencia a ampicilina, tetraciclina, gentamicina, kanamicina, estreptomicina, cloranfenicol y sulfametoxazol, sintetizando los enzimas modificantes de aminoglicósido3-acetiltransferasa, 3'-fosfotransferasa y 3"-nucleotidiltransferasa, y una beta-lactamasa TEM-1. Estos resultados y los hallazgos anteriores demuestran que existe una familia de plásmidos de resistencia a gentamicina entre bacterias gram-negativas de este Hospital. La resistencia a gentamicina en todos estos plásmidos está asociada a la formación de 3-N-acetiltransferasa.

The incidence of nosocomial infections caused by gram-negative bacilli, resistant to multiple antibiotics, has increased in recent years. This resistance increase poses serious epidemiological and therapeutic problems for clinicians. At the University Hospital (U.H.) of Zaragoza extensive efforts have been made to define both the dissemination and the evolution of antibiotic resistance by studying the transferable R plasmids from clinical isolates obtained from hospitalized patients. It was previously demonstrated the existence of an endemic R plasmid disseminated into multiple strains and species of *Enterobacteriaceae* which confers resistance to ampicillin, tetracycline, gentamicin, tobramycin, dibekacin and netilmicin by synthesizing a TEM-1 beta-lactamase and an aminoglycoside-

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acetyltransferase AAC (3)-V (9). This report shows the existence of another plasmid-specified resistance pattern frequently observed in the gram-negative hospital flora. This pattern has been studied with two aims: to show whether the multiple resistance in all the strains was determined by one plasmid species and, if that was the case, to investigate the relationship and evolution of the hospital environment plasmids.

Nine clinical strains of gram-negative bacilli isolated from patients hospitalized in different wards were chosen for this study. These strains were selected among a total of 6.339 R plasmid-harbouring strains isolated at the U.H. over 7-year period (1976-1983).

These isolates were identified by a characteristic resistance pattern transferred to *Escherichia coli* K-12 J62 (F^-, Nal^r, pro, his, lac) by conjugation (Table 1). The MIC's of several antibiotics for *E. coli* K-12 transconjugants were: gentamicin (Gm) and sisomicin (Ss) > 32 μg/ml, kanamycin (Km), neomycin (Nm), and lividomycin (Lv), 256 μg/ml; butirosin (Bu), 2 μg/ml; amikacin (Ak) and tobramycin (Tm), 1 μg/ml; streptomycin (Sm) and chloramphenicol (Cm) > 64 μg/ml.

Plasmid DNA from each *E. coli* K-12 transconjugant was purified, and the molecular weight determined as described (9). All the *E. coli* strains carried a single plasmid of 73 kb (Fig. 1) and these plasmids were found to belong to the Incompatibility group P as demonstrated by the test for incompatibility (1).

Enzyme assays were performed to determine the mechanisms of plasmid-mediated resistance to antibiotics.

The phosphocellulose paper binding assay (4) indicated that these plasmids mediate aminoglycoside resistance by determining the production of aminoglycoside-modifying enzymes. Each *E. coli* K-12 transconjugant showed acetyltransferase, phosphotransferase and nucleotidyltransferase activities. The substrate profiles suggested that acetylation was mediated by an AAC (3)-I with affinity for Gm and Ss, phosphorylation by an APH (3')-I with affinity for Km, Nm and Lv, and nucleotidylation by an ANT (3") with affinity for Sm and spectinomycin (Sp).

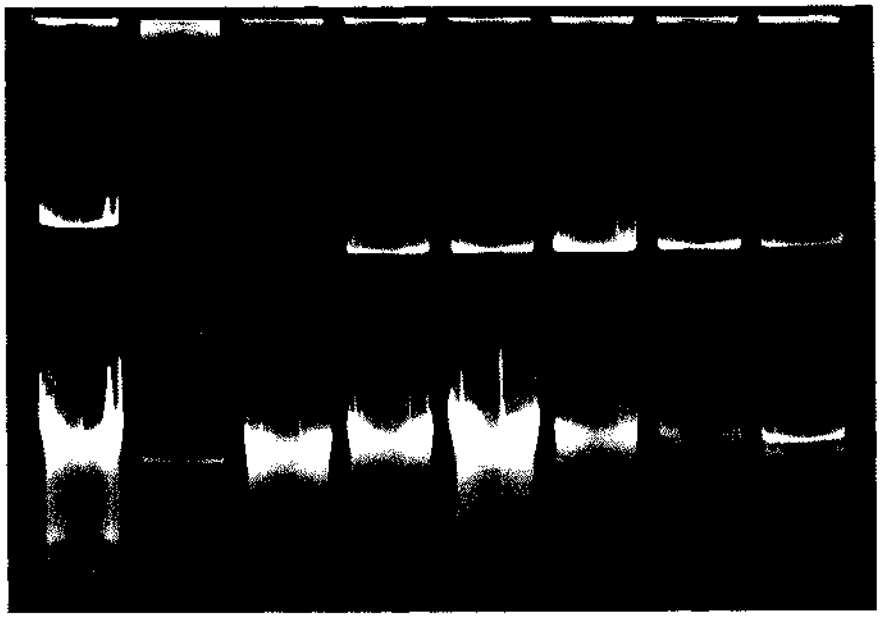

Fig. 1. Agarose gel electrophoresis of plasmid DNA from *E. coli* K-12 transconjugants. (1) Reference plasmid R144 (93 kb). (2) Reference plasmid S-a (45 kb). (3) pUZ 1070. (4) pUZ 7. (5) pUZ 10. (6) pUZ 14. (7) pUZ 15. (8) pUZ 350. Chr, chromosomal DNA.

TABLE 1
CLINICAL ISOLATES ANALYSED FOR R PLASMIDS

Donor strain	Source	Ward	Transferred resistance pattern *	Plasmid
S. marcescens 965	Exudate	Surgery	Ap Tc Gm Km Sm Cm Su	pUZ 7
K. pneumoniae 21938	Exudate	Surgery	Ap Tc Gm Km Sm Cm Su	pUZ 9
E. coli 16306	Urine	Int. Medicine	Ap Tc Gm Km Sm Cm Su	pUZ 10
K. pneumoniae 19932	Urine	Urology	Ap Tc Gm Km Sm Cm Su	pUZ 14
K. pneumoniae 19990	Urine	Urology	Ap Tc Gm Km Sm Cm Su	pUZ 15
S. marcescens 785	Cerebrospinalfluid	Neurosurgery	Ap Tc Gm Km Sm Cm Su	pUZ 270
S. marcescens 1018	Urine	Urology	Ap Tc Gm Km Sm Cm Su	pUZ 350
S. marcescens 1706	Gastric juice	Paediatrics	Ap Tc Gm Km Sm Cm Su	pUZ 613
E. coli 2966	Urine	Urology	Ap Tc Gm Km Sm Cm Su	pUZ 1070

* Abbreviations: Ap, ampicillin; Tc, tetracycline; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Cm, chloramphenicol; Su, sulfamethoxazole.

Beta-lactamase assays (7, 8) demonstrated that resistance to beta-lactam antibiotics was due to the synthesis of a TEM-1 beta-lactamase with an isoelectric point of 5.4

Resistance to Cm was studied by the radioenzymatic assay (10). No chloramphenicol acetyltransferase (CAT) activity was present in extracts from any isolate.

Based on these results it was concluded that these pUZ plasmids encoded aminoglycoside-modifying enzymes with identical site specificity and indistinguishable substrate profiles, conferred resistance to beta-lactams by the same type of beta-lactamase, and mediated Cm resistance by a mechanism which did not involve drug modification by acetylation. To be certain that these plasmids were identical, each of them was analyzed by using restriction endonucleases and agarose gel electrophoresis. The similar fragment patterns obtained by digesting all plasmids with *Eco*RI (Fig. 2) and with *Bam*HI and *Hae*II confirmed this identity. The epidemiological data indicated that this plasmid was first found in an *E. coli* clinical isolate *(E. coli* 16306/pUZ10) and approximately one month later (May 1976) appeared with an extended host range (Table 1). This report demonstrates their dissemination and persistence in gram-negative hospital flora.

It is interesting to mention that, in early 1974, a selftransmissible plasmid (pUZ1, formerly R1033) was isolated from a *Pseudomonas aeruginosa* strain at a nearby hospital (12). This plasmid mediated a spectrum of aminoglycoside resistance pattern associated with the synthesis of aminoglycoside-modifying enzymes qualitatively and quantitatively similar to those isolated at the U.H. Analogous similarities were found for resistance to beta-lactams and Cm. Moreover, evidence of considerable genetic relatedness was found among these plasmids by using restriction endonucleases analysis (data not shown). Finally it should be noted that a similar antibiotic resistance pattern had been detected later among *P. aeruginosa* clinical isolates at the U.H. These findings support the hypothesis of plasmid dissemination in close geographical area. In order to test this hypothesis it will be of interest to determine the structures of these various *P. aeruginosa* plasmids and to compare them with that of the *Enterobacteriaceae* strains.

Focusing the attention on the study of the mechanism of plasmid-mediated resistance to Gm in U.H., it is known that this resistance is mediated by the synthesis of two acetylating enzymes, namely AAC (3)-I and AAC (3)-V, as it has been already published (2, 3). These previous results are now explained by the detection in the hospital of two endemic plasmids each carrying the genes which code for these acetyl-transferases. The mentioned plasmids belong to different incompatibility groups, do not have common DNA sequences, as demonstrated by endonucleases restriction analysis (Fig. 2) and DNA-DNA hybridization experiments (unpublished data), and differ in the mechanism

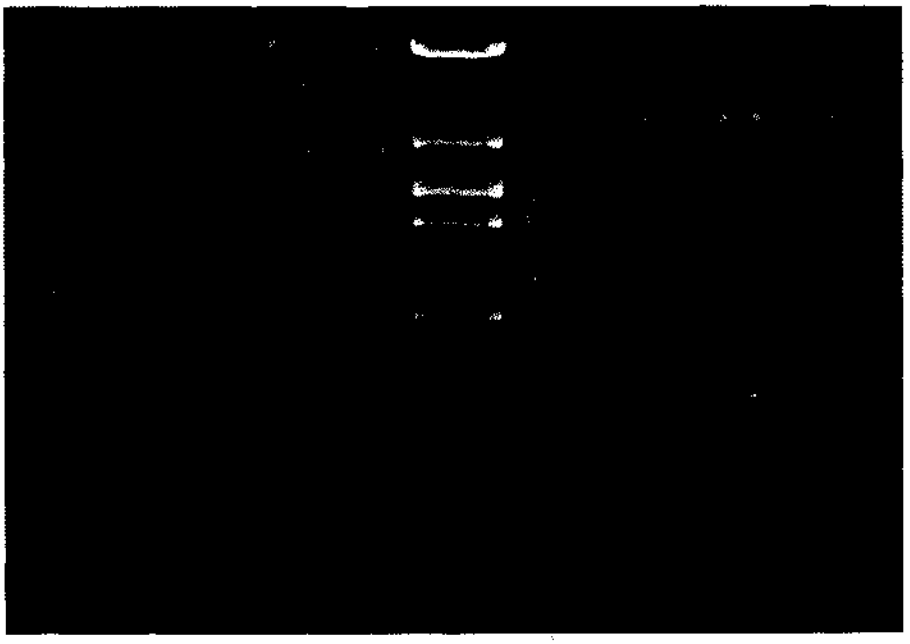

Fig. 2. Analysis by agarose gel electrophoresis of DNA from Inc P and Inc M (I) plasmids digested with *Eco* R1. Electrophoresis was carried out in 1 % agarose vertical slab gel for 9 h. at 60 V. (1) pUZ 10 (Inc P). (2) pUZ 15 (Inc P). (3) pUZ 270 (Inc P). (4) λ DNA. (5) pUZ 2 (Inc M). (6) pUZ 1321 (Inc M). (7) pUZ 1894 (Inc M).

for transposition of Gm resistance genes. The *aac*C1 are located on the Tn1696 multiresistance transposon, described by Rubens *et al.* in pUZ1 plasmid (11), whereas the *aac*C5 genes are transposed by the presence of two copies of the insertion sequence IS 15 Δ (5) recently described (6). All of these data confirm the existence of a new plasmid species although differing only in 5 kb in size from the previously detected endemic plasmid.

The study of Gm resistance plasmids with different antibiotic resistance phenotypes, frequently isolated at the U.H., and the presence of either the Tn1696 or IS 15Δ in these plasmids may cast some new light on relationship and evolution of R plasmids in a hospital environment.

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MICROBIOLOGIA SEM 4 (1988), 61-64 **NOTAS**

Distribución del potasio y sodio en vacuola y citoplasma de *Saccharomyces cerevisiae*

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Summary

In Na^+ grown yeast cells the vacuole is the main Na^+ reservoir, maintaining higher Na^+/K^+ ratios than those in the cytoplasm. This asymmetric distribution may enhance Na^+ tolerance. However, in growing cells the effect is of low significance.

Key words: Sodio, potasio, vacuola, citoplasma, *Saccharomyces cerevisiae.*

Resumen

Cuando la levadura crece en presencia de Na^+, la relación Na^+/K^+ en la vacuola supera considerablemente el valor que se alcanza en el citoplasma. Esta distribución asimétrica puede incidir positivamente en la tolerancia al sodio. Sin embargo, en células en crecimiento el efecto no es significativo.

El K^+ es el catión más abundante en todas las células vivas y el que contribuye en mayor medida a neutralizar las cargas negativas de los aniones celulares. El Na^+, en cambio, es normalmente excluido de la célula, dándose por ello la circunstancia de que los gradientes de Na^+ y K^+ a través de la membrana tienen sentidos contrarios (2). En condiciones extremas, sin embargo, la concentración externa de Na^+ puede ser demasiado alta para que la célula pueda mantener la concentración interna de sodio en un nivel bajo. En estas condiciones, la supervivencia del organismo depende de su capacidad para excluir Na^+, conservando la capacidad de concentrar el K^+. En *Saccharomyces cerevisiae*, la velocidad de crecimiento no disminuye apreciablemente hasta que el contenido en Na^+ alcanza un valor aproximadamente igual al del K^+, lo que tiene lugar a concentraciones externas de K^+ y Na^+ que varían en función de otros factores, como son el pH y la concentración de amonio en el medio (2, 6). En cualquier caso, el mecanismo de bombeo de Na^+ (5) es el mecanismo aparentemente más eficaz para evitar la toxicidad de este catión, pero, debido a la compartimentación celular, las medidas globales del contenido en Na^+ y K^+ podrían no reflejar la realidad de la concentración de iones en el citoplasma, por lo que el mecanismo de bombeo, a su vez, podría no ser el único implicado en la tolerancia al Na^+. Por estas razones, en el presente trabajo he estudiado la distribución de cationes en la vacuola y en el citoplasma, en células de *Saccharomyces* que se encontraban en dos condiciones diferentes: acumulando Na^+ al ser expuestas por primera vez a este catión y durante la desintoxicación de las células que han crecido en concentraciones altas del mismo.

La cepa de *Saccharomyces cervisiae* utilizada en el presente trabajo ha sido la X.2180.1B (*α, SUC2, mal, gal2, CUP1)* procedente del Yeast Genetic Stock Center. Las células se crecieron duran-

te toda la noche en matraces de 250 ml con 100 ml de medio sintético KNa (1) al que se adicionó ClK y, en su caso, ClNa, hasta alcanzar las concentraciones deseadas en cada ensayo (ver pie Fig. 1). La determinación de la distribución del contenido de cationes entre vacuola y citoplasma se realizó, como se indica a continuación, siguiendo los métodos descritos por otros autores (3, 4) con algunas modificaciones. Aproximadamente 10 mg de células se centrifugaron y lavaron 3 veces con medio de ensayo (manitol 0,9 mM, HEPES 1 mM, $MgSO_4$ 5mM, $CaCl_2$. $2H_2O$ 10 μM, llevado a pH 7,5 con L-arginina), diseñado para proteger la integridad de las vacuolas. Tras retirar el sobrenadante se adicionaron 50 μl de medio de ensayo con un 2 % de citocromo c (el citocromo c hace a las membranas citoplasmáticas permeables a los iones y compuestos de bajo peso molecular, pero no afecta a las vacuolas) más 18 μg ml^{-1} de antimicina A y 5 mM de 2-deoxi-D-glucosa (la inhibición del metabolismo energético fue necesaria para evitar que las células que no se hubieran roto pudieran tomar el K^+ liberado por las rotas). Tras 15 min de incubación a temperatura ambiente, las células fueron centrifugadas y lavadas 3 veces con medio de ensayo más antimicina A y 2-deoxi-D-glucosa, obteniéndose de estas extracciones el K^+ y Na^+ citoplásmico. El sedimento de células se suspendió entonces en 5 ml de agua destilada durante 10 min a temperatura ambiente, para de esta forma, romper las vacuolas por choque osmótico. Tras centrifugar, el extracto se sometió a 2 nuevos lavados (3 ml de agua durante 5 min cada uno) para extraer todo el K^+ y Na^+ celular. Finalmente los cationes de las células que no se rompieron se extrajeron con una solución de HCl 0,2 M y $MgCl_2$ 10 mM. Para calcular el contenido en Na^+ y K^+ del citoplasma y de la vacuola, así como de las células que no se habían roto, todos los sobrenadantes se analizaron por espectrofotometría de absorción atómica.

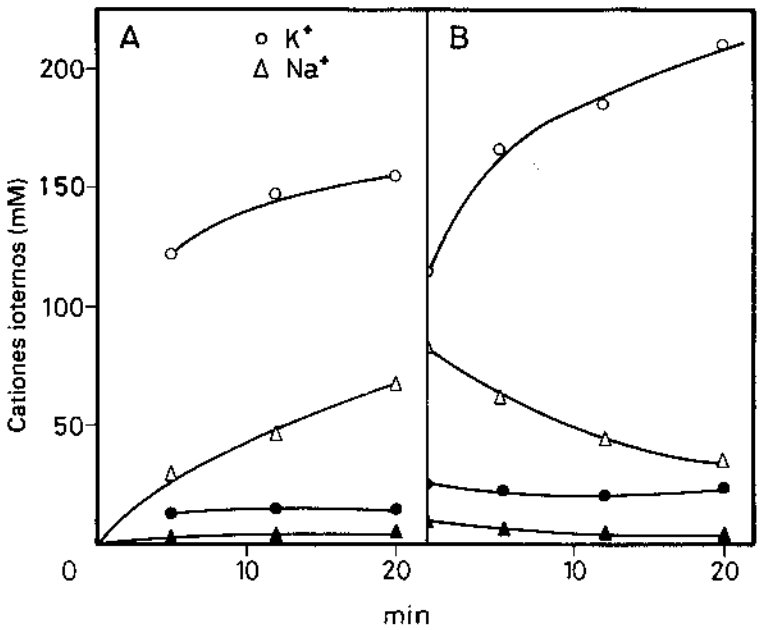

Fig. 1. Concentración de Na^+ y K^+ en el citoplasma y vacuola. (A) Células crecidas en medio sintético con 0,5 mM de K^+ y sin Na^+ se centrifugaron y, a tiempo cero, se suspendieron en medio sintético con 0,5 mM de K^+ y 100 mM de Na^+; a tiempos se determinó el contenido de Na^+ y K^+ en el citoplasma y vacuola. (B) Células crecidas en medio sintético con K^+ 0,5 mM y Na^+ 100 mM se centrifugaron y lavaron dos veces con agua destilada y, a tiempo cero, se suspendieron en medio sintético con K^+ 100 mM; a tiempos se determinó el contenido de Na^+ y K^+ en el citoplasma y vacuola. Símbolos: (▲) Na^+ en citoplasma (△) Na^+ en vacuola, (●) K^+ en citoplasma y (○) K^+ en vacuola.

La cepa de levadura estudiada, antes de ser expuesta a Na^+, mostró un contenido de K^+ más alto en la vacuola que en el citoplasma, lo que en parte se justifica por el mayor volumen de la vacuola (observaciones microscópicas no cuantificadas). Al transferir las células crecidas en el medio con 0,5 mM de K^+, al medio con 0,5 mM de K^+ y 100 mM de Na^+, la acumulación de Na^+ empezó inmediatamente, tanto en la vacuola como en el citoplasma, de tal forma que en la primera muestra (5 min) la relación Na^+/K^+ era la misma en la vacuola y en el citoplasma (Figs. 1 y 2). A partir de los 5 min la relación Na^+/K^+ permaneció constante en el citoplasma (alrededor de 0,2) mientras aumentaba constantemente en la vacuola. Es interesante observar que la acumulación de Na^+ en la vacuola estimuló la acumulación de K^+, aunque a una velocidad mucho más lenta (Fig. 1).

En el otro experimento, las células se cargaron de Na^+ cultivándolas en un medio con este catión durante toda la noche y midiéndose su salida al ser suprimido del medio de cultivo. La primera observación en este ensayo, previa a la supresión del Na^+ del medio de cultivo, es que el K^+ vacuolar estaba en el nivel normal de las células no expuestas a Na^+. Por el contrario, el K^+ citoplásmico era más alto. A pesar de ello, la relación Na^+/K^+ en el citoplasma era alta debida a la gran acumulación de Na^+ (Fig. 2). Al eliminar el Na^+, el K^+ fue sustituyendo al Na^+, tanto en la vacuola como en el citoplasma, pero la velocidad de sustitución fue notablemente más alta en la vacuola (Fig. 1). Así, en 20 min la relación Na^+/K^+ en la vacuola pasó de 0,70 a 0,17, mientras que en el citoplasma pasó de 0,37 a 0,13 (Fig. 2). Esta facilidad para intercambiar el Na^+ vacuolar con el K^+ citoplásmico contrastó con la tenacidad con que las vacuolas retenían los cationes durante los lavados, lo que sugiere que los cationes no se pierden sino que se cambian, probablemente en un proceso que depende de ATP.

Estos resultados ponen de manifiesto que, en células de *Saccharomyces cerevisiae* en crecimiento, la vacuola juega un papel significativo, aunque no muy eficaz, en la tolerancia al Na^+. El Na^+ sale rápidamente de la vacuola y, por ello, se puede considerar movilizable (Fig. 1), lo que hace suponer un rápido intercambio entre citoplasma y vacuola. En una célula en crecimiento, a una

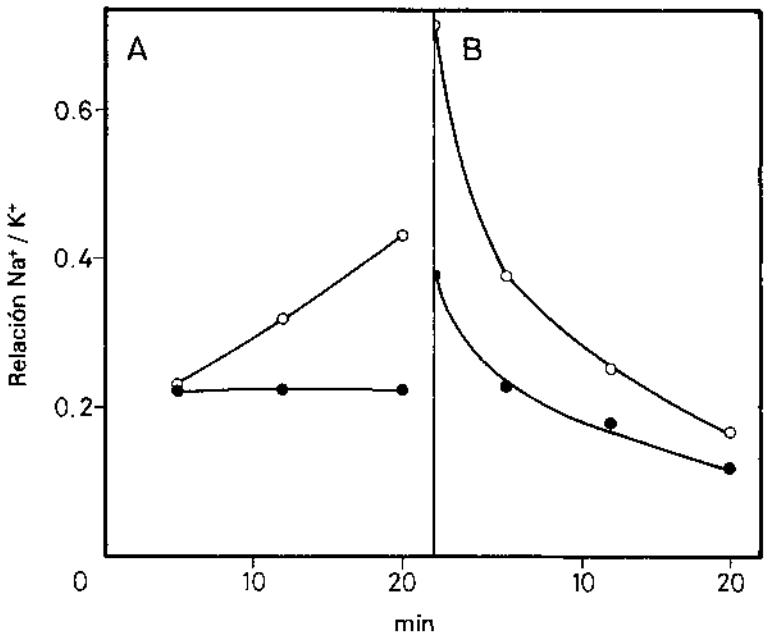

Fig. 2. Relación Na^+/K^+ en citoplasma y vacuola deducida de la Fig. 1. (A) Relación Na^+/K^+ durante la acumulación de Na^+. (B) Relación Na^+/K^+ durante la salida de Na^+. Símbolos: (●) Na^+/K^+ en citoplasma y (○) Na^+/K^+ en vacuola.

concentración alta de Na^+ en el exterior, el Na^+ entra con el K^+, y mientras el K^+ es retenido, el Na^+ es extraído a una cierta velocidad por un sistema específico (5). Un efecto equivalente a la extracción se produce por el crecimiento, el cual compensa por el incremento del volumen una cierta fracción del sodio que entra. Así, el nivel de Na^+ se establece como resultado de los flujos de entrada y salida y del efecto de incremento del volumen. Como el aporte para compensar el crecimiento es igual a la constante cinética de crecimiento por el contenido de cada compartimento, la distribución asimétrica del catión entre vacuola y citoplasma hace que la mayor parte del Na^+ que se transporta a través de la membrana citoplásmica acabe situada en la vacuola. En otras palabras, a la velocidad de salida del Na^+ del citoplasma, que es una cinética de primer orden (5), hay que añadir la velocidad de entrada del Na^+ a la vacuola, que es igual a su contenido por la constante cinética de crecimiento. Sin embargo, siendo la constante cinética de salida de Na^+ (0,11 min^{-1}, ver ref. 5) mucho mayor que la constante cinética de crecimiento de μ (0,004 min^{-1}), las diferencias de contenido entre la vacuola y el citoplasma no llegan a hacer que el efecto de crecimiento sea importante frente a la salida.

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Errata

Aislamiento extraintestinal de *Salmonella* en gallinas: estudio epidemiológico de dos brotes de salmonelosis por consumo de huevo crudo

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En este trabajo que apareció en el volumen 3, 209-211, debió figurar el siguiente Summary:

Summary

Two *Salmonella* oubtreaks which had occurred in two small family farms were investigated. *S. enterica I* serotypes *typhimurium* and *enteritidis* were isolated from cloaca, liver oviduct and ovary of hens, and in one case from the egg yolk. These observations suggest that serotypes of *Salmonella* may be carried within egg shells following contamination during early oogenesis.

Key words: S. enteritidis, S. typhimurium, *Eggs, Ovary, Foodborne.*

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