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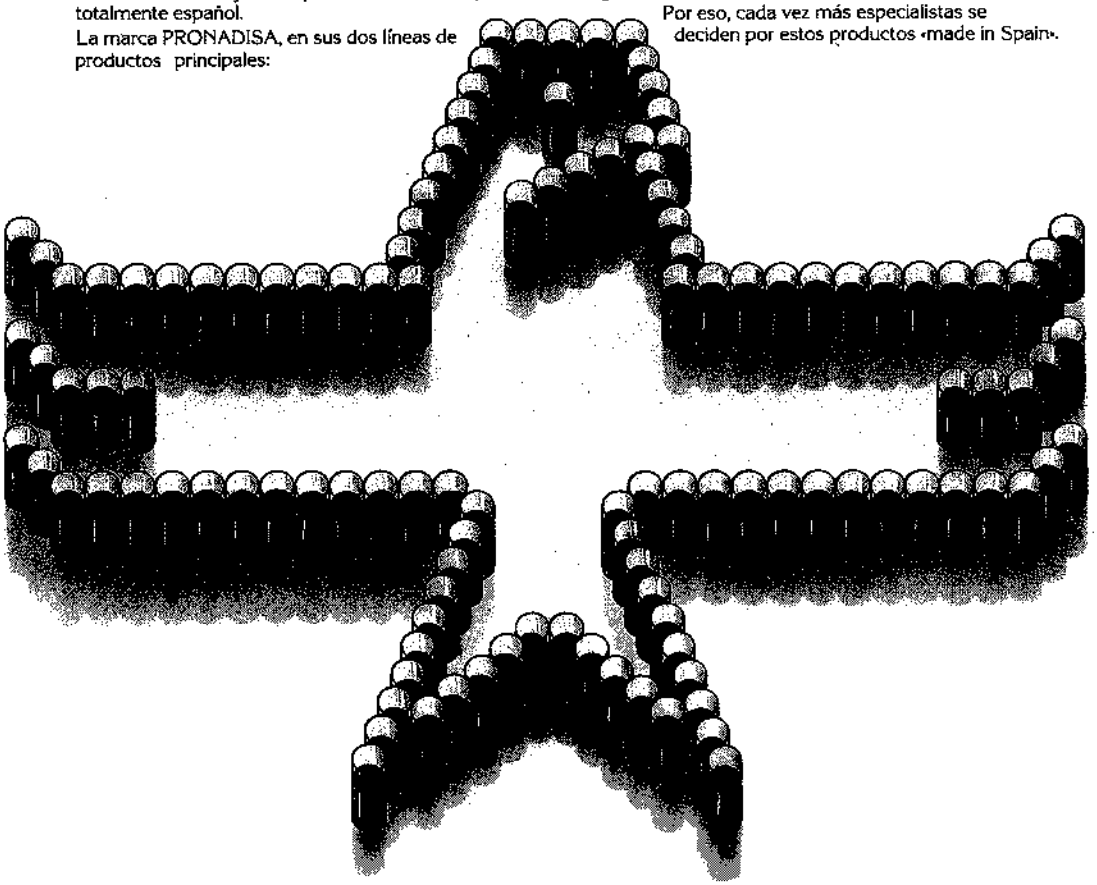

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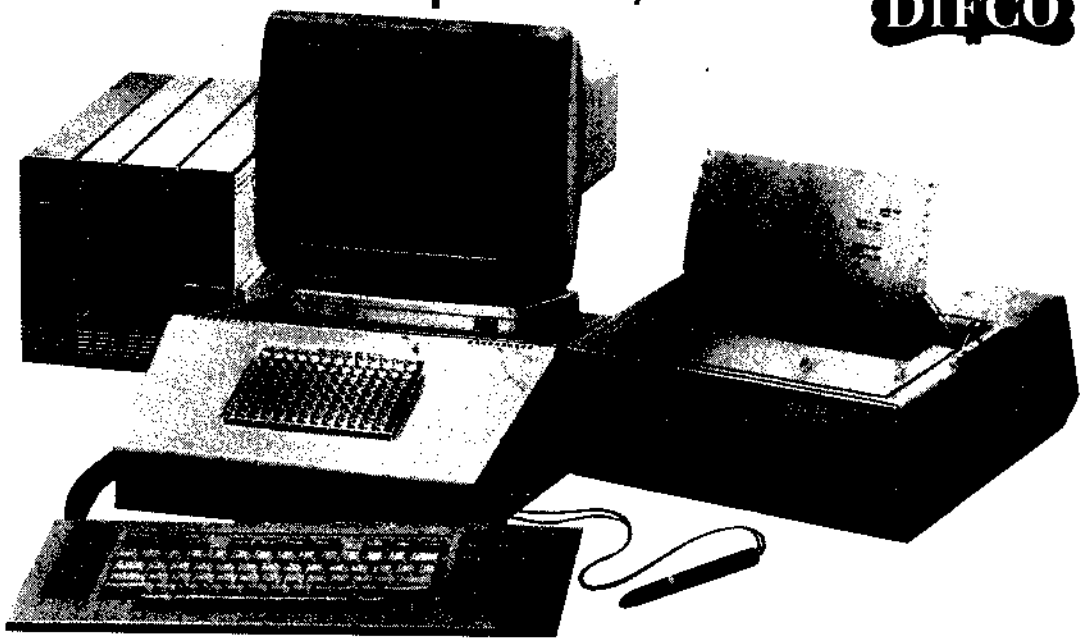

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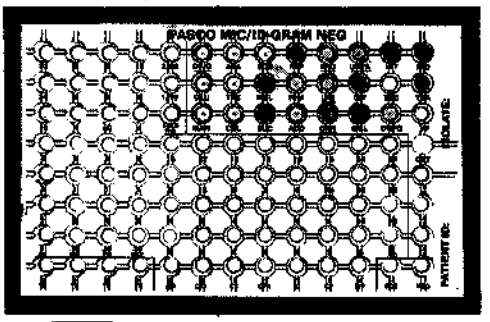

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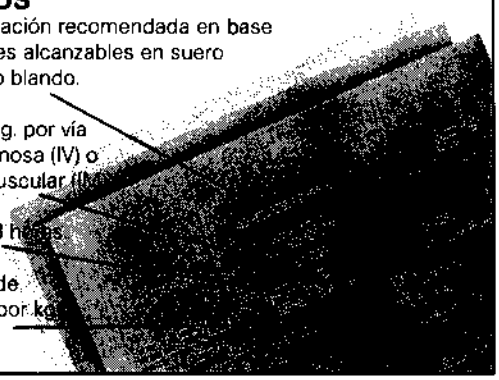

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Potassium ion accumulation in cells of different halobacteria

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Summary

Halobacteria live in extremely hypersaline environments accumulating K^+ as compatible solute. We have studied the accumulation of intracellular potassium in recently isolated halobacteria with relatively moderate salt response, and compared it with the classical halobacteria with very extreme salt response. Significant differences have been found, the more moderate group having lower intracellular K^+ concentrations. Some experiments have been carried out concerning the enrgy dependence of this K^+ accumulation, which indicate that the moderate group seems to be less dependent on metabolic energy. The results are consistent with existence of different degress of halophilism among halobacteria, probably corresponding to adaptation to different habitats.

Key words: Halobacteria, compatible solute, halophilic bacteria, potassium intracellular concentration.

Resumen

Las halobacterias viven en ambientes extremadamente hipersalinos acumulando potasio intracelularmente como soluto compatible. Hemos estudiado la acumulación de potasio intracelular en halobacterias aisladas recientemente con una respuesta a la sal relativamente moderada, y las hemos comparado con aquellas cepas clásicas de halobacterias que poseen una respuesta a la sal muy extrema. Se han encontrado diferencias significativas, así, el grupo más moderado contiene concentraciones de potasio más bajas. Se han realizado algunos experimentos relativos a la dependencia energética de esta acumulación de potasio, estos experimentos indican que el grupo moderado parece ser menos dependiente de la energía metabólica. Los resultados corroboran la existencia de diferentes grados de halofilismo entre halobacterias, probablemente debido a la adaptación a hábitats diferentes.

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Introduction

The members of the *Halobacteriaceae* are extremely halophilic archaebacteria which can grow in nearly saturated NaCl solutions. To compensate for the extremely high extracellular solute concentration these organisms accumulate K^+ intracellularly, concentrating this ion to hundreds of times the external level, whilst keeping Na^+ and Cl^- at lower concentrations than outside. The intracellular concentration of K^+ varies according to the extracellular NaCl concentration, K^+ is thus acting as a compatible solute (4).

Halobacteria have been considered for many years as a rather homogeneous group containing very few species of rather similar organisms (2). However, recently, this has been shown to be a mistake resulting from the use of a single type of enrichment culture and similar type of samples, and many different types of halobacteria have been described. In particular, some new species present a more moderate response to salt concentrations, requering much less requirements to grow and survive (7, 9, 10). However, all work done on intracellular concentrations of ions had been done only with species of the more extremely halophilic type, where an intracellular concentration of potassium around 5M (1, 3) or 3.7M (3) was found in media containing 25 % NaCl. We decided to investigate the accumulation of potassium in the new types including the ones with lower salt requirements. In fact, there appear to be two groups of halobacteria with distinct intracellular levels of K^+, one corresponding to the species with more extreme salt response and the other to the more moderate ones.

Materials and methods

Strains and culture conditions

The following collection strains were used: *Halobacterium halobium* CCM 3090, *H. mediterranei* ATCC 33500, *H. trapanicum* ATCC 33960, *H. saccharovorum* ATCC 29252, *H. volcanii* NCMB 2012 and *H. gibbonssii* ATCC 33959. The media used contained a mixture of salts corresponding in proportions to those found in sea water, which will be referred to as SW, at the final total concentrations (W/V) indicated: 17, 25 or 30 %. Media were supplemented with yeast extract (Difco) 0.5 % (W/V). The cells were grown in 100 ml batches in 500 ml Erlenmeyer flasks incubated at 37° C in an orbital shaker.

Determination of the intracellular K^+ concentrations.

One hundred ml of late logarithmic cultures of the strains with OD at 520 nm *ca* 1.0 were harvested by centrifugation and resuspended in 10 ml of culture media that were then incubated in 250 ml Erlenmeyer flasks at 37° C and with intense orbital shaking for 1 hour. Then 1 ml of this thick cell suspension was deposited into each of four Eppendorf 1.5 ml microfuge tubes. 50 µl ^{14}C-Dextran Carboxil 0.5 mCi/g (New England Nuclear) or 5 µl ^{3}H-water 4.5 mCi/mol (New England Nuclear) was added to two of the tubes. The tubes were shaken for 10 minutes and then centrifuged at 3500xg in a TDX microfuge for 5 minutes. From the tubes containing radioactive compounds 100 µl of the supernatants were transferred to scintillation vials, the rest was withdrawn and the walls of the

tubes carefully dried with filter paper to leave the pelled as dry as possible. Then the pellets were resuspended in 200 μl of 25 % sterile salt solution and 100 μl were transferred to scintillation vials. After adding scintillation liquid (HP Beckman) the vials were counted in a Beckman-2800 scintillation counter. Cell volume in the pellet was found after subtracting from the total water space the extracellular space accessible to Dextran (12). The pellets of the remaining two tubes were used to determine the K^+ content and the total protein. For the potassium determination, the pellet was resuspended in 1 ml 10 % TCA, centrifuged again and then the K^+ concentration in the supernatant was measured by flame emission spectrophotometry in a Perkin-Elmer atomic absortion spectrophotometer. The last pellet was washed twice with 15 % salts solution and then the protein determined by the Lowry method (6).

K^+/Protein ratio determinations.

A variable amount of cell suspension was filtered through 45 mm diameter 0,45 μm pore size HA Millipore filters until clogging of the filter made the filtration slow. The amount was very variable depending on the density of the cell suspension. The filter was then washed three times with 5 ml of a solution of NaCl analytical grade (Merck) of the same concentration (% W/V) as the total salts in the cell suspension. The filters were then transferred to tubes with 5 ml of deionized water and sonicated for 20 minutes in an ultrasonic bath of the types used for cleaning glassware. This treatment produced the lysis of most of the cells attached to the filters. After shaking thoroughly, the filters were withdrawn from the tubes with forceps and then the concentration of K^+ and protein in the tubes determined as above.

The Optical Density of the cultures was followed using a Spectronic-20 spectrophotometer at a wavelenght of 520 nm.

Results

The intracellular K^+ concentrations found varied considerably among the different species studied (Table 1). Two groups were apparent, the strains of *H. halobium, H. saccharovorum* and *H. trapanicum* had high intracellular concentrations reaching 5 M at 15 % SW, as had been described for *H. salinarium* (1). The second group, comprising the strains *H. mediterranei, H. volcanii* and *H. gibbonsii,* had much lower values at this concentration. The first group maintained higher concentrations than would correspond to a linear variation according to the extracellular concentration, whilst in the second group the concentrations decreased accordingly.

Considering that these differences could reflect a different physiological response to the environmental salt concentration we have studied some aspects of the salt response in representatives of each group. The potassium cell content was estimated by the K^+/protein ratio in order to simplify the experimental procedure. In general a good correlation was found between this ratio and the intracellular concentration. A marked effect of the phase of growth on the K^+ cell content has been described (4, 1). Therefore, we studied the values of the K^+/protein ratio along the growth curve of the strains shown in

TABLE 1

INTRACELLULAR CONCENTRATIONS (MOLARITY)[a] AND K^+/PROTEIN[a] RATIO OF DIFFERENT HALOBACTERIA

Organism	Type of range	Total salts in the medium (Na^+ - M)					
		17 % (2.3)		25 % (3.3)		30 % (4)	
		M	K^+/Prot.	M	K^+/Prot.	M	K^+/Prot.
H. halobium	Extreme	3.0	1.5	4.3	2.1	5.6	3.3
H. saccharovorum	Extreme	3.4	1.3	4.8	2.2	5.2	3.0
H. trapanicum	Extreme	3.2	1.7	4.9	2.5	5.5	3.5
H. volcanii	Moderate	2.1	0.9	3.4	1.5	4.0	2.2
H. mediterranei	Moderate	1.9	0.7	2.3	1.1	3.2	1.8
H. gibbonsii	Moderate	2.3	1.0	3.0	1.3	4.0	2.1

[a] All the values are, at least, the mean of three determinations.

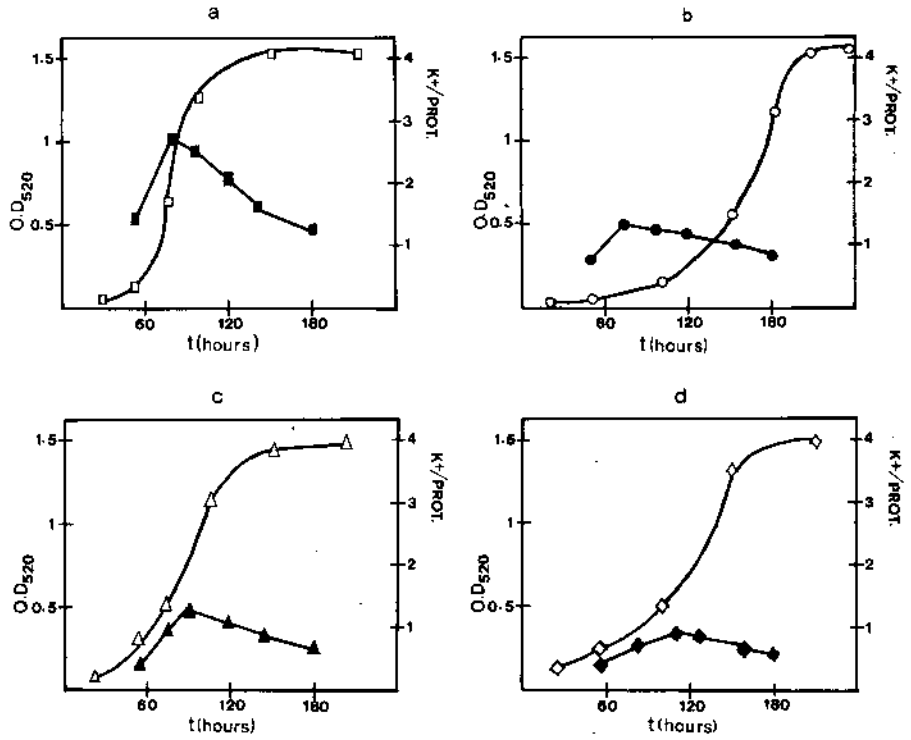

Fig. 1. Growth curves and K⁺/Protein ratio of *H. mediterranei* and *H. halobium* at different salt concentrations: a) □, OD_{520} *H. halobium* at 30 % SW; ■, K⁺/Protein ratio of *H. halobium* at 30 % SW. b) ○ OD_{520} *H. halobium* at 17 % SW; ●, K⁺/Protein ratio of *H. halobium* at *17 % SW. c)* △ OD_{520} *H. mediterranei* at 30 % SW; ▲, K⁺/Protein ratio of *H. mediterranei* at 30 % SW. d) ◇ OD_{520} *H. mediterranei* at 17 % SW; ◆, K⁺/Protein ratio of *H. mediterranei* at 17 % SW.

Fig. 1. As has been described, all the organisms showed an increase of cellular K^+ during the logarithmic phase. However, this increase was much sharper for the strains with higher concentrations (Fig. 1). This behaviour during the logarithmic phase is generally assumed to be due to the higher energy availability that is characteristic of this period. Therefore, the differences between the two groups could be due to a higher energy-dependent ion exchange in the group with higher concentrations. To check this point we studied the K^+/protein ratio of populations subjected to three levels of energy availability, firstly a medium supplied with a metabolizable substrate and oxygen, secondly without substrate but aereated, so that internal reserves could be utilized, and thirdly without substrate or oxygen so that very little metabolic energy could be generated (only the fermentation of some endogenous substrates which would be rapidly depleted) (5). Energy starvation produced in all groups a drop in the K^+/protein values which continued to decrease during all the period studied. Nevertheless, even after 69 hours of incubation in these conditions (Table 2) the group with higher concentrations had higher values than the others, although the differences appeared to be reduced. When the cells were preincubated anaerobically with light to induce purple membrane formation (8), *H. halobium* maintained higher levels of intracellular potassium (Fig. 2).

The effect of extracellular potassium on the K^+/protein ratio of *H. halobium* and *H. mediterranei* is shown in Table 3. The K^+ cell content seems independent of the extracellular K^+ concentration in both strains, although a slight but consistent difference appeared in *H. halobium* with 2 % extracellular K^+ that had somewhat higher values.

TABLE 2

EFFECT OF DIFFERENT INCUBATION CONDITIONS ON K^+/PROTEIN RATIO[a]

Time (hours)	Growth condition		
	Aerobically		Anaerobically[b]
	Glutamic acid Mineral salts	Mineral salts	Mineral salts Dark
	H. halobium		
21	1.9	1.0	0.8
45	1.7	1.0	0.8
69	1.0	0.7	0.5
92	0.8	0.6	0.5
	H. mediterranei		
21	1.3	1.0	0.8
45	1.3	0.8	0.5
69	1.3	0.5	0.3
92	0.8	0.5	0.3

[a] All the experiments were done at 25 % final salt concentrations.

[b] Anaerobic conditions were obtained bubbling nitrogen through the medium.

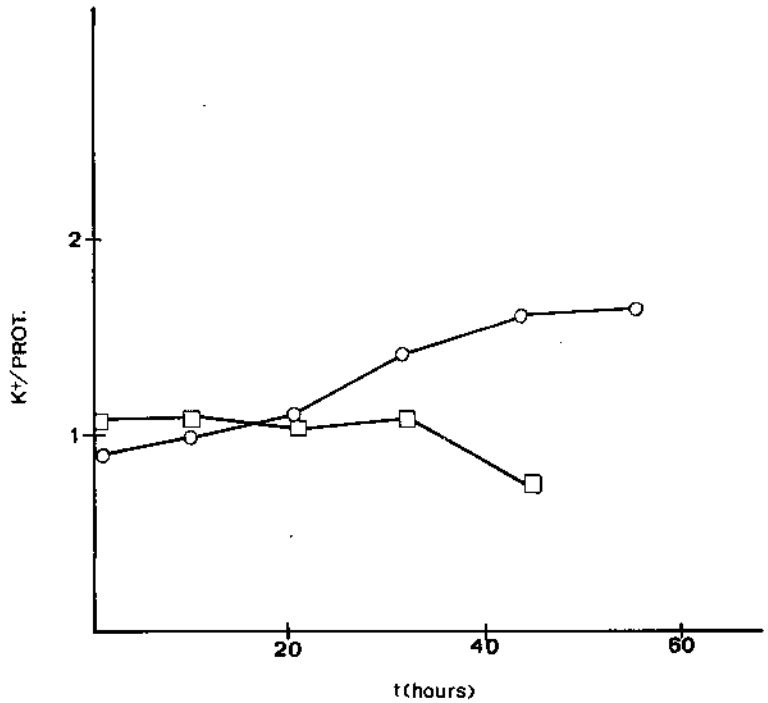

Fig. 2. Effect of purple membrane induction on K^+/Protein ratio; ○, *H. halobium* after preincubation anaerobically with light; □, *H. halobium* after preincubation aerobically.

TABLE 3

EFFECT OF EXTRACELLULAR POTASSIUM ON THE K^+/PROTEIN RATIO

Time (hours)	25 % SW without K^+	25 % SW	25 % SW + 2 % K^+
		H. halobium	
29	1.9	1.8	1.8
46	1.8	1.8	1.9
76	2.0	1.9	2.1
		H. mediterranei	
29	0.8	0.7	0.6
46	0.9	0.8	0.7
76	0.9	0.8	0.7

Discussion

There appear to be two groups within the halobacteria regarding the intracellular concentration of their compatible solute K^+. The group with lower concentrations also corresponds to organisms with more moderate salt range for growth and survival (7, 10). In both groups K^+ intracellular concentrations vary according to the extracellular NaCl concentration. In fact, the more moderate group kept the K^+ concentrations proporcional to the extracellular ones, even at the lowest concentration used in the medium, whilst the more extreme, in contrast, maintained a higher value in this medium. The question that arises is whether the moderate halobacteria have an additional compatible solute of if the extreme group have higher concentrations of K^+ than those merely required to compensate the extracellular solutes.

Although the evidence in this respect is not conclusive, it has been generally assumed that K^+ is accumulated intracellularly by means of an H^+ dependent uniport. However, K^+ is also maintained at much higher intracellular concentration, when no energy is available, due to a selective permeability of the membrane, which is 20 times more permeable to K^+ than Na^+ whilst the exit of potassium from the cell is strongly impeded (14). In both groups the accumulation of potassium seemed virtually independt of the extracellular concentrations of this ion, which shows that in both groups the K^+ accumulation mechanisms are extremely efficient. However, it seems as though the moderate group had a lower activity of the energy-dependent mechanisms although the passive ones were similarly efficient in both groups, which would explain the lower relative decrease experimented for them under energy-starvation conditions.

One indirect piece of evidence of the lower K^+ content of the moderate group is that protein synthesis *in vitro* requires very little or no potassium in *H. mediterranei* (R. Amils, personal communication).

The results presented here are consistent with ecological and taxonomical data obtained earlier that point towards the existence among halobacteria of two groups adapted to different conditions and with profound differences at several levels (14, 13). The group of *H. halobium, H. trapanicum* and *H. saccharovorum,* organisms with lower growth rates and complex nutrient requirements, live in the more concentrated hypersaline environments and maintain very high levels of intracellular potassium, spending probably considerable amounts of energy. The group of *H. mediterranei* and others with low intracellular content, have high growth rates, simple nutrient requirements and high nutritional versatility (10). They live in hypersaline media of intermediate salt concentrations, where they have to compete with halophilic eubacteria (11). If their intracellular K^+ could be maintained at a higher level by passive mechanisms it would mean saving important amounts of metabolic energy.

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Plasmid profile analysis as a tool for characterization of epidemic *Shigella sonnei* strains

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Summary

The usefulness of plasmid profile analysis as a marker to determine the strain number of *Shigella sonnei* implicated in an epidemic outbreak and its prevalency in our community was studied. We could define two multirresistant strains and determine that the drug resistance was plasmid mediated in both. One strain carried a *tra*$^-$ plasmid which encoded (Sm, Sd)^r, the other one carried two *tra*$^+$ plasmids which encoded (Ap-Cb, Sm, Sd)^r and (Sm, Km-Nm, Sd, Tp)^r respectively. These strains and some coisolates collected during the later phase of the outbreak carried cryptic plasmids which showed similar electrophoretic mobilities.

Key words: Shigella sonnei, plasmid, epidemic outbreak, epidemiological marker.

Resumen

Se ha analizado la utilidad del perfil de plásmidos como marcador para determinar las cepas de *Shigella sonnei* implicadas en un brote epidémico, así como su mantenimiento en la población a lo largo de los diez meses siguientes al brote. Se ha establecido la presencia de dos cepas multirresistentes determinándose que las resistencias son codificadas por plásmidos. Una de las cepas porta un plásmido *tra*$^-$ que codifica para (Sm, Sd)^r y la otra dos plásmidos *tra*$^+$ que codifican (Ap-Cb, Sm, Sd)^r y (Sm, Km-Nm, Tp)^r, respectivamente. Ambas cepas, así como algunos de los coaislamientos recogidos durante la cola prosodémica del brote portan plásmidos crípticos de similar movilidad electroforética.

Introduction

In Asturias, the shigellosis is a sporadically diagnosed illness which mainly affects *children from areas with inadequate sanitation. It is usually transmited from person to* person or from contaminated water.

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During the first weeks of February, 1982, an unusual outbreak of dysentery took place in Oviedo, affecting over 1000 persons, mainly children. The outbreak was due to a temporary fail in the chlorination of one of the water supply deposits of the city. The responsible bacterium was isolated from feces of the hospitalized patients and outpatients in several laboratories and it was biochemically and serologically identified as *Shigella sonnei.* All the primoisolates under study showed resistance to streptomycin and sulfadiazine, and some of them to other antibiotics as well.

This work was enterprised with the following aims: i) to use the plasmid prolife analysis as a tool to verify if there were one or more varieties of *S. sonnei* involved in the outbreak; ii) the epidemiological pursuit of the strains responsible for the outbreak; iii) the study of the pattern of resistance and the genetic nature of the multiple antibiotic resistance.

A preliminary study of this outbreak has been presented previously (Mendoza, M. C., Llaneza, J., Méndez, F. J., Mayo, B. and Hardisson, C., 1983. Proceeding III Mediterranean Congr. of Chemother. Dubrovnik, 1982, pp. 102-103).

Materials and methods

Bacterial strains and plasmids

A total of 44 isolates of *S. sonnei* from 45 different patients with diarrheal illness were studied. Of these, 21 were obtained during the first days of the epidemic outbreak, 14 of them in the «Hospital Nuestra Señora de Covadonga» Laboratory and 7 in other non hospital laboratories. The rest were obtained during the 10 months after the outbreak, from patients with diarrheal illness in the Hospital Laboratory. Strains of *Escherichia coli* K12 (W3110 Rif^r and 185 Nal^r) were used as recipients in mating experiments and *the strain 803 recA* in genetic transformation experiments.

The plasmids R40a (96 MDa); R65 (62 MDa); RP4 (34 MDa), pBR322 (2.7 MDa) and pVA517 A-H (35.8; 4.8; 3.7; 3.4; 2.6; 2.0; 1.8 and 1.4 MDa) carried by *E. coli,* were used as molecular size standards. They were kindly supplied by Esther M. Lederberg, Plasmid Reference Center, California, USA.

Antibiotic susceptibility

Antibiotic resistance was studied by paper disk diffusion and agar dilution standard methods, on Mueller Hinton agar (Difco Lab.).

Genetic plasmid transfer

Matings were performed in liquid medium as previously described (8) and transformations were carried out by the method of Mandel and Higa (6).

Plasmid analysis

Plasmid DNA was isolated by the cleared lysate method of Clewell and Helinsky (2), followed by CsCl-ethidium bromide centrifugation. For small scale preparation, a rapid variant of the single colony minicleared lysate (1) was used. The restriction endonuclease digestions, using enzymes supplied by Boehringer Mannheim, were carried out according to the manufacturer's suggestions. Analysis of plasmids and plasmid DNA restriction fragments were performed by electrophoresis in 90 mM Tris borate-2 mM EDTA (pH 8) horizontal slab gels of 0.7 % agarose. Gels were run at 50-100 V for several hours and stained with 0.2 μg/ml ethidium bromide. Plasmid size was estimated by including molecular weight standard plasmids in the gels, and restriction fragment sizes by relation to *Hin*dIII-digested λ DNA (7).

Results

The susceptibility of the 44 primoisolates was assayed with 20 antimicrobial drugs which are commonly used either alone or combined in our community to control gram-negative infections, including those recommended for gastroenteric infections. All the 21 primoisolates collected during the former phase of the outbreak were susceptible to cephalothin, cefoxitin, gentamicin, tobramycin, amikacin, spectinomycin, tetracycline, chloramphenicol, fosfomycin, nalidixic acid, norfloxacin, rifampicin and eritromycin and resistant to streptomycin (Sm) and sulphadiazine (Su). In addition, 9 of the isolates showed resistance to ampicillin (Ap), carbenicillin (Cb), kanamycin (Km), neomycin

TABLE 1

CHARACTERISTICS OF THE *SHIGELLA SONNEI* EPIDEMIC PRIMOISOLATES AND OF THEIR R-PLASMIDS

Type	Number of isolates studied	R-phenotype (MIC in μg/ml)	Plasmid profile	R-plasmids harboured			
				Transfer mechanisms	R-phenotype	Size (MDa)	Denomination
I	12 from former outbreak phase	$Sm_{>256}$, $Su_{>1024}$,	Fig. 1	Transformation	Sm, Su	7.1	pUO122
	10 from later outbreak phase	$Sm_{>256}$, $Su_{>1024}$,	Fig. 2	Transformation	Sm, Su	7.1	pUO122
II	9 from former outbreak phase	$Sm_{>256}$, $Su_{>1024}$, $TP_{>64}$, $Ap\text{-}Cb_{>1204}$, $Km\text{-}Nm_{>256}$	Fig. 1	Conjugation	Ap-Cb, Sm, Su	60	pUO120
				Conjugation and Transformation	SM, Km-NM, Tp	53	pUO121
				Transformation	Km-Nm, Tp	53	pUO121

Sm: streptomycin; Su: sulphadiazine and other sulphonamides; Tp: trimethoprim; Ap: ampicillin; Cb: carbenicillin; Km: kanamycin; Nm: neomycin.

(Nm) and the combination of trimethoprim (Tp) and sulphamethoxazole. The levels of resistance (MICs, in μg/ml) are shown in Table 1. Both patterns of resistance were found in hospitalized and non hospitalized patients. It is worthwhile to underline that isolates of *S. sonnei* showing both patterns of resistance were found in one hospitalized child. Plasmid DNA from these 21 primoisolates was analyzed by electrophoresis in agarose gels. Figure 1 shows that all the primoisolates carry several plasmids, and that some of them have different electrophoretic mobilities. According to the pattern of resistance and the profile of plasmids, these primoisolates were separated into two groups: Type I, isolates showing Sm and Su resistance, which carried at least four or five small plasmids with sizes between 1.4 and 10 MDa (Fig. 1) and Type II, isolates with resistance to Sm, Su, Tp, Ap-Cb and Km-Nm, which carried at least two large plasmids of about 53 and 60 MDa respectively as well as 4 small plasmids (Fig. 1).

The R-phenotype and the plasmid profile of the 23 isolates obtained from diarrheical patients who requested hospital care during the following months to the outbreak were also studied. We found that 10 of them showed the same R-phenotype and plasmid profile as the isolates of Type I and we assumed that they corresponded to cases of secondary infections; the rest of the isolates showed the same or a different R-phenotype and their plasmid profiles were different among them and from those of Type I of Type II isolates, thus we assumed that they belonged to other strains (Fig. 2).

To verify if the multiple drug resistance was self-transferable, we carried out mating experiments using *S. sonnei* primoisolates as donors and *E. coli* K12 W3110 Rif^r as a recipient strain. No transfer of resistance was observed in crosses with primoisolates of Type I, butt all the primoisolates of Type II transferred there patterns of resistance with different frequencies. The transconjugat clones with pattern-1 were resistant to Ap-Cb, Sm and Su; those with pattern-2 were resistant to Sm, Km-Nm and Tp and those with pattern-3 were resistant to all the markers included in patterns 1 and 2. Pattern-3 appeared with

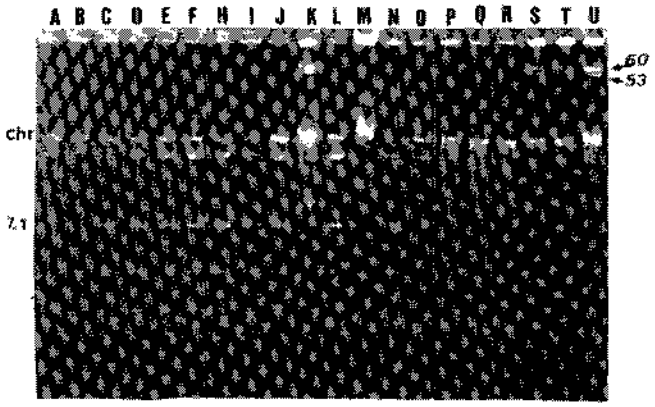

Fig. 1. Plasmid profile of *S. sonnei* strains from the former phase of the outbreak. Isolates shown in wells A, B, C, D, E, F, H, I, J and L which carry five small plasmids, together with N and O, which carry one plasmid less were all included in Type I strain. Isolates shown in wells K, P, Q, R, S, T and U exhibited the same plasmid profile composed of two large (60 and 53 MDa) and four smaller plasmids, they were included in Type II strain. M: *E. coli* pUA517 (molecular weight standards). Chr, chromosome. The numbers indicate MDa.

Fig. 2. Plasmid profile of *S. sonnei* isolates from the later phase of the outbreak. Agarose gel electrophoresis of 15 isolates collected during the 10 months after the start of the outbreak. Lysates in wells A and B showed the same plasmid profile than Type I strains. The rest of the isolates showed a different plasmid profile among them and with respect to Type I and II strains. Chr, chromosome.

the highest frequency and included all the resistances of Type II primoisolates. Different transconjugant clones resulting from mating different primoisolates were selected and their plasmid content analyzed. All the clones showing pattern-1 carried a 60 MDa plasmid and 2 or 4 small plasmids. The clones showing pattern-2 also carried 2 or 4 small plasmids in addition to a 53 MDa plasmid; finally, pattern-3 clones carried the small plasmids along with two large plasmids of 53 and 60 MDa. Plasmid size was estimate estimated by including molecular weight standards in the gels.

New conjugation experiments were carried out to obtain clones harbouring only one plasmid. Transconjugant clones of W3110 Rif^r were used as donors and 185 Nal^r as a recipient. We obtained some clones carrying only the 60 MDa plasmid which showed resistance to Ap-Cb, Sm and Su; and clones carrying only the 53 MDa plasmid with resistance to Sm, Km-Nm and Tp.

Transformation experiments were carried out to verify if some of the small plasmids, present in all the primoisolates carried resistance determinants. These small plasmids were mobilized by larger self-transferable plasmids in the isolates of Type II. Donor DNA was obtained from representative primoisolates of each group and *E. Coli* 803 was used as a recipient. Selection was carried out with those antibiotics to which the donor DNA codified resistance. With DNA from Type I primoisolates, transformant cells which showed Sm and Su resistance and carried a plasmid of about 7.1 MDa were obtained. With DNA from Type II primoisolates, transformant clones showing Sm, Km-Nm, Tp resistance or only Km-Nm, Tp resistance were obtained. Both carried a plasmid of 53 MDa.

To establish whether the plasmids from different primoisolates of *S. sonnei showing* a similar R-phenotype and molecular size in different, transconjugant or transformant clones showed the same or a different restriction pattern, DNAs purified from different clones were digested with several restriction enzymes. All the plasmids of 60 MDa and phenotype (Ap-Cb, Sm, Su)^r gave 5 fragments when they were treated with *Hin*dIII, 8 with *Cla*I and 10 with *Bam*HI IFig. 3a); these plasmids were designated as pUO120. The plasmids of 53 MDa and pnenotype (Sm, Km-Nm, Tp)^r or (Km-Nm, Tp)^r gave only 1 fragment with *Eco*RI and *Bam*HI; 2 fragments with *Hind*III and *Pst*I and 6 whit *Cla*I (Fig. 3b); they were designated as pUO121. The plasmids of 7.1 MDa and phenotype (Sm, Su) gave 2 fragments with *Bam*HI, 4 with *Eco*RI and 4 with *Hin*DIII (Fig. 3c), independently that the plasmid came from isolates of the former or later phase of the outbreak. They were designated as pUO122.

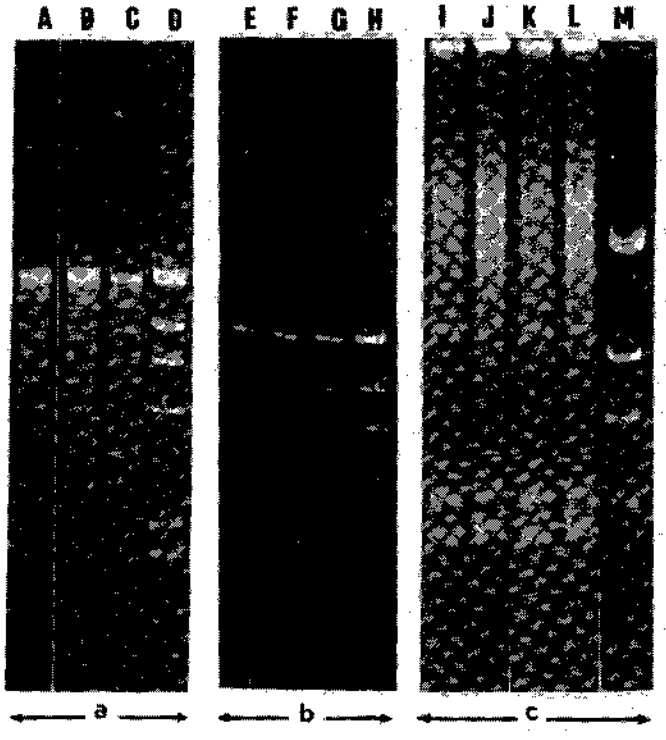

Fig. 3. Agarose gel electrophoresis of the three R-plasmids isolated from *S. sonnei* epidemic strains. Each well contains DNA from a different isolate. a) pUO120 digested with *Bam*HI; b) pUO121 digested with *Cla*I from Sm^r clones (E and F) and from a Sm^s clone (G); c) pUO122 digested with *Hin*dIII. Wells D, H and M contain DNA digested with *Hin*dIII.

Discussion

Shigella species usually harbour a heterogeneous population of plasmids, some of them are cryptic, others are responsible for resistance to different antibacterial drugs (4, 9, 12) and others encode certain essential properties for virulence and contribute to pathogenicity (5, 10, 11).

By using the plasmid profile analysis, we have characterized the types of *S. sonnei* strains responsible for the outbreak and we have done their epidemiological surveillance during the months following the outbreak. The outbreak could be defined as of a mixed type. In the former phase was holomiantic, from hydric origin, caused by two varieties of *S. sonnei* which showed a characteristic and different plasmid prolife and were defined as two different strains (Type I and II). In a next phase, it was prosodemic propagated by infection chain, and from the feces of diarrheical patients who requested hospital care, cells of only one of the *S. sonnei* strains (Type I) were isolated. Moreover, the diversity of plasmid profiles found among the coisolates collected during the 10 months of surveillance allowed us to assume that our community is bearing a large number of different *S. sonnei* strains.

The genetic nature of the multiple antibiotic resistance showed by the two *S. sonnei* strains which caused the outbreak was established. All the resistance markers analyzed from the different isolates were plasmid-mediated. Resistance to streptomycin and sulphonamides in isolates of Type I was encoded by a non-conjugative small plasmid, named pUO122. Streptomycin resistance linked with resistance to sulphonamides represent a common group of plasmid-borne durg resistance pattern. It has been described in species of *Shigella* as well as in other enteric bacteria isolated in different geographic areas (3, 9). In the isolates of Type II, multiple drug resistance was due to the joint expression of determinants located in two different replicons. The first one (pUO121), of 53 MDa, encoded resistance to streptomycin, kanamycin-neomycin and trimethoprim; streptomycin resistance was not expressed in many transformant clones, but molecular weight and restriction patterns were similar both in Sm sensitive and resistant clones. The second plasmid (pUO120) of 60 MDa, encoded resistance to semisynthetic penicillins (Ap, Cb), streptomycin and sulphonamides. We must underline that, in our community, trimethoprim is used combined with sulphamethoxazol, but, in spite of it, the resistance determinants for each of these drugs were carried in different plasmids. The plasmids pUO120 and pUO121 are conjugative and capable of mobilizing small cryptic plasmids which coexist in *S. sonnei* strains.

Both *S. sonnei* epidemic strains carried several small cryptic plasmids, three of them of similar electrophoretic mobility. Cryptic plasmids with similar size were also detected among non epidemic strains. We conclude that, in our study, the plasmid profile was a useful tool to define the epidemic strains and their circulation and maintenance in the community.

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Estudio de las actividades nitrogenasa, hidrogenasa y nitrato reductasa, consumo de oxígeno y contenido en ATP de los nódulos formados por las razas de *Rhizobium leguminosarum* 128C53 y 300 en simbiosis con guisante

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Summary

The nitrogenase activity, nitrate reductase activity and oxygen uptake as well as the hydrogen incorporation and ATP content were examined in the root nodules and bacteroids, respectively, formed by *Rhizobium leguminosarum* strains 128C53 (hydrogenase positive) and 300 (hydrogenase negative) in symbioses with *Pisum sativum* plants grown in the presence of 2 mM KNO_3. The strain 128C53 showed the greatest values for all parameters analyzed, except for the nitrate reductase activity, which was higher for the strain 300. Similarly, nodule nitrate reductase activity in strain 300 was greater than that in strain 128C53 when plants grew in the absence of combined nitrogen. In general, the highest values were obtained when determinations were made after 7 hours of plant illumination. However, the hydrogenase activity of strain 128C53 and the nitrate reductase activities of both strains increased with the light period, reaching a maximun after 14 hours of illumination. These results suggest that the benefits derived from the superior symbiotic properties and from the presence of hydrogenase activity in strain 128C53 could be counteracted by the higher rates of the nodule nitrate reductase activity in strain 300.

Key words: Nitrogenase, hydrogenase, nitrate reductase, Rhizobium-legume synbiosis.

Resumen

Se han estudiado las actividades nitrogenasa, nitrato reductasa y consumo de oxígeno así como la actividad hidrogenasa y el contenido en ATP de los nódulos y bacteroides, respectivamente, formados por las razas de *R. leguminosarum* 128C53 (fenotipo

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hidrogenasa positivo) y 300 (fenotipo hidrogenasa negativo) en simbiosis con plantas de guisante *P. sativum* crecidas en presencia de 2 mM de NO_3K. La raza 128C53 mostró valores más elevados en todos los parámetros analizados, excepto en la actividad nitrato reductasa, que fue mayor en la raza 300. Igualmente, la actividad nitrato reductasa de los nódulos de la raza 300 fue superior a los de la raza 128C53 cuando las plantas se cultivaron en ausencia de nitrógeno combinado. En general, los mejores resultados se obtuvieron cuando las determinaciones se efectuaron después de 7 horas de iluminación. Sin embargo, la actividad hidrogenasa de la raza 128C53 y las actividades nitrato reductasa de ambas razas aumentaron desde el comienzo de la fase luminosa, alcanzándose el máximo de actividad en las medidas realizadas después de 14 horas del inicio del periodo de iluminación. Los resultados obtenidos sugieren que el efecto beneficioso sobre el crecimiento de las plantas, que pueden derivarse de las superiores propiedades simbióticas y de la presencia de actividad hidrogenasa en la raza 128C53, puede quedar contrarrestado en la raza 300, por una mayor actividad nitrato reductasa.

Introducción

La fijación biológica de nitrógeno es el proceso por el cual el nitrógeno atmosférico (N_2) es reducido a amonio (NH_4^+). Esta reacción está catalizada por la enzima denominada nitrogenasa que acopla la reducción del N_2 a la de protones (H^+) a hidrógeno (H_2) (3). La producción de H_2 por los nódulos de las leguminosas representa una pérdida de energía (ATP) y poder reductor (electrones), que disminuye la eficacia de la fijación de N_2 (21).

Algunas especies de *Rhizobium* han desarrollado un sistema enzimático con actividad hidrogenasa (sistema Hup) capaz de oxidar todo o parte del H_2 producido por la nitrogenasa (6), lo que resulta, a su vez, en la recuperación de electrones que pueden ser utilizados por la nitrogenasa o destinados a la producción de ATP (7). Además la presencia de actividad hidrogenasa en *Rhizobium* puede eliminar el H_2 liberado por la nitrogenasa, prevenir la inhibición de la nitrogenasa por el oxígeno y proporcionar poder reductor a la nitrogenasa (20).

En soja *(Glycine max),* se ha establecido que la inoculación de estas plantas con razas de *R. japonicum* que poseen actividad hidrogenasa (fenotipo Hup^+) produjo un incremento de la fijación de N_2 y, consecuentemente, un aumento del peso seco y contenido en nitrógeno de las plantas en relación a aquellas que fueron inoculadas con razas que carecían de actividad hidrogenasa (fenotipo Hup^-) (9). Sin embargo, no se ha podido demostrar que el empleo como inoculantes de razas Hup^+ y Hup^- de *R. leguminosarum* produzcan en guisantes *(Pisum sativum)* efectos similares a los descritos en soja (4).

Este trabajo se inició con el objeto de examinar una serie de parámetros-actividad nitrogenasa, actividad hidrogenasa, actividad nitrato reductasa, consumo de oxígeno y producción de ATP- de los nódulos y bacteroides producidos por la raza Hup^+ y otra Hup^- de *R. leguminosarum* en simbiosis con *P. sativum.* La finalidad de estos experimentos ha sido estudiar las posibles diferencias que pudieran existir entre ambos tipos de razas y que pudieran explicar la ausencia de los efectos beneficiosos atribuidos a la expresión de los genes *hup* y demostrados en otras asociaciones *Rhizobium*-leguminosa.

Materiales y métodos

Plantas y microorganismos

Se ha utilizado guisante (*Pisum sativum* L.), var. Alaska. Las semillas se esterilizaron con alcohol durante 3 minutos, se lavaron abundantemente con agua estéril y se germinaron en placas Petri que contenían papel de filtro húmedo. Las plántulas se colocaron en jarras Leonard provistas de la solución mineral descrita por Rigaud y Puppo (18) adicionadas de 2 mM de NO_3K. En el momento de la siembra, cada plántula se inoculó, aisladamente con 1 ml de una suspensión bacteriana (10^8 cels/ml) de *R. leguminosarum* razas 128C53 (Hup^+) (19) y 300 (Hup^-) (2). Las plantas se cultivaron en cámaras de crecimiento bajo las siguientes condiciones: ciclo día/noche de 16/8 horas, 21/15° C, 50 % humedad relativa y una intensidad luminosa (400-700 nm) de 500 nmol m^{-2} s^{-1}.

Estudios fisiológicos

La actividad nitrogenasa se ensayó mediante la técnica de la reducción del acetileno a etileno. Las plantas se decapitaron a la altura de los cotiledones y las raíces noduladas se colocaron en tubos provistos de tapón de rosca perforable, sustituyéndose el 10 % del volumen interior del tubo por acetileno. El etileno producido se determinó por cromatografía gaseosa a los 5 y 10 minutos, en las condiciones previamente descritas (1).

La actividad nitrato reductasa de los nódulos se estudió mediante ensayos *in vivo* siguiendo la técnica de infiltración al vacío (11). Para ello, los nódulos (0,20 g) se sumergieron en 2,5 ml de tampón fosfato potásico (pH 7,5) adicionado de EDTA 1 mM y NO_3K 50 mM. Los nódulos se cosecharon de plantas crecidas en presencia de la solución mineral descrita previamente que contenía o no 2 mM de NO_3K. La infiltración del tejido nodular se efectuó realizando un vacío de 40 cm de Hg durante 10 minutos, y posterior descompresión. Los nódulos se incubaron en oscuridad, sin agitación, a 30° C durante tres horas. La producción de nitrito (NO_2^-) se determinó en alícuotas de 1 ml mediante la reacción de diazotación descrita por Hageman y Hucklesby (10), midiéndose la absorbancia a 540 nm después de 20 min. de la adición de los reactivos.

El consumo de oxígeno por nódulos se determinó mediante las técnicas amperométricas (5), utilizando 0,20 g de nódulos en 2 ml de tampón HEPES (Sigma) 100 mM (pH 7,5).

El contenido de ATP de los bacteroides se midió previo aislamiento de los mismos según la metodología descrita por Emerich *et al.* (8), aunque en condiciones de aerobiosis. Los bacteroides se incubaron, en presencia y ausencia de 0,02 atm. de H_2 en la fase gaseosa, a 30° C durante 5 min. El ATP fue extraído mediante la adición de 0,5 ml de las diferentes suspensiones de bacteroides a 4,5 ml de tampón HEPES 100 mM (pH 7,5) y calentando la mezcla durante 90 s a 100° C (16). El contenido en ATP del extracto obtenido después de la centrifugación a 20.000 I ×g 10 min se determinó por el método de la luciferin-luciferasa (15), midiéndose la emisión de luminiscencia en un contador de centelleo (Packard, TriCarb).

La actividad hidrogenasa de los bacteroides se determinó registrándose amperométricamente el consumo de H_2 cuando a la suspensión de bacteroides se les suministró H_2

y un aceptor final de electrones de acuerdo con la técnica descrita por López *et al.* (13). El contenido en proteínas se determinó mediante el procedimiento de Lowry *et al.* (14) utilizando albúmina como estándar.

En todos los casos, los parámetros antes mencionados se ensayaron a las 0,0, 0,7 y 14,0 horas del intervalo luminoso del fotoperíodo. El peso seco de los bacteroides se calculó después de filtrar las muestras a través de la membrana Millipore de 0,25 μm de tamaño de poro y secándolos a 70° C durante 48 horas. El contenido en nitrógeno se analizó por el método Kjeldhal.

Resultados

En la Tabla 1 se presentan los resultados correspondientes a la actividad nitrogenasa de las razas 128C53 y 300 de *R. leguminosarum*. En ambos casos, el máximo de actividad se obtuvo a las 7 horas de iluminación. Aunque no se observaron diferencias al inicio de la fase luminosa (tiempo 0), la actividad nitrogenasa de la raza 128C53 fue superior a la de la raza 300 en las otras dos medidas realizadas (tiempos 7 y 14 horas).

La raza 128C53 también mostró valores más elevados de consumo de oxígeno que la raza 300 en las tres determinaciones efectuadas, y al igual que la actividad nitrogenasa, la respiración de los nódulos fue mayor cuando este parámetro se analizó despues de 7 horas de régimen luminoso (Tabla 2).

TABLA 1

ACTIVIDAD NITROGENASA DE LOS NODULOS FORMADOS POR *R. LEGUMINOSARUM* EN SIMBIOSIS CON *P. SATIVUM*

Raza de	Tiempo de iluminación (horas)		
Rhizobium	0	7	14
300	4,9 ± 0,4	10,6 ± 0,3	6,7 ± 0,6
128C53	5,2 ± 0,4	12,3 ± 0,3	8,6 ± 0,4

Las plantas crecieron en presencia de 2 mM de NO_3K.
Los valores de actividad son la media ± SE de seis repeticiones y se expresan en μmol de C_2H_4/planta/hora.

TABLA 2

CONSUMO DE OXIGENO DE LOS NODULOS FORMADOS POR *R. LEGUMINOSARUM* EN SIMBIOSIS CON *P. SATIVUM*

Raza de	Tiempo de iluminación (horas)		
Rhizobium	0	7	14
300	7,2 ± 0.7	14,4 ± 1,1	15,5 ± 1,0
128C53	12,8 ± 0,1	18,5 ± 1,2	16,5 ± 1,2

Las plantas crecieron en presencia de 2 mM de NO_3K.
Los valores de consumo de oxígeno son la media ± SE de seis repeticiones y se expresan en μmol de O_2/g peso fresco de nódulos/h.

El contenido en ATP de los bacteroides fue igualmente superior en la raza 128C53 y tanto en esta raza como en la 300, los máximos valores de ATP se obtuvieron en las medidas que se realizaron hacia la mitad del período luminoso (Tabla 3). No se encontraron diferencias, respecto al contenido en ATP, cuando los bacteroides se incubaron en presencia o ausencia de H_2 (Tabla 3).

TABLA 3

CONTENIDO CON ATP DE LOS BACTEROIDES DE LOS NODULOS FORMADOS POR *R. LEGUMINOSARUM* EN SIMBIOSIS CON *P. SATIVUM.*

Raza de	Tiempo de iluminación (horas)		
Rhizobium	0	7	14
300	0,67 ± 0,03	0,97 ± 0,05	0,88 ± 0,03
300 + H_2*	0,65 ± 0,02	0,95 ± 0,04	0,88 ± 0,04
128C53	0,71 ± 0,03	1,14 ± 0,05	0,95 ± 0,05
128C53 + H_2*	0,66 ± 0,03	1,02 ± 0,05	0,96 ± 0,03

Las plantas crecieron en presencia de 2 mM de NO_3K.
Los valores de ATP son la media ±SE de seis repeticiones y se expresan en nmol de ATP/mg peso seco de bacteroides.
* Las suspensiones de bacteroides se incubaron en ausencia y presencia de 0,02 atm. de H_2 en la fase gaseosa.

Por el contrario, la actividad hidrogenasa de la raza 128C53 incrementó desde el comienzo de la fase de iluminación, obteniéndose mayores tasas de consumo de H_2 cuando esta actividad se determinó a las 14 horas del inicio de la etapa luminosa. Los valores de actividad hidrogenasa correspondientes a los tiempos 0,0, 7,0 y 14,0 horas son los siguientes: 0,3 ± 0,01, 1,14 ± 0,05 y 1,49 ± 0,08 μmoles de H_2/mg proteína/hora.

De forma similar a la actividad hidrogenasa, la actividad nitrato reductasa incrementó con el tiempo de iluminación, obteniéndose los valores más elevados hacia el final de la parte luminosa del fotoperiodo (tiempo 14 horas) (Fig. 1). Aunque los nódulos formados por las razas 128C53 y 300 presentaron niveles muy similares de actividad nitrato reductasa al comienzo del período de iluminación (tiempo 0), los de la raza 300 exhibieron mayor actividad para reducir nitrato, a lo largo del tiempo, que los de la raza 128C53. Este hecho ocurrió tanto en los ensayos realizados con nódulos procedentes de plantas que habían crecido en presencia como en ausencia de nitrógeno combinado (Fig. 1).

Los datos de peso seco y contenido en nitrógeno correspondientes a las plantas inoculadas con cada una de las razas en estudio se presentan en la Tabla 4, donde pueden observarse que no existen diferencias entre tales parámetros fisiológicos, tanto en plantas crecidas en presencia como en ausencia de nitrato.

Discusión

La presencia de actividad hidrogenasa en los nódulos de las raíces de las leguminosas representa una serie de ventajas que pueden traducirse en una mayor capacidad de fi-

TABLA 4

PESO SECO Y CONTENIDO EN NITROGENO DE PLANTAS DE *P. SATIVUM* EN SIMBIOSIS CON *R. LEGUMINOSARUM*

Raza de *Rhizobium*	Plantas + NO_3K		Plantas − NO_3K	
	Peso seco	Contenido en N	Peso seco	Contenido en N
300	0,52 ± 0,07	26,0 ± 0,32	0,37 ± 0,05	17,0 ± 0,21
128C53	0,53 ± 0,07	25,7 ± 0,31	0,37 ± 0,05	16,8 ± 0,20

Los valores de peso seco y contenido en nitrógeno son la media ± SE de seis repeticiones y se expresan en g/planta y mg/planta, respectivamente.

jar N_2 (7). Evans *et al.* (9) han demostrado en la simbiosis *R. japonicum-G. max* que las razas Hup^+ empleadas como inoculantes incrementan el peso seco y el contenido de nitrógeno de las plantas respecto a las inoculadas con razas Hup^-.

De los resultados obtenidos en este estudio, puede concluirse que una raza de *R. leguminosarum* que posee actividad hidrogenasa (128C53), presentó valores más elevados de reducción de acetileno que otra que no posee tal actividadd (300) cuando las plantas estaban cultivadas en un medio que contenía 2 mM de nitrato (Tabla 1). Este hecho, sin embargo, no estuvo asociado a un incremento en el rendimiento de cosecha (Tabla 4). Resultados similares han sido descritos recientemente (4) utilizando razas isogénicas $Hup^{+/-}$ de la raza 128C53 obtenidas por Kagan y Brewin (12) mediante mutagénesis con el transposón Tn 5.

Las diferencias en actividad hidrogenasa entre las razas 128C53 y 300 podrían derivarse de la presencia de actividad hidrogenasa en la raza 128C53. Aunque nuestros resultados indican un mayor consumo de oxígeno (Tabla 2) y contenido en ATP (Tabla 3) de los nódulos y bacteroides de la raza 128C53, Ruiz-Argüeso *et al.* (19) no encontraron relación entre consumo de oxígeno y reducción del acetileno en un estudio con 15 razas Hup^+ y Hup^- de *R. leguminosarum*. Además, en *R. leguminosarum* 128C53 la actividad hidrogenasa no está acoplada a la producción de ATP (16). Por tanto, estos parámetros no pueden considerarse como índice para seleccionar razas de *Rhizobium* con mejores características simbióticas.

Nuestros resultados, sin embargo, confirman y extienden a tres determinaciones efectuadas a distintos tiempos del fotoperiodo los obtenidos por Nelson y Salminen (16), ya que la presencia de H_2 en el medio de incubación de los bacteroides no incrementó el contenido en ATP de los mismos en ninguna de las dos razas empleadas (Tabla 3).

La actividad nitrato reductasa de los nódulos formados por la raza 300 fue superior a los de la raza 128C53 cuando los ensayos se realizaron con nódulos aislados de plantas crecidas en presencia de nitrato (Fig. 1). La mayor capacidad para reducir nitrato de la raza Hup^- fue también evidente cuando los nódulos se aislaron de plantas cultivadas en ausencia de nitrógeno combinado (Fig. 1). Estos hechos sugieren que la raza 300 puede asimilar el nitrato de forma más eficiente que la raza 128C53, contribuyendo de esta forma al mejor metabolismo nitrogenado de las plantas hospedadoras. Es posible, por tanto, que los efectos beneficiosos que sobre el crecimiento y desarrollo de las leguminosas se atribuyen a la presencia de actividad hidrogenasa en la raza 128C53 puedan quedar en-

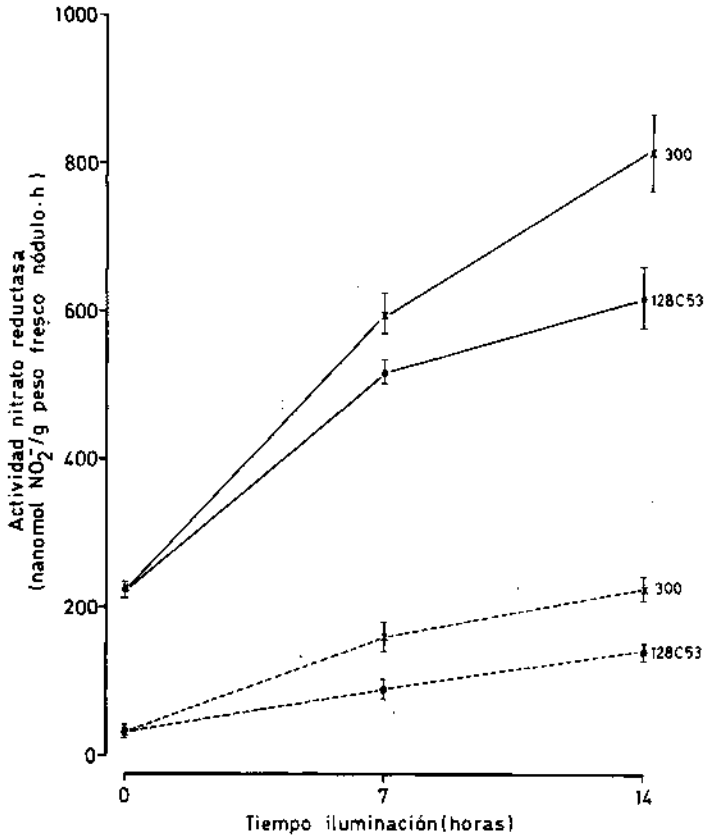

Fig. 1. Actividad nitrato reductasa de los nódulos formados por *R. leguminosarum* en simbiosis con *P. sativum*. Las plantas crecieron en presencia (-) y en ausencia (---) de 2mM de NO_3K. Los valores de actividad nitrato reductosa presentan la media ± SE de seis repeticiones.

mascarados o contrarrestados por la mayor capacidad para utilizar el nitrato de la raza 300.

Actividad nitrogenasa y nitrato reductasa no siguieron perfiles similares a lo largo del tiempo de iluminación (Tabla 1 y Fig. 1). Ambas actividades compiten por el ATP y el esqueleto carbonado necesarios para la incorporación del amonio formado en dichos procesos (17, 12), por lo que, indirectamente, la actividad hidrogenasa podría resultar afectada. La ausencia de información sobre las posibles interrelaciones que, a nivel de bacteroide, puedan ocurrir entre nitrato reductasa e hidrogenasa, hace difícil establecer la contribución relativa de cada una de estas actividades al metabolismo nitrogenado de las leguminosas. La obtención de razas de *R. leguminosarum* que representan un fenotipo Hup⁺/NR⁻ podría ayudar a resolver estos problemas.

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Production of some enzymes in the autolysis of the white-rot fungus *Coriolus versicolor* in fermenter

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Summary

The autolysis and production of some extracellular enzymes by *Coriolus versicolor* was studied in submerged cultures. After 48 days of incubation the fungus lost 31 % of its maximum dry weight. 1.3-β-glucanase was excreted at the begining of autolysis and proteases were present during the course of the experiment. On the other hand, laccase was produced in very small amount in the first days of incubation, reaching the maximum activity at the 8th-day of autolysis.

Key words: Extracellular enzymes, autolysis, Coriolus versicolor.

Resumen

Se ha estudiado la autólisis y producción de enzimas extracelulares en cultivo sumergido de *Coriolus versicolor.* Después de 48 días de incubación el hongo pierde un 31 % de su peso máximo. Al comienzo de la fase autolítica excreta 1,3-β-glucanasa, mientras que las proteasas están presentes durante todo el período de incubación y la lacasa, detectada en pequeña cantidad durante los primeros días alcanza su máxima actividad a los ocho días de autólisis.

Introduction

The wood-degrading ability of white-rot fungi has been associated with the production of extracellular enzymes. A wide range of enzymes have been demonstrate to take part in such processes, including cellulases, glucanases, phenol oxidases, lignina-ses, etc. (1, 2, 5, 121)

Although considerable attention has been given to the autolytic phase of growth in Ascomycetes and Imperfecti Fungi (24) little is known about the autolysis of Basidiomy-cetes. White-rot fungi are often used to examine lignin biodegradation. It has been found

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that in shaken cultures lignin degradation does not occur (9), and specially when pellets are formed, since the hyphal surface in contact with O_2 and with lignin is very small (27). Other authors (22) have found that *Phanerochaete chrysosporium* is capable os degrading lignins in shaken as well as in stationary cultures.

The production of extracellular laccase is common in white-rot fungi (2). This occurs when the fungus grows either in the presence of lignin or when this polymer is absent. Other enzymes, such as proteases and 1,3-β-glucanase are often produced by fungi as well (5, Gómez-Alarcón, G., Saiz, C., Lahoz, R. and O'Connor, A. 1985. Abs. Int. Symp. Plant. Prod. and the new Technol. p. 17).

The aim of this work was to study the changes in cultural parameters and enzymes produced by a strain of the white-rot fungus *Coriolus versicolor* in fermenter over extended period of time.

Materials and methods

Organism

C. versicolor (L ex Fr.) Quel. Mad-697-R was obtained from Dr. T. K. Kirk, Forest Products Laboratory, Madison, WI.

Preparation of inoculum

Cultures of *C. versicolor* were grown at 25° C for 10 days on agar-malt slants. Two 100 ml conical flasks containing 20 ml of culture medium were inoculated with 10 mm diameter plugs of *C. versicolor* and incubated for 5 days in a stationary culture and then transferred to the fermenter.

Medium

The fungus was grown in Reyes and Byrde's (23) medium, using glucose and ammonium tartrate as C and N sources, respectively. Two and half litres of this medium were prepared and poured into a 3 l capacity Pyrex glass cylindrical wessel. The lid of the vessel had screw-thread adapters and connectors for adition of water, inoculation port and sampling. The fermenter was maintained at 25° C and stirred at 120 rpm.

Sampling

Two samples (50 ml) of culture were taken at convenient intervals and the mycelium was separated by filtration through Whatman no. 1 filter paper on a Büchner funnel, washed with cold distilled water and dried at 70° C to constant weight. Filtrates were used for enzyme and chemical analyses.

Analytical methods

Total reducing substances were determined according to Somogyi (28) in conjunction with Nelson's (20) method. Soluble proteins in the filtrates were estimated by the Lowry (18) method. Conductivity was measured at 25 ±0.1° C using a Philips portable meter (model PW 9504/00 with a K = 1.46 cell).

Enzyme assays

Laccase (E.C.1.10.3.2) activity in filtrates was determined with 0.1 *M* guaiacol (Merck) at 30° C in presence of 0.05 *M* citrate-phosphate-borate buffer, pH 0.5 by the Kirk and Kelman's (14) method. One enzyme unit was defined as the amount that caused a change in absorbance of 1.0 per min. 1.3-β -glucanase (exoglucanase) (E.C.3.2.1.6) determination was assayed as described by Reyes and Byrde (23), using a solution of laminarin as substrate. One unit of enzyme was defined as the amount which releases 1 μmol glucose per min/ml. Proteases were determined using 1 mg/ml Azocoll (Calbiochem) as substrate in 0.05 M sodium acetate buffer, pH 5.0, according to Eriksson and Pettersson (5). One unit of protease activity was defined as that amount which produced a change in absorbance of 1.0 per min. The specific activity for each of the enzymes studied is equal to the enzyme activity per mg of culture-filtrate protein.

TABLE 1

CHANGES IN pH, MYCELIUM DRY WEIGHT, REDUCING SUBSTANCES AND PROTEINS OF *CORIOLUS VERSICOLOR*, GROWN AND AUTOLYSED IN SUBMERGED CULTURES

Incubation time (days)	pH	Mycelium dry wt (mg/sample*)	Reducing substances (μmol/ml)	Proteins (mg/ml)
0	5.4			
5	4.9	26.6	46.8	0.36
8	4.3	56.3	32.7	0.37
12	4.0	105.4	14.4	0.45
14	4.0	103.8	8.2	0.51
15	4.0	111.3	4.5	0.47
16	4.0	105.9	1.1	0.36
20	4.6	100.5	0.4	0.37
23	4.9	85.5	0.4	0.36
27	5.3	81.2	0.3	0.35
30	5.5	80.3	0.6	0.34
34	5.7	76.2	0.3	0.34
38	5.7	78.2	0.4	0.34
48	6.0	80.0		

* The term «Sample» indicates mg of dry mycelium in 50 ml of culture.

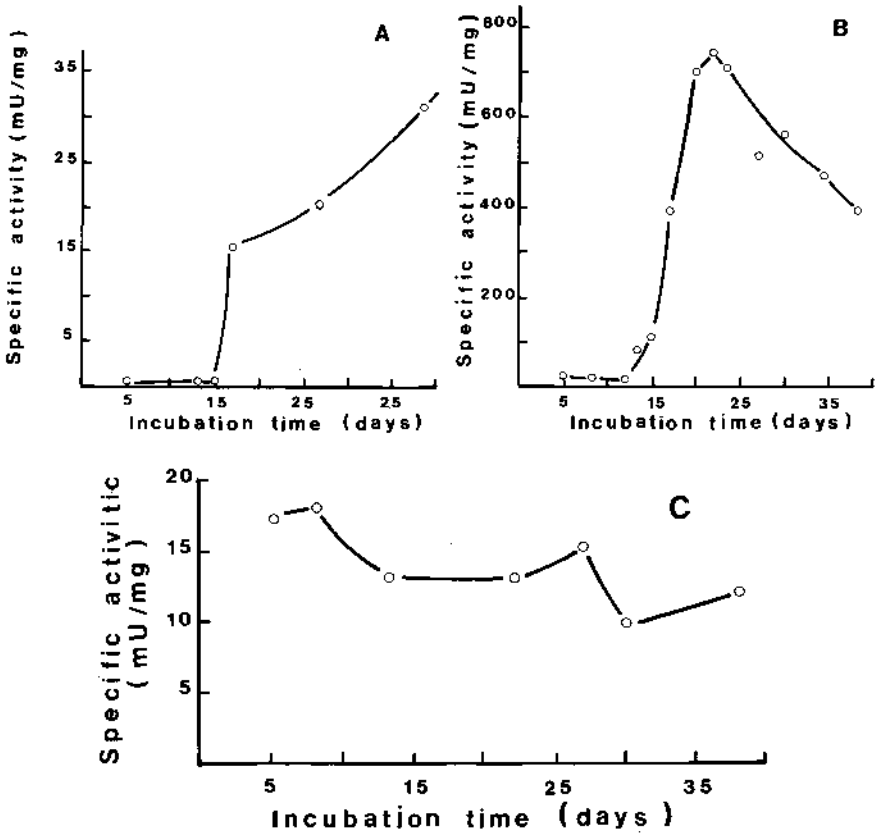

Fig. 1. Changes in the specific activity of 1,3-β-glucanase (A), lacasse (B), and proteases (C) of *Coriolus versicolor* culture fluid during 38 days of incubation in fermenter.

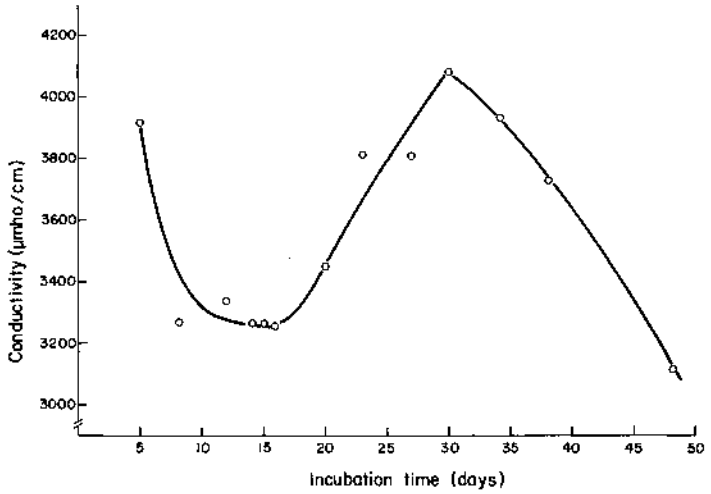

Fig. 2. Variation in the conductivity of culture fluid of *Coriolus versicolor* during growth and autolysis.

Results

Under the fermentation conditions used the fungus grew in mycelial form with no pellet formation. This process was repeated three times. In the choosen experiment to be described here the fungus reached its maximum mycelial dry weight at the 15th-day of incubation (0 day of autolysis). The degree of autolysis (percentage of dry weight mycelial loss) amounted to 31 %. Small changes in the pH value were observed, the lower values coinciding with initiation of the autolytic phase of growth. Reducing substances in the filtrate attained their minimum values at the begining of autolysis. Little changes on the amount of total proteins released into the medium were observed, but a slight rise took place with increasing mycelial dry weight (Table 1).

Laccase activity (Fig. 1B) was small until the 13th-day of incubation, gradually increasing in porcentage to attain the maximum value (743 mU/mg) 22 days after inoculation. The presence of 1,3-β-glucanase (Fig. 1A) was not observed until the initiation of autolysis, and increased steadily during the course of the experiment (48 days after inoculation). Proteases (Fig. 1C) were present throughout the whole period of incubation, with a maximun value of 17.8 mU/mg recorded before autolysis.

Electrical conductivity (Fig. 2) decreased during fungal growth reaching a minimun value upon the initiation of autolysis, increasing thereafter until the 30th-day of inoculation.

Discussion

One of the characteristic features of the autolytic phase of growth in filamentous fungi is the progressive loss of cell content. Nevertheless, this loss will vary according to the organism and the culture conditions. For *Aspergillus flavus,* grown and autolysed in a fermenter, Lahoz and Ibeas (17) found autolysis levels of 85 %, while for *Aspergillus niger* 67 % was reporter (16). The relatively low value of autolysis found for *C. versicolor* would indicate that the degradation of cytoplasmic material and specifically the cell wall degradation progressed slower.

Work on autolytic and phenol oxidase enzymes from Basidiomycetes in submerged cultures is scarce. In stationary cultures, Fähraeus (7) only detected laccase in filtrates of *Polyporus versicolor* when glucose was consumed. Grabbe et al. (10) showed that glucose depresses laccase synthesis in *P. versicolor.* These facts concur with our observations in fermenter, where laccase activity is small in the first days of incubationm when the amount of clucose in the medium is highest, thereafter when the carbon source was consumed (autolysis), the syntesis of laccase reached a maximum (Fig. 1B).

1,3-β-glucanase is often produced by fungi. The existence of 1,3-β-glucan in the hyphal walls of fungi is well documend (19). The enzyme is present at high levels in autolysed cultures of many fungi (21), including wood degrading species (3). Friebe and Holldorf (8) found in an unidentified basidiomycete that initiation of synthesis and excretion of 1,3-β-glucanase was triggered when a low concentration of a carbon source is present in the medium. The possible glucose repression has been studied in other fungi and yeasts. So, Santos et al. (26) observed in *Penicillium italicum* that in the presence of an excess of glucose the 1,3-β-glucanase system is repressed. Del Rey et al. (4) found a si-

milar fact in *Neurospora crassa,* whereas in *Trichoderma viride* and *Sacharomyces cerevisiae* the presence of glucose was accompanied by an increase in the activity of 1,3-β-glucanase. In this work, we found that *C. versicolor* synthesized this enzyme when the concentration of glucose in the medium was reduced to 0.7 μmol/ml (17 days incubation) and autolysis had been initiated. This enables us to conclude that glucose or glucose metabolite (s) represses the formation of extracellular 1,3-β-glucanase.

The function of proteases in white-rot fungi are still unclear. Eriksson and Pettersson (5) reported that the endo-1,4-β-glucanase activity of *Sporotrichum pulverulentum* is considerably enhanced if culture solutions containing these enzymes are treated with proteases, and suggested that a possible role of proteases in wood-rotting fungi may be to release the enzymes while attacking wood polymers from plant cells walls.

During the autolytic phase of growth a liberation of electrolytes from mycelium seems to take place. This increase in conductivity during autolysis has previously been observed in *Neurospora crassa* cultures (15).

Ander and Eriksson (1) and Ishihara (12) reported that laccase plays a mandatory role in lignin degradation, whereas other authors indicated that laccase exact role is not clear (6). Laccase is synthesized regardless of the presence of ligning in *C. versicolor* cultures, which indicates that the ligninolytic system may be relatively non specific, as demonstrated also for *Phanerochaete chrysosporium* (13). This low specificity is apparent as well when *C. versicolor* is incubated in the presence of kraft lignin (a heavily modified industrial lignin), which is substantially biodegraded to an extent comparable with natural lignins (Gómez-Alarcón, G., Saiz, C., Lahoz, R. and O'Connor, A. 1975. Abst. Int. Synp. Plant. Prod. and the New Technol. p. 17).

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Comparative study of two trehalases from *Candida utilis*

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Summary

Candida utilis ATCC 60459 contains two intracellular trehalase enzymes clearly distinguishable by molecular weight, behaviour in ion-exchange chromatography and kinetic properties. The high molecular weight trehalase (500 kDa trehalase) is specifically inhibited by acetate and accounts for less than 30 % of the total trehalase activity found in cell extracts. The smaller trehalase (280 kDa trehalase) exists mostly as a cryptic enzyme whose activity can be postranslationally activated by cAMP-dependent phosphorylation. The enzyme activity of the 280 kDa trehalase is strongly inhibited by Zn^{2+} and markedly enhanced in the presence of Ca^{2+} and Mn^{2+}. The activation by these cations, contrariwise to that induced by ATP and cAMP, does not imply a covalent modification of the 280 kDa enzyme. Several parameters have been determined for both enzymes. The 280 kDa enzyme has the properties shown by the so-called regulatory trehalases whereas the 500 kDa enzyme presents characteristics of a nonregulatory type of trehalase.

Key words: Trehalases, enzyme activation, Candida utilis.

Resumen

Candida utilis ATCC 60459 contiene dos trehalasas intracelulares que difieren en peso molecular, comportamiento en cromatografía de intercambio iónico y propiedades cinéticas. La trehalasa de alto peso molecular (500 kDa) se inhibe específicamente en presencia de acetato y constituye menos del 30 % de la actividad trehalásica total presente en extractos celulares. La trehalasa de peso molecular menor (280 kDa) existe en forma críptica y puede ser activada postraduccionalmente por fosforilación dependiente de AMP cíclico. Esta actividad muestra fuerte inhibición por Zn^{2+} y es activada por Ca^{2+} y Mn^{2+}. A diferencia de la activación inducida por AMP cíclico y ATP, la provocada por dichos cationes no supone una modificación covalente de la enzima. Se han determinado varios parámetros característicos de ambas trehalasas. La enzima 280 kDa presenta propiedades típicas de las denominadas trehalasas regulatorias, mientras la enzima 500 kDa parece una trehalasa de tipo no regulatorio.

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Introduction

Trehalose is a nonreducing disaccharide which accumulates in yeast cells as a reserve carbohydrate (5). The ways whereby the cells control the intracellular trehalose breakdown are of interest because of the potential role of this sugar as an energy source during development. There is a wide evidence for the existence among yeasts of at least two independent strategies to control the hydrolysis of the stored trehalose. This has been established in parallel to the demonstrations that, depending on the particular yeast species analyzed, two types of trehalase enzymes may be found in the cells, regulatory and nonregulatory trehalases (7, 11). These different types of trehalase enzymes are distinguishable on the basis of catalytic properties and mechanisms of regulation of the enzyme activity (12, 13).

Yeasts with the first type of trehalase have a cryptic hydrolase in the cystosol whose activity increases after cAMP-dependent phosphorylation of the enzyme protein and decreases by protein dephosphorylation (12). In contrast, changes in the compartmentalization of enzyme and substrate, rather than enzyme activation and inactivation, appear to regulate trehalose mobilization in species containing nonregulatory type of trehalases (12).

The ocurrence of nonregulatory trehalases among yeast cells has been shown in many genera, including and fission yeast (4, 7), whereas the demonstration of the existence of regulatory trehalases has apparently been restricted to members of the genus *Saccharomyces* (12, 14). However, we have recently described the existence of a regulatory trehalase activity in the yeast *Candida utilis* and presented evidence to suggest the copresence in this yeast of both regulatory and nonregulatory trehalase activities inside the cell (1, 2). To further characterize this system we have comparatively analysed in the present work the two trahalase activities and report here some prominent, differential properties of the two enzymes.

Materials and methods

Microorganism and culture conditions

C. utilis ATCC 60459 (formerly strain CECT 1061) was grown at 28° C in liquid medium containing 2 % glucose and 0.3 % yeast extract. The cells were collected at early stationary phase, washed and routinely resuspended in 0.1 M Na-phospate buffer pH 5.6. When appropriate, Na-acetate buffer, Zn^{++} or EDTA was added to the cell suspension at the concentrations indicated in the particular experiments. The cells were broken in a MSK Braun homogenizer with coupled cooling and the supernatants of the cell extracts obtained by centrifugation at 20,000xg for 10 min were used as enzyme source unless otherwise stated.

Enzyme assays

Total trehalase activity was measured at pH 5.6 in Na-phospate buffer as previously described (2). One enzyme unit released one nanomol of glucose per min under the assay

contidions. Because of the selective inhibition carried out by acetate and Zn^{++} the enzyme assays for regulatory trehalase contained 0.1 M acetate buffer pH 5.6 whereas the assays for nonregulatory trehalase contained 1 mM Zn^{++} in the phosphate buffer. In enzyme assays containing cations the following salts (Merck) were used: chloride for Ca^{++}, Mn^{++} and Hg^{++}, sulphate for Mg^{++} and Zn^{++}, and nitrate for Co^{++}.

In vitro trehalase activation was performed as already indicated (2). Briefly, to four volumes of enzyme solution one volume of activating mixture (AM) was added containing 4mM ATP, 9mM $mgSO_4$, 50 μM cAMP, 50 mM NaF and 5mM teophylline in 50 mM Na-phospate buffer pH 7.0 (P-buffer). Control samples were mock activated by addition of P-buffer alone. The reaction mixtures were incubated at 30° C for 10 min and used inmediately thereafter for trehalase activity determination.

Other measurements

Gel filtration and ion-exchange chromatography were performed by following the manufacturer's indications (Pharmacia). Fractions of 3.0 ml were collected in a Gilson Microcol TDC 80 and assayed for enzyme activity and protein absorbance. Proteins for molecular weight calibration were obtained from Boehringer Mannheim. To determine specific activities protein measurement was carried out according to Lowry *et al.* (10) using bovine serum albumin as standard.

Results

Characterization of two trehalase enzymes

Fig. la shows a typical elution profile of the trehalase activity from cell-free extracts after gel filtration throughout a column of Sephadex G-200. Two different soluble trehalases appear to be present. The first enzyme elutes slighty ahead of the marker protein ferritin and shows a molecular weight about 500 kD. The second trehalase enzyme shows a lower molecular weight, that is close to 280 kD as deduced from the elution pattern of proteins of known molecular weight (Fig. 1, right). The profile of the enzyme activity changes substantially when the crude enzyme preparations are preincubated with activating mixture containing cAMP and ATP before chromatography (Fig. 1b). Under these conditions, the second peak of trehalase activity results markedly inereased, in comparison to the level of activity obtained from control samples. However, the enzyme activity associated to the 500 kDal trehalase remains fairly constant after preincubation with the activating mixture.

The activation of the 280 kDa trehalase by cAMP and ATP apparently requires an additional factor which is present in unfractionated cell extracts and is lost during purification, because the trehalase activity of Sephadex G-200-filtered enzyme preparations does not increase by the sole presence of the components present in the activating mixture. This result suggests the participation of a cAMP-dependent protein kinase in the activation by those effectors. Once activated, the enzyme activation persists after the removal of the cAMP and ATP by gel filtration, indicating a covalent modification of the

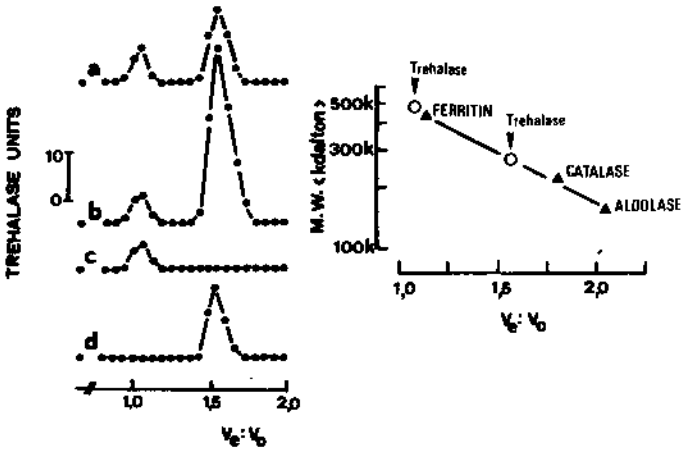

Fig. 1. Evidence for two trehalase enzymes. *Left Panel:* a) Elution profile of trehalase activity in a Sephadex G-200 column (50 ×2.0 cm); 0.1 M Na-phospHate buffer pH 5.6 was used as elution and assay buffer; b) as in a, but after preincubation of the unfractionaterd enzyme preparation with activating mixture as indicated in Materials and methods; c) as in a, but enzyme assays performed in phosphate buffer pH 5.6 containing 1 mM Zn^{++}; d) as in a, but enzyme performed in phosphate buffer pH 5.6 plus 0.1 M Na-acetate buffer pH 5.6. *Right panel:* Correlation between the molecular weight of standard proteins and the ratio elution volume/void volume (Ve:Vo) at which the marker proteins were recovered.

TABLE 1

ACTIVATION BY cAMP AND ATP OF TREHALASE ACTIVITIES IN SUPERNATANT FLUIDS FROM *C. UTILIS* STATIONARY PHASE CELLS

	Specific activity*	Activation factor
Nonregulatory trehalase:		
— Control	0.8	1.0
— Control + AM	0.9	1.1
Regulatory trehalase:		
— Control	4.3	1.0
— Control + AM	16.3	3.8

Stationary pahse cells (OD = 11.0 at 600 nm) were broken in 0.1 M Na-phosphate buffer pH 5.6 without EDTA and the enzyme preparations preincubated for 10 min at 30° C with P-buffer alone or with P-buffer containing activating mixture (AM, see Materials and methods). The enzyme activity was thereafter measured in the presence of 1 mM Zn^{++} or 0.1 M Na-acetate buffer pH 5.6 for nonregulatory and regulatory trehalase activities.

* nmoles glucose/min/mg protein.

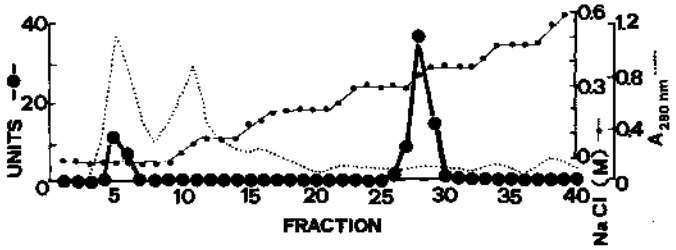

Fig. 2. Elution pattern of trehalase activity from a column of DEAE-Sephadex (20 ×1 cm). 50 mM Tris-HCl buffer pH 7.5 was used as the initial elution buffer and a stepwise gradient of NaCl was generated in the same buffer after the firs peak of trehalase activity was obtained. The recovered fractions were analyzed for enzyme activity, absorbance at 280 nm and conductivity.

enzyme (Fig. 1b). This is a primary characteristic of the so-called regulatory trehalases of fungi (12).

On the other hand, the inclusion of 1 mM Zn^{++} (Fig. 1c) or 0.1 M Na-acetate (Fig. 1d) in the enzyme assays reveals a differential inhibition of the two trehalase enzymes. The activity of the 280 kDa, regulatory trehalase is inhibited by Zn^{++} whereas that of the 500 kDa, nonregulatory trehalase is selectively inhibited by acetate.

Chromatography of crude enzyme preparations on a column of DEAE Sephadex A-50 gives further evidence for the coexistence in *C. utilis* of both regulatory and nonregulatory trehalase activities (Fig. 2). The enzyme activity of the trehalase which is not retained into the DEAE Sephadex column at pH 7.5 shows properties identical to those of the enzyme characterized as 500 kDa trehalase by gel filtration. In turn, the trehalase activity of the enzyme recovered in the eluate when the salt concentration reaches about 0.3M NaCl corresponds to the activity of the 280 kDa trehalase obtained by gel filtration since it is unaffected by the presence of 0.1 M Na-acetate, strongly inhibited by 1 mM Zn^{++} and markedly increased when the enzyme samples are preincubated with activating mixture prior to the chromatographic run.

Properties of the regulatory and nonregulatory trehalases

The existence of two distinct trehalase activities is also evident from the results of enzyme assays performed with unfractionated enzyme preparations in the presence of Zn^{++} or acetate (Tabla 1). The acetate-sensitive trehalase activity, which is measurable in the presence of Zn^{++}, is not enhanced by cAMP and ATP whereas the Zn^{++}-sensitive trehalase activity, measured in the presence of acetate, shows about a 4-fold increase when assayed after a 10 min preincubation with activating mixture.

In addition to Zn^{++} others cations also affected differently the trehalase activities. Mg^{++}, Co^{++}, and particularly Ca^{++} and Mn^{++} increased the activity of the regulatory trehalase in crude enzyme preparations but did not alter that of the nonregulatory enzyme (Table 2).

Experiments on thermal denaturation showed single exponent decays for both enzyme activities and a higher lability for the regulatory trehalase activity than for the nonregulatory type (Fig. 3). Other enzyme parameters such as Km for trehalose, optimun pH,

TABLE 2

EFFECT OF SEVERAL CATIONS ON THE TREHALASE ACTIVITIES OF *C. UTILIS*

	Enzyme activity (nmoles glucose/min/mg protein)		
	Total trehalase	Nonregulatory trehalase	Regulatory trehalase
Control	2.5 (100)	0.6 (100)	2.0 (100)
Control + 4mM Ca^{++}	7.2 (288)	0.6 (100)	6.4 (320)
Control + 4mM Co^{++}	4.0 (160)	0.6 (100)	3.3 (165)
Control + 4mM Mg^{++}	4.0 (160)	0.6 (100)	3.3 (165)
Control + 4mM Mn^{++}	6.3 (252)	0.6 (100)	5.8 (290)
Control + 4mM Hg^{++}	0.0 (0)	0.0 (0)	0.0 (0)

Early stationary phase cells (OD =10.0) were broken in 0.1 M Na-phosphate buffer pH 5.6 containing 2 mM EDTA. The enzyme activity was measured in the presence and absence of the cations shown. The assays were performed in the above buffer (total trehalase) and in the same buffer containing 4mM Zn^{++} (nonregulatory trehalase) or added 0.1 M Na-acetate buffer at the same pH (regulatory trehalase). Numbers among brackets refer to the relative enzyme activity taking the activity in the control assays as 100.

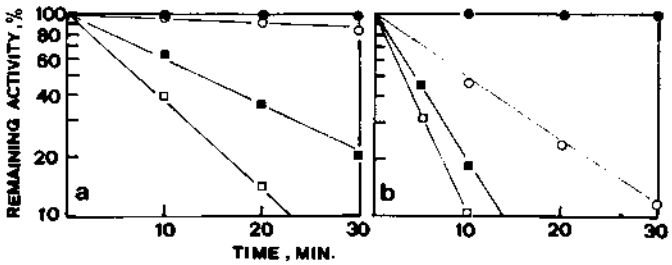

Fig. 3. Decay curves for nonregulatory (a) and regulatory (b) trehalase activities after exposure to different temperatures for the times indicated. Symbols: ●, 30° C; ○, 40° C; ■, 50° C; □, 60° C.

optimun temperature for activity et standard conditions and Ki values for Zn^{++} or acetate were determined for each trehalase enzyme. These and other properties are summarized on Table 3. The Km values shown were obtained from regression lines of Lineweaver-Burk double reciprocal plots. The type of inhibition by Zn^{++} or acetate, and the respective constants of inhibition, were deduced from Dixon-type representations at various concentrations of substrate and inhibitors at optimum pH for each enzyme activity.

Activation of the regulatory trehalase by calcium

The activating effect of Ca^{++} on the regulatory trehalase was investigated further (Table 4). Since yeast cells contain a calmodulin-like polypeptide (6) we tested the possibility that the trehalase activation by Ca^{++} might result from a Ca^{++}-dependent phos-

TABLE 3

DIFFERENTIAL PROPERTIES OF THE NONREGULATORY AND REGULATORY TREHALASES FROM *C. UTILIS*

	Nonregulatory trehalase	Regulatory trehalase
Approximate molecular weight by gel filtration	500 kDalton	280 kDalton
Binding to DEAE-Sephadex at pH 7.5	Not bound	Released at 0.3 M NaCl
Optimum pH for activity*	5.0	6.5
Optimum temperature for activity (30 min assays)	40° C	30° C
% inactivation at 50° C for 10 min	35	81
Km for trehalose	2.6×10^{-3} M	10.1×10^{-3} M
Reversible cation activation	None	Ca^{++}, Mn^{++}, Co^{++}, Mg^{++}
Covalent activation by cAMP and ATP	No	Yes
Glucose-induced *in vivo* activation**	No	Yes
Inhibition by Zn^{++}	None	Noncompetitive (Ki = 0.11 mM)
Inhibition by acetate	Noncompetitive (Ki =47 mM)	None

* Citrate-phosphate buffer.

** See reference 2.

phorylation of the enzyme protein as an alternative to the cAMP-dependent pathway of activation (2).

Experiments A and B on Table 4 demonstrate that the levels of trehalase activity in cell extracts obtained in the presence of EDTA are comparatively lower than those measured in enzyme preparations from cells broken in the absence of EDTA. Such levels are markedly increased upon addition of Ca^{++}. These data suggest that endogenous Ca^{++} released during the rupture of the cells (9) likely activates the regulatory trehalase in enzyme preparations obtained without EDTA in the breaking buffer.

However, when the effect of Ca^{++} was analyzed on the enzyme after gel filtration no significant activation was detected (experiment C, Table 4). Two interpretations were considered to account for this result. The lack of activation by Ca^{++} of partially purified regulatory trehalase was due either to the involvement in the activation process of another compound being lost from the enzyme preparations during the purification procedure or to a loss of affinity of the enzyme toward Ca^{++} during the fractionation.

Experiment D on Table 4 shows that the specific activity of Ca^{++}-preactivates trehalase is the same after gel filtration on Sephadex G-200 than that of the nonactivated enzyme subjected to gel filtration (experiment C). This result indicates that the preactivated enzyme looses the activation during the process and, therefore, that the Ca^{++}-induced activation is reversible and dependent on the continued presence of the cation. Moreover, the activity of the preactivated enzyme also decreases when Ca^{++} is sequestered by EDTA (experiment E, Table 4). Assays of the type F and G, shown in Table 4, additionally dismissed the participation of a heat-stable, calmodulin-like factor in the activation

TABLE 4

ACTIVATION OF THE REGULATORY TREHALASE BY Ca^{++}

Assay	Enzyme sample	Specific activity (nmoles gluc/min/mg protein)
A:	Cell extract without EDTA (Control)	6.0 (100)
	Control + 2mM Ca^{++}	8.6 (143)
B:	Cell extract in 2mM EDTA (Control)	2.8 (100)
	Control + 2mM Ca^{++}	9.0 (321)
C:	Cell extract in 2mM EDTA (Control)	2.8 (100)
	Control after Sephadez G-200 filtration	34.3 (1225)*
	As above + 2mM Ca^{++}	33.1 (1182)
D:	Cell extract in 2mM EDTA (Control)	2.8 (100)
	Control + 2mM Ca^{++} + 1 mM PMSF	9.0 (321)
	As above after Sephadex G-200 filtration	32.0 (1142)
E:	Cell extract in 2mM EDTA (Control)	2.8 (100)
	Control + 2mM Ca^{++}	9.0 (321)
	As above +6 mM EDTA	2.9 (103)
F:	Cell extract in 2mM EDTA (Control)	2.8 (100)
	Control + 2mM Ca^{++}	9.0 (321)
	Control + 4mM Ca^{++} + boiled extract	8.9 (317)
G:	Cell extract in 2mM EDTA (Control)	2.8 (100)
	Control after Sephadex G-200 filtration	34.3 (1225)
	As above + 2mM Ca^{++}	33.1 (1182)
	As above + 2mM Ca^{++} + boiled extract	34.0 (1214)

Early stationary phase cells (OD = 9.0) were used to obtain cell extracts. Enzyme assays were performed in 0.1 M Na-acetate buffer pH 5.6. Numbers in brackets refer to relative activity with respect to the particular controls.

* Protein values for specific activities were measured in the pooled fractions showing regulatory trehalase activity.

of the regulatory trehalase enzyme by Ca^{++} both in cell extracts and in partially purified preparations. Also, the inclusion of 20 μM trifluoperazine (Sigma), a drug known to block Ca^{++}-calmodulin mediated reactions (6, 8), did not block the enhancement of the enzyme activity in assays of trehalase activation by Ca^{++} similar to the reported in experiment F (results not shown). In addition, identical results to those shown in experiment G were obtained in assays additionally supplemented with 1 mM ATP in order to favour a potential Ca^{++}-induced, activating phosphorylation, by eliminating any possible limitation of available ATP.

Discussion

The above results indicate that *C. utilis* contains two distinct trehalases with different properties, which are summarized in Table 3. One of the enzymes shows an acid pH

optimum, relative high heat stability and no evidence of activation by cAMP-dependent protein phosphorylation. These are characteristics that fit well with the properties of the nonregulatory trehalases previously described in several yeast especies and filamentous fungi (12). A second trehalase enzyme shows a pH optimun near neutral, lower heat stability and *in vitro* activation in the presence of cAMP and ATP. Such activation also occurs under *in vivo* conditions (2). These are primary characteristics of the so-called regulatory trechalases, which up to recently had been foured only in species of zygomycetons fungi and in baker's yeast 12, 14). The regulatory enzyme in *C. utilis* also presents a higher Km than that of the nonregulatory type. This appears to be a constant feature when the regulatory trehalases so far described are compared in this respect with nonregulatory trehalases; the Km values for nonregulatory trehalases fall in the range 0.5-3 mM whereas those reported for regulatory trehalases vary from 4.5 to 55 mM (12).

The activation of the regulatory trehalase by cAMP and ATP (Table 1) is clearly a covalent modification because we can separate activated enzyme from small molecules by gel filtration (Fig. 1a, 1b). In parallel studies (3) we have shown that this activation is accompanied by the phosphorylation of a polypeptide of 70 kDa suggesting that the activation is caused by a cellular protein kinase present in cell extracts and absent in partially purified enzyme preparations. Those results also suggested that the regulatory trehalase presumably is a tetramer compossed of four identical subunits.

The activation of the regulatory trehalase by Ca^{++} does not imply, however, a covalent modification of the enzyme protein but requières the maintained presence of the cation. Notably, the regulatory trehalase of baker's yeast is also activated by Ca^{++} and Mn^{++} and the activation dissapears during precipitation by ammonium sulphate (9). Since in *C. utilis* the mechanism of trehalase activation by Ca^{++} appears to be the same than operating in the activation by Mn^{++}, Co^{++} or Mg^{++} (results not shown), it seems that the action of these cations in freshly obtained cell extracts involves their direct, reversible interation with the enzyme protein. Otherwise is should be expected that the trehalase, once activated, would retain the preinduced activation in the presence of EDTA in excess and would maintain the activity after gel filtration. The results shown in Table 4 demonstrate that this is not the case. In particular, we have found no basis to consider that the activation by Ca^{++} is related to a Ca^{++}-dependent phosphorylation of the trehalase protein. The failure to increase the activity of partially purified trahalase by Ca^{++} is apparently due to a change in the affinity of the enzyme toward this cation rather than to the loss of some specific calmodulin-controlled protein kinase.

The possibility that one of the trehalases might be a nonspecific α-glucosidase acting on trehalose has been ruled out since none of the fractions with treahalase activity obtained by gel filtration shows activity against p-nitrophenyl-α-glucoside or maltose. In *C. utilis* the α-glucosidase activity shows a lower molecular weight than the trehalases and elutes off at a Ve/Vo ratio of 2.0 in Sephadex G-200 columns (results not shown). The physiological relationship between the two trehalases reported, if any, is unclear. In view of the drastic differences in their physical and catalytic properties we think likely that they are synthesized independently without interconversions of the type precursor-product. The determination of the specific role and cellular location for each enzyme deserves further research.

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Studies on the replication of bacteriophage Cp-1 DNA in *Streptococcus pneumoniae*

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Summary

The DNA of bacteriophage Cp-1 replicates at optimal conditions when Cp-1-infected *Streptococcus pneumoniae* was incubated at 30° C. The *in vitro* formation of the initiation complex between the terminal protein and 5'-dAMP was only partially inhibited at 37° C whereas an almost complete inhibition of the DNA replication was found at this temperature *in vivo*. Aphidicolin inhibited the multiplication of phage Cp-1 but not that of Dp-4. This drug did not affect the *in vitro* formation of the initiation complex but seems to affect extensive Cp-1 DNA replication *in vivo*.

Key words: Initiation complex, diplophage, terminal protein.

Resumen

El DNA del bacteriofago Cp-1 se replicó en condiciones óptimas cuando las células de *Streptococcus pneumoniae* infectadas con dicho fago fueron incubadas a 30° C. A 37° C, se observó una inhibición casi completa de la replicación del DNA fágico *in vivo* mientras que la formación del complejo de iniciación *in vitro* se vio sólo parcialmente afectada. La adición de afidicolina inhibió la multiplicación de Cp-1, pero no la del bacteriofago Dp-4 que infecta al mismo huésped. Nuestros resultados indican que la afidicolina bloquea la replicación continuada del DNA de Cp-1 sin afectar la formación del complejo de iniciación.

Introduction

Bacteriophage Cp-1 from *Streptococcus pneumoniae* contains a double-stranded linear DNA of 18 000 base pairs (9) that has a terminal protein (TP) of 28 000 Da covalently linked at the two 5' ends (2). The presence of TPs convalently linked to the nucleic

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acids either DNA or RNA, has been reviewed recently (12). Strong evidence on the role of these TPs as primers for the initiation of DNA replication in phage ∅ 29 and in adenovirus has been obtained (for recent reviews, see 11, 14). We have also demonstrated recently that extracts from Cp-1-infected *S. pneumoniae* incubated with dATP catalize the formation of a covalent complex between the TP and 5'-dAMP; under appropriate conditions, this complex can be elongated (3). In this report, we present new data on the replication of Cp-1 DNA.

Materials and methods

Bacterial strains and phages

S. pneumoniae R6, a derivative of the Rockefeller University wild type strain R36A and the thymidine-requiring strain 470 (kindly supplied by S. Lacks) have been previously described (6). The preparation and purification of bacteriophages Cp-1 and Dp-4 have been reported (9, 10).

Media and chemicals

The media used for growing *S. pneumoniae* (CpH8) and for the preparation of phages (M3) have been described in previous publications (9, 10). (*methyl-*^{3}H) thymidine (25 Ci/mmol) was purchased from The Radiochemical Center at Amersham/Searle. 6-(*p*-hydroxyphenylazo)-uracil (HPUra) and aphidicolin were kindly provided by A. H. Todd (Imperial Chemical Industries PLC).

Conditions of infection and ^{3}H-labeling

Conditions of infection and pulse-labeling of intracellular DNA using (*methyl-*^{3}H) thymidine in the presence or in the absence of HPUra and the preparation of sucrose density gradients have been described elsewhere (4, 6).

Formation of the Cp-1 TP-dAMP complex

The standard assay for the formation of the Cp-1 TP-dAMP complex has been recently described (3).

Results and discussion

Selective replication of Cp-1 DNA in the presence of HPUra

We have previously found that the highest yield of bacteriophage Cp-1 was obtained when the infective process was carried out at 30° C. This observation was further exten-

ded to other Cp-1-related phages (7, 9). An approach to study the infective cycle of Cp-1 was to inhibit selectively host DNA synthesis without affecting bacteriophage Cp-1 DNA replication to specifically label phage DNA withous interference from bacterial DNA. As shown in Table 1, the used of HPUra fulfills these requeriments.

Incorporation of [^{3}H]-thymidine during the infective cycle at 30° C and at 37° C

Fig. 1 shows a high level of incorporation of [^{3}H]-thymidine into Cp-1-infected *S. pneumoniae* at 30° C whereas an inhibition of phage DNA replication was found when the infection was carried out at 37° C. The onset of replication showed a lag, with DNA synthesis starting at about 45 min after infection. We have recently reported (3) a decrease of about 60 % in the *in vitro* formation of the Cp-1 DNA initiation complex when the incubation was carried out at 37° C rather than at the optimal temperature (30° C) (Fig. 2). Nevertheless, the remarkable inhibition of the *in vitro* phage DNA replication at 37° C (Fig. 1) suggests that even those molecules that have been able to form the initiation complex can hardly elongate the DNA.

Effect of aphidicolin on the replication of pneumococcal bacteriophages Cp-1 and Dp-4

Another way to get new information about the replication of Cp-1 was to take advantege of the use of aphidicolin. This tetracyclic diterpenoid inhibits the activity of DNA

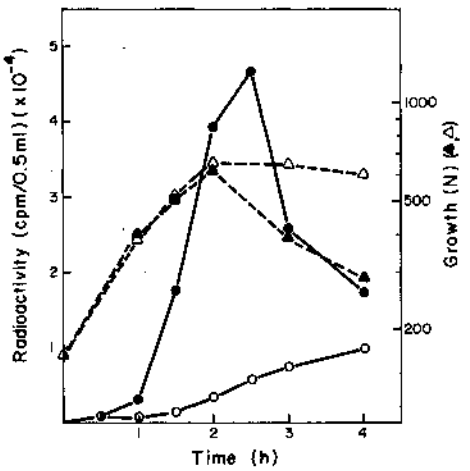

Fig. 1. DNA synthesis and growth curves of Cp-1 infected pneumococcal cells at 30° c and at 37° C. Cultures of *S. pneumoniae* strain 470 containing 6.2 ×10^7 cells/ml received 50 μ M HPUra 5 min before addition of Cp-1 at m. o. i. of 25. [^{3}H]-thymidine (5 μ Ci/ml) was added at 0 time and the culture was divided into two portions that were incubated at 30° C (solid symbols) or at 37° C (open symbols). At different times, samples (0.5 ml) were withdrawn and the TCA-precipitable radioactivity (●, ○) was determined as indicated in Table 1. Growth (and lysis (▲, △) was followed by neyphelometry (N).

TABLE I

EFFECT OF HPUra AND APHIDICOLIN ON *S. PNEUMONIAE* DNA SYNTHESIS AND ON THE PRODUCTION OF PHAGE Cp-1

Treatment	Host DNA synthesis (%)[a]	Phage titer (p. f. u./ml)[b] (%)
None (control)	100	100
HPUra (15 μg/ml)	28	110
(25 μg/ml)	20	138
(50 μg/ml)	9	100
(100 μg/ml)	6	85
(25 μg/ml)	—	30
(50 μg/ml)	—	5
(100 μg/ml)	100	1

[a] Strain 470 (6.2×10^7 colony-forming units/ml) in TS broth received 5 μ Ci of [^{3}H] (2) thymidine/ml in the presence of the indicated concentrations of the drugs. Incubation was continued for 120 min at 30° C. Samples (0.5 ml) of the cultures received 2 mg unlabeled thymidine/ml and were centrifuged (10 000 ×g, 2 min), resuspended, precipitated with 5 % trichloroacetic acid (TCA) and collected onto Whatman GF/A filter disks, dried and counted (100 % = 85 381 cpm).

[b] Strain 470 (6.2×10^7 c. f. u. ml) in TS broth was infected with Cp-1 at m. o. i. of 10 the presence of the different drugs. Incubation was continued for 4 h at 10° C. Samples (1 ml) were centrifuged (10 000 ×g, 10 min) and phages were assayed in the supernatants using R6 as indicator strain (100 % = 3.4×10^9 p. f. u./ml). Aphidicolin was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/ml.

polymerase α in eukaryotic cells (13). It has also been reported that this drug inhibited replication of small *B. subtilis* phages such as ∅ 29 and M2 that contain protein covalently linked to the DNA whereas other phages like SPP1 or SP50 were not affected in their replication (5). When samples of a culture of *S. pneumoniae* infected with Cp-1 were incubated in the presence of increasing concentrations of aphidicolin we found a total inhibition in the production of viable virus particles (Table 1). In addition, Figure 3A shows that the synthesis of phage DNA was decreased to a high extent when a Cp-1-infected culture was incubated in the presence of 100 μg/ml of aphidicolin. In similar experiments, the production of Dp-4 particles and the replication of its DNA were not affected by the presence of the drug (Fig. 3B). This bacteriophage does not contain protein linked to the DNA (2).

The experiments presented in Fig. 2 show that aphidicolin (100 μg/ml)does not inhibit to a high extent the *in vitro* formation of the Cp-1 initiation complex in agreement with the results obtained in the ∅ 29 system (1, 15). Nevertheless, when aphidicolin was added to a Cp-1-infected culture of *S. pneumoniae*, we found a noticeable reduction in the incorporation of [^{3}H]-thymidine. Futhermore, when we analyzed the distribution of Cp-1 DNA labeled in 5 min pulses of [^{3}H] thymidine by using alkaline sucrose gradients, we found that fragments smaller than the unit-length phage DNA remained in relatively large amounts in the presence of aphidicolin (data not shown).

We have reported that bacteriophage Cp-1, as well as other related phages (7, 9), multiplied under optimal conditions when incubated at 30° C. The results presented here provide experimental support to explain this behaviour since the *in vitro* formation of the initiation complex between the TP and 5'-dAMP was partially affected (Fig. 2) and the

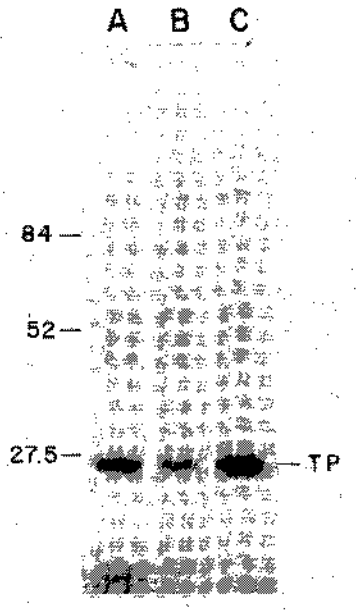

Fig. 2. Influence of different experimental conditions on the labeling of the TP by incubation of extracts from Cp-1-infected cells with (α^{32}P) dATP. Extracts from Cp-1-infected cells were incubated with (α^{32}P)dAPT as previously described (3) and subjected to SDS-polyacrylamide gel electrophoresis. Lane A: extracts incubated in the presence of aphidicolin (100 μg/ml) at 30° C; lane B: extracts incubated at 37° C; lane C: extracts incubated at 30° C. Numbers at the left indicate the $M_r \times 10^{3}$ of the ^{35}S-labeled Ø 29 structural proteins. TP: terminal protein.

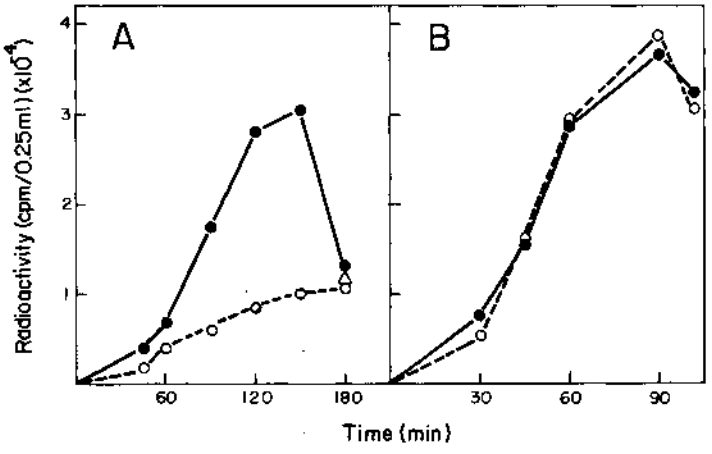

Fig. 3. Effect of aphidicolin on DNA synthesis in Cp-1- and Dp-4-infected pneumococcal cells. Cultures of *S. pneumoniae* strain 470 containing 6.2×10^{7} cells/ml received 50 μ M HPUra 5 min before addition of Cp-1 at m. o. i. of 25 (A) or Dp-4 at m. o. i. of 3 (B). [^{3}H]-Thymidine (5 μ Ci/ml) was added at 0 time and the cultures were divided into two portions that were incubated with (open symbols) or without (closed symbols) aphidicolin (100 μg/ml). At different times, samples (0.25 ml) were withdrawn and the TCA-precipitable radioactivity was determined as indicated in Table 1. Control cultures (closed symbols) contained DMSO instead of aphidicolin.

in vivo replication of the phage DNA was strongly reduced (Fig. 1) when Cp-1-infected pneumococci were incubated at 37° C. On the other hand, HPUra blocks the replication of the DNA of the host cells without affecting the multiplication of the phages (Table 1) whereas aphidicolin specifically inhibited Cp-1 DNA synthesis. Our results suggest that aphidicolin could interfere in the elongation of Cp-1 DNA fragments (Fig. 3) as it has been desmonstrated in the small *Bacillus* phages (8). The experimental conditions developed in this work should allow a more detailed characterization of the Cp-1 DNA replication machinery.

Note added in proof. Matsumoto *et al.* (Virology **152**, 32, 1986) have recently reported the isolation of aphidicolin-resistant mutans of bacteriophage ϕ29. The mutations were mapped in gene 2 which codes for ϕ29 DNA polymerase producing an altered form of the polymerase with a reduced sensitivity to aphidicolin. In addition, Blanco and Salas (Virology **153**, 179, 1986) also have demonstrated that the ϕ29 DNA polymerasa itself is the target of aphidicolin and of other nucleotide analogs.

Acknowledgements

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Homología ADN-ADN y caracterización fenotípica de cepas ambientales de *Vibrio parahaemolyticus* y *Vibrio pelagius*

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Summary

A taxonomic study by means of DNA homology and the most recent phenetic criteria has been carried out in 11 environmental strains of *Vibrio parahaemolyticus* and 4 of *Vibrio pelagius,* previously adscribed to the respective phenons in a numerical taxonomy study. The strains of *V. parahaemolyticus* showed genetic homogeneity, a guanine plus cytosine content (G + C) of 46 % and 61 % homology with *V. parahaemolyticus* type strain ATTC 17802. They exhibited phenetic atypicalities but could be adscribed to the species *V. parahaemolyticus.* The strains of *V. pelagius* behaved similarly in regard to the phenotype, but were genetically heterogeneous; they showed a % G +C of 44-46 and were asigned to *V. splendidus-V pelagius* homology group.

Key words: G + C content; Vibrio taxonomy; ADN-ADN hybridization.

Resumen

Once cepas ambientales de *Vibrio parahaemolyticus* y cuatro de *Vibrio pelagius,* caracterizadas en un estudio previo mediante taxonomía numerica como pertenecientes a los respectivos fenones, han sido sometidas a un estudio taxonómico mediante hibridación ADN-ADN y caracterización fenotipica de acuerdo con los criterios más recientes. Las cepas de *V. parahaemolyticus* han mostrado homogeneidad genética entre sí, un porcentaje guanina más citosina (% G + C) de 46 y un 61 % de homologia ADN-ADN con la cepa de *V. parahaemolyticus (ATCC 17802),* además de diversas atipicidades fenotipicas. Las cepas de *V. pelagius,* al igual que las anteriores, han presentado alguna atipicidad, pero han resultado genéticamente heterogéneas, muestran valores de % G + C de 44-46 y se encuentran genéticamente relacionadas con el grupo de homología de *V. splendidus-V. pelagius* según los datos de hibridación ADN-ADN.

* A quien se dirigira la correspondencia.

Introducción

De las, aproximadamente, treinta especies que comprende en la actualidad el género *Vibrio*, la mayoría son habitantes autóctonos de ecosistemas marinos, en los cuales desempeñan importantes funciones: reciclaje de materiales tales como la quinina (14), fijación de nitrógeno molecular (8), luminiscencia simbiótica y parasimbiótica (10), asociaciones específicas con organismos planctónicos (11, 25) y patogenicidad para un variado rango de la fauna marina (7, 9, 16). Su actividad patógena afecta, asimismo, al hombre a través de la ingestión de alimentos de origen marino, o de infecciones oportunistas por contacto (4, 5). Sin embargo, el estudio sistemático de tales especies, particularmente bajo un enfoque ambiental, se ha visto gravemente dificultado por la complejidad taxonómica del género, que parece abarcar límites más amplios que los que engloban a familias tales como *Enterobacteriaceae* (17). Los rangos de variabilidad fenotípica de cada especie son, por tanto, relativamente amplios, y se encuentran en gran parte, aún por determinar.

En un estudio previo, realizado mediante técnicas numéricas sobre cepas ambientales de Vibrio procedentes de diversos tipos de muestras marinas, fueron identificadas las especies *Vibrio alginolyticus, V. parahaemolyticus, V. splendidus, V. harveyi, V. campbellii* y *V. pelagius* (19). El fenón 10, no identificado, ha sido adscrito, tras un estudio de homología genética, a la nueva especie *V. mediterranei* (20). Los fenones 4 y 9, que corresponden a las especies *V. parahaemolyticus* y *V. pelagius*, respectivamente, fueron seleccionados para la realización de un estudio de homología genética (determinación del porcentaje en bases guanina más citosina e hibridación ADN-ADN), al tiempo que se procedió a completar la caracterización fenética según los criterios taxonómicos más recientes (4). En el presente trabajo se presentan los datos obtenidos en ambas aproximaciones.

Materiales y métodos

Cepas bacterianas.

Las cepas bacterianas utilizadas se indican en la Tabla 1. Fueron conservadas en Agar Marino (Difco) a temperatura ambiente y resembradas con una periodicidad bimensual. Las cepas se conservaron también en forma de liófilos.

Caracterización fenotípicas.

A los datos fenotípicos obtenidos en el estudio previo (19) se añadió la batería mínima de pruebas de identificación de género y especies (4). Las pruebas realizadas fueron: prueba O/F (con o sin producción de gas a partir de glucosa), oxidasa, reducción de nitratos, pigmentación, motilidad por deslizamiento («swarming»), producción de acetoína, crecimiento a 40° C, arginina dihidrolasa y utilización de sacarosa, celobiosa, D-gluconato, gamma-aminobutirato y putrescina como fuentes únicas de carbono y energía; todas ellas se llevaron a cabo siguiendo la metodología recomendada. Las cepas integrantes del fenón 9 fueron sometidas, además, a la prueba de degradación de alginato (3).

TABLA 1
CEPAS BACTERIANAS

Especie	Origen
Vibrio parahaemolyticus	ATCC 17802^T
cepa 10	Agua de mar. Valencia.
cepa 11	Agua de mar. Valencia.
cepa 65	Agua de mar. Valencia.
cepa 66	Agua de mar. Valencia.
cepa 67	Agua de mar. Valencia.
cepa 68	Agua de mar. Valencia.
cepa 69	Agua de mar. Valencia.
cepa 70	Agua de mar. Valencia.
cepa 79	Agua de mar. Valencia.
cepa 130	Plancton marino. Valencia.
cepa 131	Plancton marino. Valencia.
V. alginolyticus	NCMB 1903^T
cepa 22	Agua de mar. Valencia.
V. harveyi	NCMB 1280^T
cepa 158	Agua de mar. Valencia.
V. campbellii	NCMB 1894^T
V. splendidus	NCMB 1^T
V. ordalii	NCMB 2167^T
V. nereis	NCMB 1897^T
V. natriengens	NCMB
V. pelagius	
cepa 54	Sedimento marino. Valencia.
cepa 55	Sedimento marino. Valencia.
cepa 56	Sedimento marino. Valencia.
cepa 57	Sedimento marino. Valencia.
Escherichia coli cepa B	CECT 101

Aislamiento y purificación del ADN.

El ADN de las cepas estudiadas se obtuvo a partir de cultivos sobre medio sólido —agar extracto de levadura agua de mar (14)— excepto en el caso de *V. ordalii* NCMB 2167, que se cultivó según las recomendaciones de Schiewe y col. (23). Se procedió al aislamiento del ADN siguiendo el método de Marmur descrito por Johnson (12) con las siguientes modificaciones: en la primera desproteinización el tratamiento con perclorato fue sustituido por un tratamiento con fenol saturado con agua, tras lo cual se dializó el sobrenadante durante toda la noche en 0,1 SSC (SSC: tampón citrato salino estándard). Se llevó a cabo un tratamiento con ARNasa durante una hora, seguido por la adición de Pronasa (Sigma Co.) predigerida hasta una concentración final de 100 μg/ml. La incubación se prolongó durante dos horas a 37° C, y toda la noche a 28° C. La pureza de las soluciones de ADN se controló haciendo uso de las razones de absorbancia 260/280 y 260/230, que deben situarse por encima de 1,80 y 2,30, respectivamente (12).

Porcentaje en bases guanina más citosina (G +C).

El porcentaje en bases G +C del ADN de las cepas 10, 55 y 56 se determinó a partir de la temperatura media de fusión (T_m), haciendo uso del ADN de *Escherichia coli* (% G+C =51) como control, y aplicando la fórmula de Owen y Hill (18).

Marcaje radiactivo del ADN.

El ADN fue marcado radioactivamente mediante «nick translation» (22) con desoxicitidina trifosfato tritiada (Amersham TRK.621) y el equipo para «nick translation» (Amersham N 5000), siguiendo las instrucciones del fabricante. El ADN marcado se aisló mediante paso por columna de Sephadex G-50 (Pharmacia Fine Chemicals) de 10 cm. Se recogieron las 20 primeras fracciones (aproximadamente 200 μ l/fracción), cuya radioactividad total y precipitable (con ácido tricloroacético) fue seguida mediante contador de centelleo líquido. Las dos primeras fracciones radiactivas se seleccionaron para las experiencias de hidridación.

Hibridación de los ADNs.

Se siguió el método de hibridación competitiva sobre membrana de nitrocelulosa (12). Los experimentos se realizaron por quintuplicado (las excepciones se indican en las Tablas). Las mezclas de hibridación se prepararon con un 20 % de formamida (Sigma Co.) y la temperatura de hibridación se ajustó en cada caso a ma T_m-25° C (temperatura óptima). La adsorción inespecífica de radioactividad a las membranas se evaluó mediante la realización paralela de hibridaciones con ADN de salmón ligado a membrana en viales de hibridación no competitiva.

La evaluación de la homología genética se realizó haciendo uso de la fórmula (A-C/A-B) 100, en las que A corresponde a las desintegraciones por minuto (dpm) retenidas en las membranas de hibridación no competitiva, B las dpm retenidas en las hibridaciones con competidor homólogo y C las retenidas en presencia de competidor heterólogo.

TABLA 2

RESULTADOS DE LA CARACTERIZACION FENOTIPICA DE LAS CEPAS AMBIENTALES

CEPA[a]	O/F	Gas de glucosa	«Swarming»	Oxidasa	Pigmento	Arginina dihidrolasa	Voges-Proskauer	Crecimiento a 40° C	Reduccion de nitratos	Utilización de Sacarosa	Utilización de Celobiosa	Utilización de D-glucoonato	GABA[c]	Putrescina
10	F	–	–	+	–	–	–	+	+	–	–	+	–	+
11	F	–	–	+	–	–	–	+	+	–	–	–	–	–
65	F	–	–	+	–	–	–	+	+	–	–	+	–	+
66	F	–	–	+	–	–	–	+	+	–	–	+	+	+
67	F	–	–	+	–	–	–	+	+	–	–	+	+	+
68	F	–	–	+	–	–	–	+	+	–	–	+	–	+
69	F	–	–	+	–	–	–	+	+	–	–	+	–	+
70	F	–	–	+	–	–	–	+	+	–	–	+	–	+
79	F	–	–	+	–	–	–	+	+	–	–	+	–	+
130	F	–	–	+	–	–	–	+	+	–	–	+	–	+
131	F	–	–	+	–	–	–	+	+	–	–	+	–	+
54	F	–	–	+	–	–	–	–	NC[b]	+	–	+	+	+
55	F	–	–	+	–	–	–	–	NC	+	–	+	+	+
56	F	–	–	+	–	–	–	–	+	–	–	+	+	+
57	F	–	–	+	–	–	–	–	+	–	–	–	+	+

[a] Las cepas 10 a 131 pertenecen al fenón 4 *(V. parahaemolyticus)*; las cepas 54 a 57 pertenecen al fenón 9 *(V. pelagius)*.

[b] NC ausencia de crecimiento.

[c] GABA gamma-aminobutirato.

Resultados y discusión

Las cepas pertenecientes a los fenones 4 y 9 presentan, tanto en el estudio fenético previo (19), como en la caracterización fenotípica realizada en el presente trabajo (Tabla 2), el perfil fenotípico propio de *V. parahaemolyticus* y *V. pelagius*, respectivamente.

Las once cepas de *V. parahaemolyticus*, además de cumplir con los criterios mínimos para la adscripción al género, presentan las siguientes características: son positivas en las pruebas de la oxidasa, lisina descarboxilasa, crecimiento a 42° C, crecimiento en 8 % NaCl, producción de ácido a partir de arabinosa, sensibilidad a 0/129 y utilización de citrato, y son negativas para descarboxilación de arginina, producción de acetoína, ureasa, luminiscencia, producción de ácidos a partir de sacarosa y movilidad por deslizamiento. Todas ellas son respuestas típicas de la especie. De entre los 135 caracteres previamente estudiados, sólo resultaron atípicas la respuesta negativa a la descarboxilación de ornitina, la variabilidad de la prueba del indol y la capacidad de crecimiento en 10 % NaCl (19). Entre los resultados reflejados en la Tabla 2 sólo se dieron atipicidades ocasionales en la cepa 11 y en la utilización de gamma-aminobutirato por parte de las cepas 66 y 67.

Los resultados de la hibridación competitiva de la cepa 10, elegida como representante de su fenón, con miembros representativos del mismo y cepas de referencia del grupo de homología genética al que la especie pertenece (2, 21), quedan reflejados en la Tabla 3. Los valores de competencia entre los ADNs de los miembros del fenón 4 y la cepa 10 se sitúan por encima del 80 %, excepto para la cepa 130, que presenta un 74 %, lo que confirma la homogeneidad genética del grupo previamente definido en términos fenéticos (19). La cepa 10 muestra un 61 % de homología con la cepa de *V. parahaemolyticus* ATCC 17802, un valor quizá bajo para la adscripción específica según los criterios más estrictos(4). Cabe destacar, sin embargo, que no existe unanimidad en la aplicación de estos criterios, y así, mientras que algunos investigadores consideran suficientes homologías del orden del 60 % (13, 24), otros separan en especies distintas cepas con homologías de 65 %, como en el caso de *V. campbelli* y *V. harveyi* (4). Dentro del mismo gé-

TABLA 3

HOMOLOGIA ADN/ADN ENTRE LA CEPA 10, REPRESENTANTES DEL FENON 4 Y CEPAS TIPO DE ALGUNAS ESPECIES DE *VIBRIO*[a]

ADN competidor de:	% competencia	N.º de réplica
Cepa 10	100	5
Cepa 11	104	1
Cepa 67	82	4
Cepa 130	74	4
V. parahaemolyticus ATCC 17802^T	61	5
V. alginolyticus 22	48	5
V. alginolyticus NCMB 1903^T	40	1
V. campbellii NCMB 1894^T	45	5
V. harveyi NCMB 1280^T	20	4

[a] Retención inespecífica de radioactividad: 3.7 %.

nero se da el caso de cepas que, presentando valores medios de homología de 67 % (lo que incluye casos de 60 % de homología) se encuentran como biotipos de la misma especie - biotipos I y II de *V. splendidus* (4, 21). La homología que la cepa 10 presenta con el resto de especies del grupo de *V. campbellii* es proporcionalmente más baja que la que presenta *V. parahaemolyticus,* pero las distancias relativas se mantienen: las relaciones descritas para *V. alginolyticus* y *V. parahaemolyticus* son de 61-66 % con el método de hibridación en membrana (21) y de 68 % con el método de la hidroxiapatita (6); entre ambos y el grupo *V. harveyi-V. campbellii* los porcentajes oscilan entre 47-49 y 46-52 %, según el método (6, 21). En nuestro estudio los valores son más bajos, lo que puede indicar una excentricidad de nuestras cepas respecto al grupo de homología tomado como conjunto.

A la vista de la semejanza fenotípica, la coincidencia en el porcentaje en bases G + C, que es de 46 % para la cepa 10, y teniendo en cuenta que la máxima homología genética se ha dado con *V. parahaemolyticus,* nos inclinamos por la conservación de estas cepas dentro de la mencionada especie, quizá como una variante geográfica atípica o como núcleo de un nuevo biotipo.

Las cepas correspondientes al fenón 9 (cepas 54, 55, 56 y 57) presentaron el perfil fenotípico de *V. pelagius:* fueron positivas para las pruebas de la oxidasa, sensibilidad a 0/129, reducción de nitratos, hidrólisis de gelatina, alginato y esculina, producción de ácidos a partir de sacarosa, manitol, manosa, trehalosa y utilización de D-gluconato, gamma-aminobutirato y putrescina. Fueron negativas en su capacidad de crecimiento a 40° C, producción de acetoína, descarboxilación de arginina, lisina y ornitina, producción de gas de glucosa, crecimiento en 10 % NaCl, producción de indol, luminiscencia, ureasa y ácidos de arabinosa. Las cepas fueron atípicas en la prueba de utilización de citrato, y una de ellas en la incapacidad de utilización de D-gluconato (cepa 57, que posteriormente mostraría falta de relación genética con la cepa 55). Con la excepción de estos dos casos, todas las respuestas son propias de *V. pelagius.* Asímismo los valores de G + C obtenidos para las cepas 55 y 56, que son de 44 y 46 %, están en concordancia con los de la especie (4).

Las relaciones de homología de la cepa 55, elegida como representante del fenón 9,

TABLA 4

HOMOLOGIA ADN/ADN ENTRE LA CEPA 55, REPRESENTANTES DEL FENON 9 Y ALGUNAS ESPECIES DE *VIBRIO*[a]

ADN competidor de:	% competencia	N.º de réplicas
Cepa 55	100	5
Cepa 54	95	3
Cepa 57	0	1
V. splendidus NCMB 1[T]	40	5
V. harveyi 158	25	2
V. ordalii NCMB 2167[T]	17	1
V. natriengens NCMB	16	5
V. nereis NCMB 1897[T]	12	5

[a] Retención inespecífica de radioactividad: 3.6 %.

con cepas pertenecientes al mismo y cepas de diversas especies del género se muestran en la Tabla 4. Resalta, en primer lugar la heterogeneidad genética de la cepa 57, que en el estudio previo había mostrado una similaridad fenética del 90 % con la cepa 55 (19), con respecto a la misma. En cambio, el valor de la homología entre las cepas 55 y 54 se encuentra en el rango propio de la especie.

Los datos sobre la homología genética de la cepa 55 con otras especies de *Vibrio (V. splendidus, V. ordalii, V. natriengens, V. harveyi, V. nereis)* no permiten en ningún caso la inclusión de las cepas en dichas especies. La única relación significativa es la que se obtiene con *V. splendidus* NCMB 1, y esta se encuentra en el nivel de los grupos de homología genética actualmente definidos en el género *Vibrio*, grupos que Baumann y Baumann definen a partir del 40 % (2) y Brenner y cols. a partir del 30 % (6).

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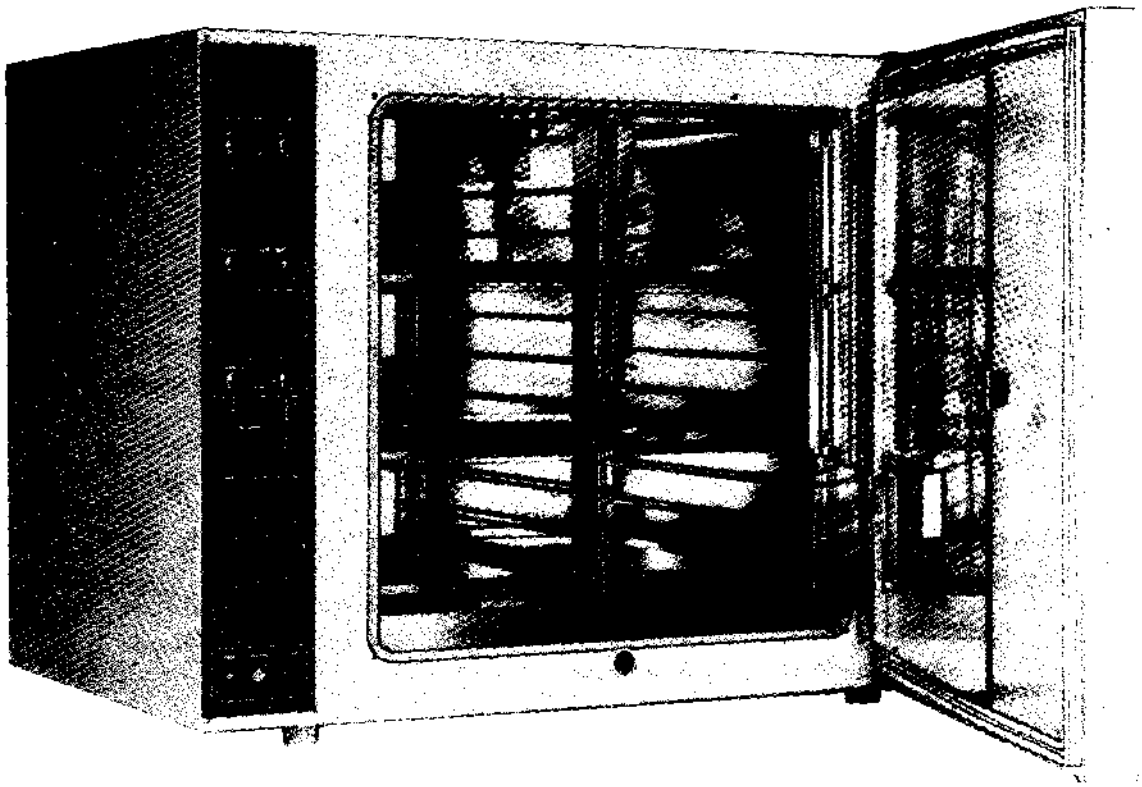

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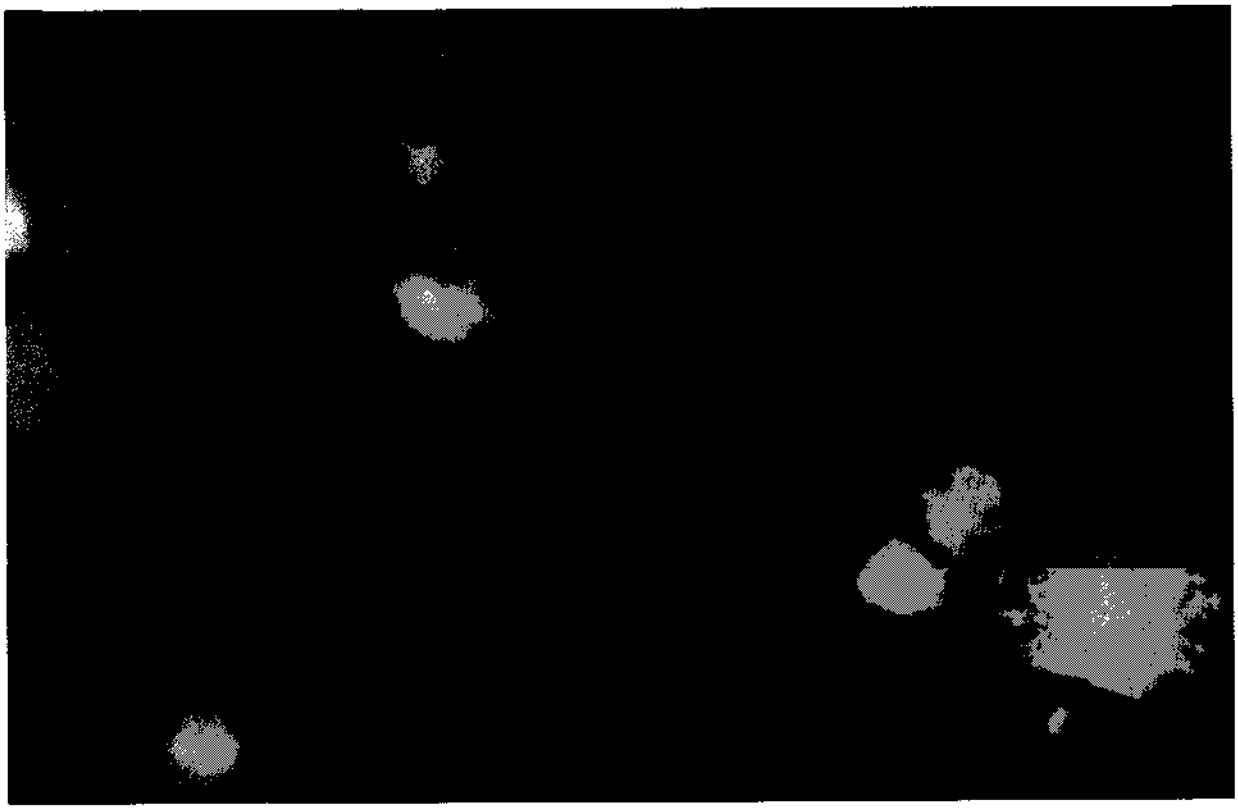

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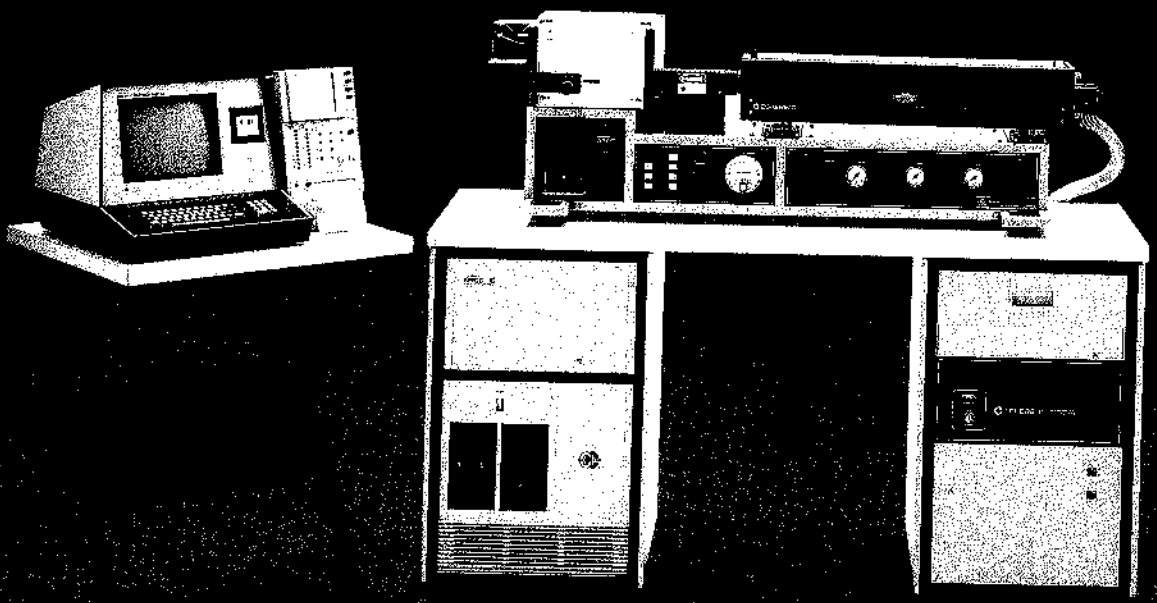

- Detección e identificación de especies.
- Aislamiento de individuos.
- Estudio de ciclo vital.
- Permeabilidad de membrana.
- Resistencia a antibióticos, etc.

Las aplicaciones presentes y futuras sólo están limitadas por la imaginación del investigador.
Pregúntenos sobre el EPICS C si desea conectar con la investigación del futuro.

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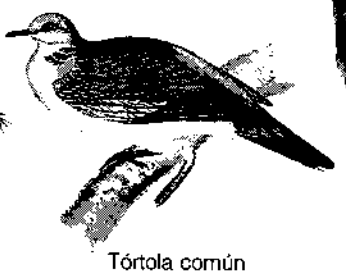
Urraca

Serín

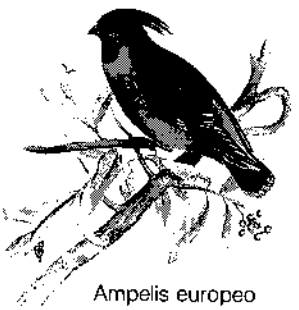


Tórtola común

Arrendajo

Oropéndola

Tarabilla común

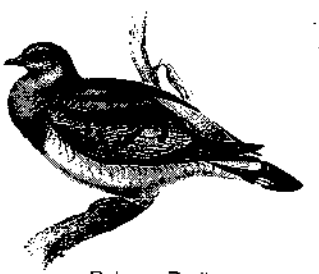


Ampelis europeo

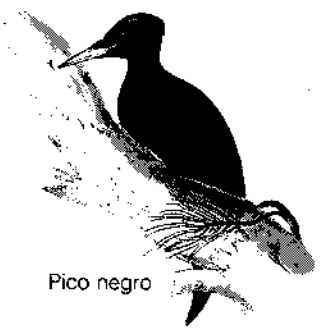
Zorzal común

Estornino pinto

Verderón común



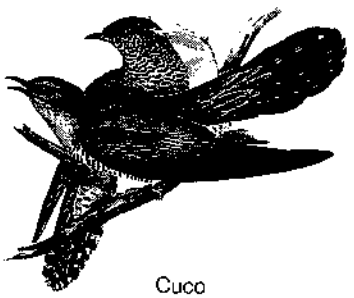
Paloma Zurita



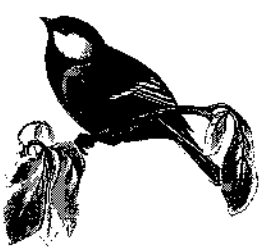
Pico negro

Mirlo común

Roquero rojo



Cuco



Carbonero común