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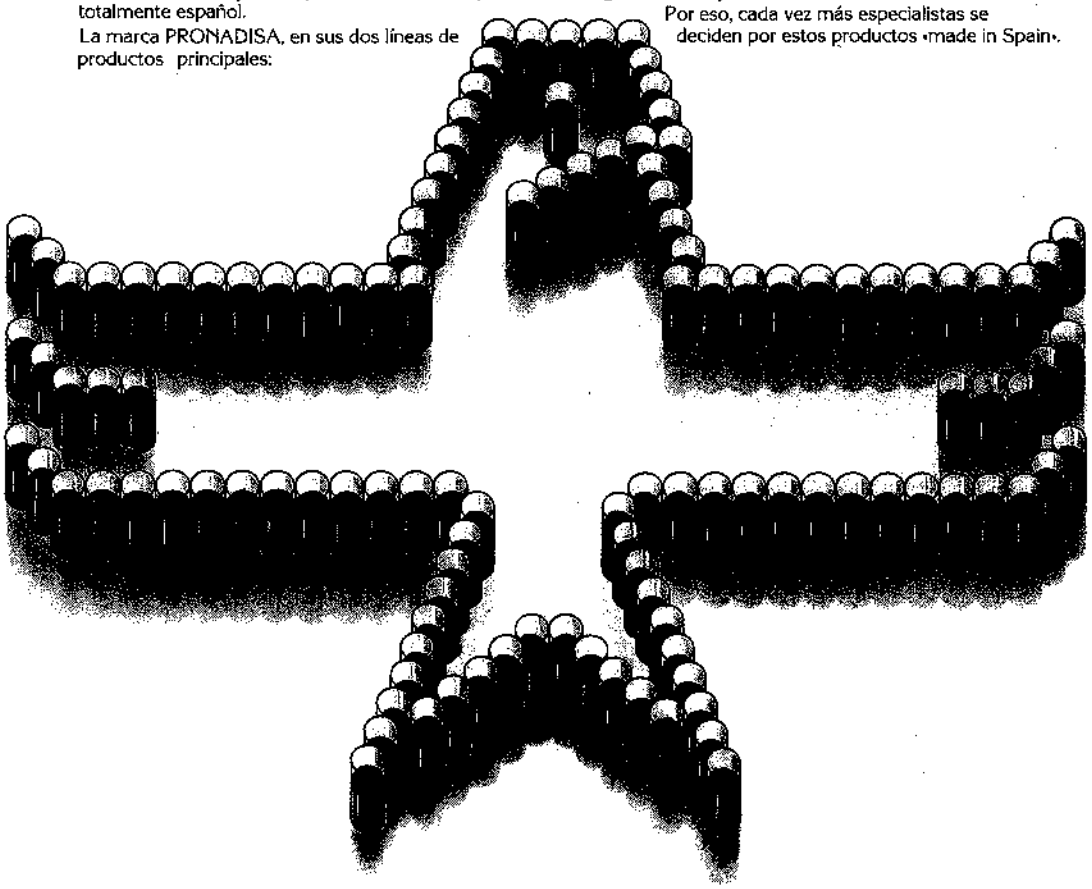

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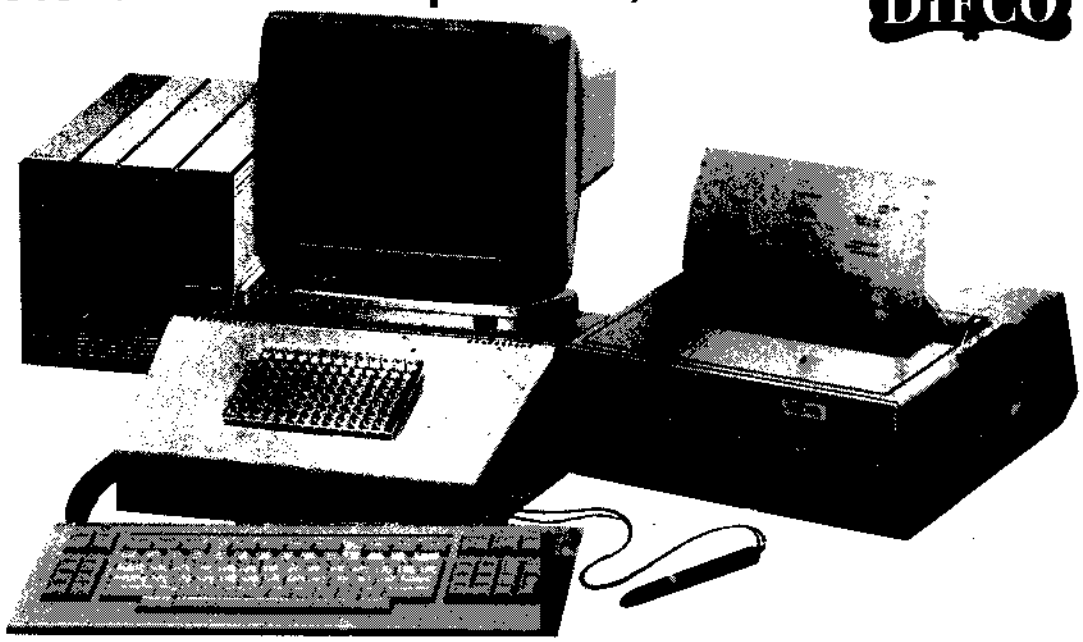

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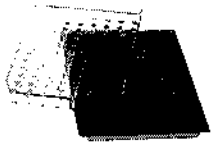

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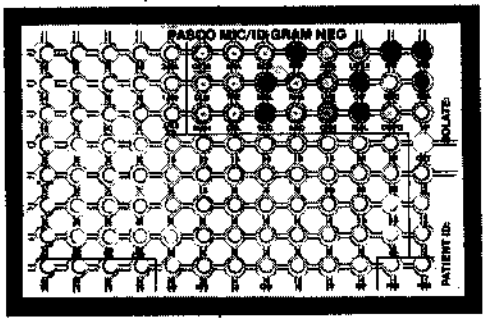

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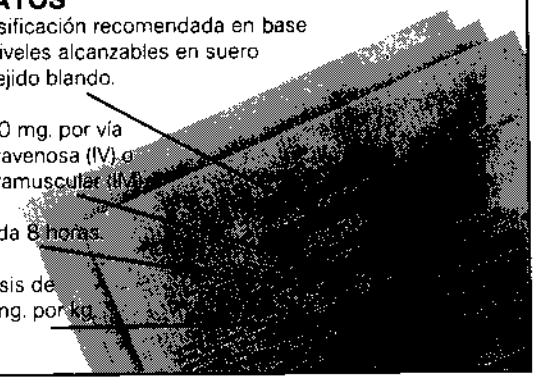

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MINIREVIEW

Phototrophic bacteria (an incoherent group of prokaryotes). A taxonomic versus phylogenetic survey *

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(Received May 15, 1987)

Summary

In spite of their apparently consistent classical systematic scheme the phototropic bacteria are, as 16S-rRNA oligonucleotide cataloguing and sequencing have shown, deeply split into phylogenetic divisions of very little relationships between one another. Phototrophy as a mode of energy metabolism occurs in the phylogenetic divisions of a) «Gram-positive eubacteria», b) «Cyanobacteria/Chloroplasts», c) «Green Sulfur Bacteria», d) «*Chloroflexus* and related taxa», and e) «Purple Bacteria and related taxa», i.e. in five of the nine phylogenetic divisions of eubacteria. The arising disagreements are discussed and an attempt is made towards a stepwise reconciliation of taxonomy with phylogeny. The strong and the weak points in the taxonomy of phototrophic eubacteria are pointed out within the existing families. Emphasis is given to areas where taxonomic studies are urgently needed.

Key words: phototrophic bacteria, taxonomy, phylogeny.

Resumen

A pesar de que las bacterias fototróficas presentan un esquema sistemático de consistencia aparentemente clásica, la catalogación de los oligonucleótidos 16S-rRNA y la secuenciación, han establecido una profunda ruptura de este grupo en divisiones filogenéticas con escasas similitudes entre las mismas. La fototrofia, como una forma de metabolismo energético, tiene lugar en las divisiones filogenéticas de: a) «Eubacterias Gram-positivas», b) «Cianobacterias/Cloroplastos», c) «Bacterias verde sulfurosas», d) «*Cloroflexus* y taxones relacionados» y e) «Bacterias purpúreas y taxones relacionados», es decir, en cinco de las nueve divisiones filogenéticas de eubacterias. Se discuten los desacuerdos que han surgido y se realiza un esfuerzo para conseguir una armonización paulatina entre la taxonomía y la filogenia. Se analizan conceptos fuertemente y débilmente

(*) Dedicated to Holger W. Jannasch, Woods Hole Oceanographic Institution, on the occasion of his 60th birthday.

establecidos en la taxonomía de las diferentes familias de bacterias fototróficas. Asimismo, se presta una especial atención a aquellas áreas donde se requieren urgentemente estudios taxonómicos.

Introduction

Numerous chemical and biochemical compounds undergo changes when they are illuminated, i.e., take up light energy.

«Phototrophy» *sensu stricto* is the capability of many living organisms to utilize the energy of light for growth and maintenance. Phototrophy therefore includes complicated biophysical reactions taking place within specifically developed structural arrangements that are localized in and at membranes. These structures contain light-harvesting (i.e. antenna) pigments (chlorophylls, phycobilins, carotenoids), reaction centers including reaction center pigments, primary electron acceptors and several other electron transport components such as iron-sulfur proteins, quinones, cytochromes, etc. The possession of such structures and their functioning certainly require a greater number of genes to express phototrophy in an organism. It appeared therefore reasonable that in classical bacterial taxonomy phototrophic prokaryotes have been considered as more or less closely related by the common possession of the multiply-coded property «phototrophy», and as principally different from and only distantly related with nonphototrophic bacteria (61, 91, 63, 21).

After it has been generally accepted by microbiologists and a large proportion of botanists that the Cyanophyceae (blue-green algae) —being prokaryotes— belong to the bacteria and therefore should not be dealt with as «algae» in algal (i.e. botanical) taxonomy together with eukaryotic algae, and the term «cyanobacteria» is generally used for this group by now, it seemed appropriate to include this group in the taxonomic hierarchy of phototrophic prokaryotes. Gibbons and Murray (21) thus arranged the phototrophic prokaryotes as Photobacteria (Table 1) and set them aside from all other, nonphototrophic bacteria, the Scotobacteria.

TABLE 1
HIGHER TAXA OF PHOTOTROPHIC PROKARYOTES
After Gibbons and Murray (22)

Class:	Photobacteria, Gibbons and Murray (22)
Subclass I:	Oxyphotobacteriae, Gibbons and Murray (22)
	Order I: Cyanobacteriales, Gibbons and Murray (22)
	Order II: Prochlorales Lewin (50)
Subclass II:	Anoxyphotobacteriae Gibbons and Murray (22)
	Order I: Rhodospirillales Pfennig and Trüper (62)
	Family I: Rhodospirillaceae Pfennig and Trüper (62)
	Family II: Chromatiaceae Bavendamm (2)
	Family III: Ectothiorhodospiraceae Imhoff (37)
	Order II: Chlorobiales Gibbons and Murray (22). Trüper (89)
	Family I: Chlorobiaceae Copeland (9)
	Family II: Chloroflexaceae Trüper (89)

Families of the Anoxyphotobacteria were added from the Literature as indicated.

The systematics of the cyanobacteria that consequently should follow the International Code of Nomenclature of Bacteria (49), ICNB, i.e. should be based on living type cultures, is by historical reasons through predominantly botanical/planctological studies, based upon descriptions of habitat specimens and documented —if at all— by herbarium (!) specimens.

The energetic efforts of the late Roger Y. Stanier and his coworkers have led to a new taxonomy of the cyanobacteria based upon pure cultures in the sense of bacteriology: at the genus level Stanier and coworkers have provided a workable scheme (68, 69, 85) from which species differentiation as well as taxa above the level of genus will be reliably worked out in a foreseeable future (90).

In view of this situation the Oxyphotobacteria will not be discussed in further detail here.

The Anoxyphotobacteriae at present comprise the three families of «purple bacteria» (Rhodospirillales), the two families of «green bacteria» (Chlorobiales) as listed in Table 1, and several new isolates —as yet *incertae sedis*— that will be discussed below. So far, also the classification of this group has been based on phenotypic characters (e.g., by Trüper and Pfennig, 92), although the thorough consideration of new chemotaxonomic data (64) has led to first steps in reorganizing the family of the Rhodospirillaceae (40) and proposing the Ectothiorhodospiraceae as a new family of purple sulfur bacteria besides the Chromatiaceae (37).

The pioneering work of Carl R. Woese and his coworkers who introduced 16S-rRNA oligonucleotide cataloguing and 16S-rRNA total sequencing as new powerful tools of chemotaxonomy has produced the strongest impact on classical bacterial taxonomy so far, in that it for the first time laid open phylogenetic relationships and lines of evolution in prokaryotes. And these lines reveal that the major portion of higher taxa (family and higher) and even several phenotypically «well defined» genera (e.g. *Pseudomonas, Rhodopseudomonas, Rhodospirillum, Clostridium, Bacillus*) in the classical system are not phylogenetic units.

As indicated above, this also holds for the phototrophic bacteria. In a recent general treatise on the eubacterial hierarchic system, Stackebrandt (80) has pointed out that on the basis of 16S-rRNA data the eubacteria at present may be divided into 9 «Divisions» (this designation was adapted from eukaryote systematics) that show rather low phylogenetic relationships to one another (Table 2).

The occurrence of phototrophy is spread over Divisions 2, 6, 7, 8 and 9, i.e., it is manifested in prokaryotes of extremely low phylogenetic relationship. Like the structure and function-related properties of «gliding mortality» or «budding type of cell division», not to speak of plain morphology like «spirilloid», «coccoid», etc., the property «photosynthesis», although coded in numerous genes, is not qualified as a basic principle for the construction of systematic hierarchies as depicted by Table 1.

The wide distribution of photosynthesis has been interpreted as a sign of its high age, i.e. all eubacteria evolved from an originally phototrophic ancestor (18, 84). Also, lateral gene transfer during evolution is being discussed in the literature (12, 98). As long as the relevant sufficient evidence through molecular biological data is lacking, these questions remain open.

Phototrophic bacteria thus are an incoherent group of prokaryotes.

Certainly the higher taxa of eubacteria will undergo extensive rearrangements on the

basis of 16S-rRNA sequences in the near future. In physiological or ecological laboratory work taxa above the rank of family, however, are very seldom of importance. Thus it is most important to keep the taxa «species», «genus» and «family» in a workable order.

In the following I shall discuss the situation in the presently existing families of phototrophic bacteria (excluding the phototrophic organisms and organelles of Division 6, Table 2). I shall also propose changes, however, without claiming priority in the sense of the International Code of Nomenclature of Bacteria (49).

The family Chlorobiaceae (Green Sulfur Bacteria)

So far, Division 7 (Table 2) is represented by *Chlorobium vibrioforme, C. limicola* (f. *thiosulfatophilum*), *Prosthecochloris aestuarii* and *Chloroherpeton thalassium* only, because no other species of the presently recognized green sulfur bacteria (Table 3) have been studied with respect to 16S-rRNA. Further, so far no nonphototrophic genera have been found that would belong into this Division.

TABLE 2
THE NINE DIVISIONS OF EUBACTERIA.
After Stackebrandt (80)

Nr.	Division	Groups contained/Special properties	References
1	Planctomyces/Pirella	Protein cell walls, low S_{AB} to other eubacteria.	82
2	Gram-positive eubacteria (except Deinococcus)	a) Clostridium and relatives, low G + C. b) Actinomycetes and relatives, high G + C.	18, 83, 84, 97, 99
3	Deinococcus/Thermus	Two rather different phenotypes, both with ornithin in their peptidoglycan.	7
4	Spirochetes	Spirochetes with uniform morphology; and Haloanaerobium.	60
5	Cytophaga/Bacteroides	Bacteroides, cytophagas, flavobacteria, flexibacteria, Saprospira, Haliscomenobacter.	59
6	Cyanobacteria/chloroplasts	Cyanobacteria, Prochlorales, chloroplasts.	59
7	Green Sulfur Bacteria	Chlorobium, Chloroherpeton	22
8	Chloroflexus and relatived taxa	Chloroflexus, Herpetosiphon, Thermomicrobium; low S_{AB} to other eubacteria.	22, 58
9	Purple Bacteria and related taxa	4 separate subdivisions (α-δ): α: Acetobacter, Agrobacterium, Rhodobacter, Rhodospirillum, etc. β: Alcaligenes, Nitrosococcus, Pseudomonas, Rhodocyclus, etc. γ: Enterobacteriaceae, Legionella, Pseudomonas, Chromatiaceae, Ectothiorhodospiraceae, etc. δ: Desulfovibrio, Desulfuromonas, Myxococcus, etc.	16, 17, 84, 100, 101, 102

The Chlorobiaceae are characterized by bacteriochlorophyll *a* as reaction center pigment and bacteriochlorophylls *c*, *d*, or *e* as light-harvesting chlorophylls as well as by carotenoids of the chlorobactene, renieratene and isorenieratene types. Their light-harvesting pigments are located in chlorosomes attached to the inner side of the cytoplasmic membrane, whereas their photosynthetic reaction centers are located in the cytoplasmic membrane.

TABLE 3
SPECIES OF THE FAMILY CHLOROBIACEAE
Modified after Trüper and Pfennig (92); T: Type species

Ancalochloris	*perfilievii* (T)
Chlorobium	*chlorovibrioides*
	limicola (T)
	phaeobacteroides
	phaeovibrioides
	vibrioforme
Chloroherpeton	*thalassium* (T) (23)
Pelodictyon	*clathratiforme*
	luteolum
	phaeum
Prothecochloris	*aestuarii* (T)
	phaeoasteroidea

So far all species of the Chlorobiaceae are obligate photolithoautotrophic bacteria requiring reduced sulfur compounds as photosynthetic electron donors and probably all fix carbon dioxide via a reverse tricarbocylic acid cycle (15, 19, 44). Except for *Chloroherpeton thalassium* all species are nonmotile. Flagella do not occur; *C. thalassium* shows gliding motility. In this respect the taxonomic family description needs to be emended.

From 16S-rRNA oligonucleotide catalogue comparison of 4 species Gibson et al. (22) concluded that these, i.e. the Chlorobiaceae, for a moderately ancient group, because their lowest similarity coefficients were about 0.45, i.e. in a range comparable to that occurring within the *Clostridium* subdivision of Division 2 between the genera *Bacillus, Lactobacillus, Streptococcus* (18).

As only part of the Chlorobiaceae species have been included it remains open whether such statements will hold in general. A thorough taxonomic study of the Chlorobiaceae seems necessary and is justified by the rather wide ranges observed in DNA base ratios within several species, the assignment of strains with different antenna bacteriochlorophylls to one species and the questionable rank of the subspecific formae «*thiosulfatophilum*» of *Chlorobium limicola* and *C. vibrioforme*.

The family Chloroflexaceae

This family was proposed (89) to contain *Chloroflexus* and similar bacteria, that could not be grouped with the family Chlorobiaceae. The Chloroflexaceae are defined as

phototrophic bacteria containing chlorosomes, antenna bacteriochlorophyll *c* or *d*, besides reaction center bacteriochlorophyll *a*; cells have gliding motility, cell walls are flexible, growth is filamentous. Although the family was proposed on the basis of the properties of *Chloroflexus aurantiacus*, a photoorganoheterotrophic thermophilic bacterium, the author explicitly did not include these properties in the family description (89). Mesophilic (26), and very recently also photolithoautotrophic *Chloroflexus*-like bacteria were described (25). Therefore it is not justified to talk of «Green nonsulfur bacteria» when Chloroflexaceae are meant as rRNA chemotaxonomists keep doing (e.g. 58).

On the basis of morphology, motility, fine structure (possession of chlorosomes), and pigments, further species (Table 4) were assigned to the Chloroflexaceae by Gorlenko and coworkers (13, 27,29). *Oscillochloris chrysea*, originally described as a blue green alga *(Oscillatoria coerulescens)* was found to contain chlorosomes located on cristae-like structures (29).

TABLE 4
SPECIES OF THE FAMILY CHLOROFLEXACEAE
(T: Type species)

Chloroflexus aurantiacus (T)	(65)
Chloronema giganteum (T)	(13)
spiroideum	(13)
Oscillochloris chrysea (T)	(29)
trichoides	(27)
Heliothrix oregonensis (T)	(66)

When *Chloroherpeton thalassium* was described, it would —on the basis of flexible cells and gliding motility —have been grouped with the Chloroflexaceae. But 16S-RNA data clearly proved its much closer relation to *Chlorobium* (22) and consequently *Chloroherpeton* was grouped with the Chlorobiaceae.

Therefore, as long as 16S-rRNA data are lacking on *Chloronema* and *Oscillochloris* it remains open whether these genera are grouped correctly with the Chloroflexaceae, or perhaps belong into the Chlorobiaceae.

Another unsolved problem is posed by the red, extremely thermophilic, filamentous gliding phototrophic bacterium *Heliothrix oregonensis* (66).

This organism lacks chlorosomes and antenna bacteriochlorophylls *c, d,* or *e*, but contains only bacteriochlorophyll *a* in the cytoplasmic membrane. I.e., it lacks an important basic property of the Chloroflexaceae and in fine structure and photopigments it rather resembles the Rhodospirillaceae. Pierson et al. (66), however, assigned it to the Chloroflexaceae on the basis of 5S-rRNA sequence comparisons. 16S-rRNA studies are still lacking. An emendation of the description of Chloroflexaceae will become necessary to include *Heliothrix*.

Thorough studies on the primary photochemistry of *Chloroflexus aurantiacus* have shown that it is basically different from that of the Chlorobiaceae and that it closely

resembles that of the purple bacteria (i.e. the Rhodospirillaceae), (4, 8). Again, at this point the question of lateral gene transfer must arise, i.e. the question whether at an early stage of evolution the genes for chlorosome synthesis and function were transfered to an «early purple bacterium» giving rise to *Chloroflexus*.

Very recently, a new type of gliding, filamentous, purple phototrophic bacterium was reported although not yet in pure culture and not taxonomically named, that lives together with the filamentous cyanobacterium *Microcoleus chthnoplastes* inside a common sheath in hypersaline cyanobacterial mats (10). The cells of the purple bacterium contain large membrane stacks like the Ectothiorhodospiraceae. Pure culture studies are necessary before this new organism can be assigned taxonomically.

The phylogenetic Division 8 (Table 2) has been named after *Chloroflexus*, although this is still the only phototrophic bacterium assigned to Division 8. The nonphototrophic Genera of Division 8 are the thermophilic *Thermomicrobium* and the mesophilic gliding filamentous bacterium *Herpetosiphon* (22). The latter organism has been compared with other gliding eubacteria of different affiliations (including cyanobacteria) with the result that gliding is a phenomenon without phylogenetic weight (67) with the exception of the myxobacteria (51). This finding is supported by the close relationship between *Chlorobium* (nongliding) and *Chloroherpeton* (gliding), as mentioned above, and on the other hand may be considered as a warning with respect to the present family description of Chloroflexaceae (89). Division 8 is phylogenetically deep but entirely distinct from all other eubacterial Divisions (Table 2). Oyaizu et al. (58) consider Division 8 significantly older than either Division 7 (green sulfur bacteria) or Division 6 (cyanobacteria); therefore it appears likely that the oldest stromatolites, 3.5×10^9 years old, were formed by *Chloroflexus*-like bacteria rather than by cyanobacteria (58). This view is supported by the fact that *Chloroflexus aurantiacus* is thermophilic, and that with respect to the higher ambient temperatures of the Earth's surface 3.5×10^9 years ago, a thermophilic ancestor of the eubacteria seems likely (1).

The family Rhodospirillaceae

The Rhodospirillaceae as a family are characterized by the possession of either bacteriochlorophyll *a* or *b* as reaction center and antenna chlorophyll, localized in different types of intracytoplasmic membrane systems as extensions of the cytoplasmic membrane. Photoorganoheterotrophic growth (under anaerobic conditions) is usually preferred, many species are capable of respiratory metabolism under aerobic or microaerobic conditions.

Often the vernacular name «nonsulfur purple bacteria» is used for this family. This name is confusing as some people base it on the inability to utilize reduced sulfur compounds as photosynthetic electron donors, others on lacking intracellular sulfur globules. Both usages are misleading because on one hand some of the Rhodospirillaceae do use sulfide and thiosulfate (a few even elemental sulfur) as electron donors, and on the other hand, the Ectothiorhodospiraceae are also purple bacteria that lack intracellular sulfur globules.

The present arrangement of genera and species of the Rhodospirillaceae (Table 5) is the most modern one (40) of all families of phototrophic bacteria, because it is not

predominantly based on morphology (as the Chromatiaceae and the Chlorobiaceae) but on ultrastructure and on chemotaxonomic data such as 16S-rRNA data, DNA/rRNA hybridization, cytochrome c structures, lipid composition, lipopolysaccharide structure, quinone composition, and pathways of sulfate assimilation. The necessity for this new arrangement had been discussed in detail by Pfennig and Trüper (64).

TABLE 5
SPECIES OF THE RHODOSPIRILLACEAE
Modified after Imhoff et al. (40).
T: Type species

Rhodobacter	*adriaticus*
	capsulatus (T)
	euryhalinus (46)
	sphaeroides
	sulfidophilus
	veldkampii (32)
Rhodomicrobium	*vannielii* (T)
Rhodopila	*globiformis* (T)
Rhodopseudomonas	*acidophila*
	blastica
	marina
	palustris (T)
	rutila
	sulfoviridis
	viridis
Rhodospirillum	*fulvum*
	mediosalinum (47)
	molischianum
	photometricum
	rubrum (T)
	salexigens
	salinarum (54)
Rhodocyclus	*gelatinosus*
	purpureus (T)
	tenuis

Except for the genus *Rhodocyclus*, all genera of the Rhodospirillaceae belong into the α-subdivision of Division 9 (Table 2), whereas *Rhodocyclus* belongs into the β-subdivision (24, 100, 102). Within these subdivisions they occur intermingled with a variety of nonphototrophic species from genera such as *Agrobacterium, Aquaspirillum, Azospirillum, Erythrobacter* (cf. below), *Nitrobacter, Paracoccus, Phenylobacterium, Rhizobium* (α-subdivision) and *Alcaligenes, Aquaspirillum, Chromobacterium, Comamonas, Nitrosovibrio, Nitrosospira, Nitrosolobus, Nitrosomonas, Nitrosococcus, Pseudomonas, Sphaerotilus, Spirillum, Thiobacillus, Vitreoscilla* (β-subdivision) (100, 102).

In several cases there are closer relationships between phototrophic and nonphototrophic species of the same taxonomic genus, e.g., between *Rhodopseudomonas*

palustris and *Nitrobacter winogradskyi* (73), or between *Rhodocyclus gelatinosus* and *Sphaerotilus natans* (24).

The rather deep branching between the α-, β- (and γ-) subdivisions of Division 9 (S_{AB} about 0.35) probably warrants a separate family for the three species now in the single genus *Rhodocyclus (R. purpureus, R. gelatinosus, R. tenuis)*. Such a family then should be named «Rhodocyclaceae».

Within the β-subdivision Woese et al. (102) have further formed the groups β-1, β-2 and β-2a, with the consequence that *R. gelatinosus* belongs into β-1, but *R. tenuis* into β-2. These differences probably warrant different genera for these two species. As long as the type species of the genus *Rhodocyclus, R. purpureus*, has not been analyzed with respect to 16S-rRNA, however, it is not clear, which of the two former species would retain the old genus name, or whether even three genera are necessary. With respect to quinone composition a family Rhodocyclaceae would be as coherent as the Chromatiaceae (38).

Also the α-subdivision has been further subdivided by Woese et al. (100) into the groups α-1 (containing *Rhodospirillum rubrum, R. photometricum, R. molischianum* and *Rhodopila globiformis*), α-2 (containing *Rhodopseudomonas palustris, R. acidophila, R. viridis* and *Rhodomicrobium vannielli* (i.e., so far only species that divide by budding) and α-3 (containing *Rhodobacter capsulatus* and *R. sphaeroides*). Unfortunately no further species (cf. Table 5) have been included in 16S-rRNA studies yet.

With respect to carotenoid and quinone composition only the genus *Rhodobacter* gives a uniform picture (38, 92) whereas the genera *Rhodospirillum* and *Rhodopseudomonas* are —in spite of being easily recognizable by morphology (spiral or budding, respectively)— rather heterogeneous with respect to carotenoids (92), quinones (38) and polar lipids (39).

DNA-DNA hybridization studies have been carried out with species of the genera *Rhodobacter, Rhodopseudomonas, Rhodospirillum* and *Rhodomicrobium* (11, 93). The results obtained are in general agreement with the present taxonomic scheme.

The rather low S_{AB} value of 0.43 between the groups α-1, α-2 and α-3, could in the future lead to a splitting of the Rhodospirillaceae into three respective families, perhaps the Rhodospirillaceae, «Rhodopseudomonadaceae» and «Rhodobacteraceae». As a consequence, splitting of the genera *Rhodospirillum* and *Rhodopseudomonas* into more genera could be imagined.

The family Chromatiaceae

The typical properties of the Chromatiaceae (Table 6) are the possession of either bacteriochlorophyll *a* or *b* as reaction center *and* antenna chlorophyll, localized in intracytoplasmic membrane systems (usually of the vesicular type) as extensions of the cytoplasmic membrane. The basic morphological difference between these and the Rhodospirillaceae is the occurrence of elemental sulfur globules in the cells of Chromatiaceae, due to the basic physiological difference of predominant anaerobic photolithoautotrophic metabolism in the latter. The vernacular name «purple sulfur

bacteria» for the Chromatiaceae is correct in both respects of its meaning (cf. above under Rhodospirillaceae).

With respect to 16S-rRNA oligonucleotide cataloguing the Chromatiaceae are the family best studied so far, because 11 species have been studied (16, 24, 101). The species are: *Amoebobacter pendens, A. roseus, Chromatium minus, C. vinosum, C. warmingii, C. weissei, Lamprocystis roseopersicina, Thiocapsa roseopersicina, Thiocystis gelatinosa, T. violacea, Thiodictyon elegans, Thiospirillum jenense.* In addition, the bacteriochlorophyll *b* containing *Thiocapsa pfennigii* has been 16S-rRNA catalogued and shown to be related closely to *Thiodictyon elegans* (personal communication by Dr. N. Pfennig, Konstanz, FRG). They form a group of closely to moderately related bacteria with S_{AB} values above 0.66. The whole group is located in the γ-subdivision of Division 9 (Table 2). This subdivision has been further divided into γ-1, γ-2, γ-3 by Woese et al. (101). The Chromatiaceae, together with the Ectothiorhodospiraceae and *Nitrosococcus oceanus* form the γ-1 branch, the γ-2 branch contains the Legionellaceae, the γ-3 branch the Enterobacteriaceae, vibrios, oceanospirilla, pseudomonads, *Leucothrix, Thiomicrospira, Halomonas, Beggiatoa* and others (101). Within the Chromatiaceae *Amoebobacter pendens, A. roseus* (both containing gas vacuoles) and *Thiocapsa roseopersicina* (no gas vacuoles) are very closely related, i.e. by S_{AB} values of 0.93 or higher. A second subgroup of closer relationship is formed by *Chromatium minus, Thiocystis violacea*, and *T. gelatinosa* (S_{AB} about 0.82). Together with *Thiodictyon elegans* (and *Thiocapsa pfennigii*) these two subgroups form the main cluster within the family. *Chromatium vinosum* and *C. warmingii* are only moderately related to each other ($S_{AB} = 0.76$) and *Thiospirillum jenense* and *Chromatium weissei* are only distantly related to any of the other species (S_{AB} of about 0.68 and 0.65, respectively). The genus *Chromatium* is obviously not at all coherent (16).

These results already suggest a number of possible changes if one assumed an S_{AB} value of 0.8 to differentiate between genera and species in this family. Then 1) the type species of *Chromatium, C. okenii* [of which most probably *C. weissei* is just a variety in size (92)] would have to retain the genus name; 2) *C. minus* would become *Thiocystis minus*; 3) *C. vinosum* and *C. warmingii* would each have to receive new genus names; 4) the genera *Thiocapsa* and *Amoebobacter* would have to be united, probably under the common name *Thiocapsa* (i.e. gas vacuoles become taxonomically unimportant properties); 5) all other species studied would retain their generic affiliation.

It appears questionable whether such rigorous changes are desirable now. As all of the species listed in Table 6 are in pure culture it should be possible to obtain the 16S-rRNA data of the 12 lacking species in the near future. Then, however, a careful rearrangement of the genera and species should be taken into consideration.

TABLE 6
SPECIES OF THE CHROMATIACEAE
Modified after Trüper and Pfennig (92) - T: Type species

Amoebobacter	*pediformis*	(14)
	pendens	
	roseus (T)	
Chromatium	*buderi*	
	gracile	
	minus	
	minutissimum	
	okenii (T)	
	purpureum	
	tepidum	(52)
	vinosum	
	violascens	
	warmingii	
	weissei	
Lamprobacter	*modestohalophilus* (T)	(28)
Lamprocystis	*roseopersicina* (T)	
Thiocapsa	*roseopersicina* (T)	
Thiocystis	*gelatinosa*	
	violacea (T)	
Thiodictyon	*bacillosum*	
	elegans (T)	
Thiopedia	*rosea* (T)	
Thiospirillum	*jenense* (T)	

The family Ectothiorhodospiraceae

The genus *Ectothiorhodospira* was originally grouped with the Chromatiaceae (63). Based on ecophysiological properties, lipid composition, quinones, cytochrome-c-551 amino-acid sequences and other data, Tindall (Tindall, B. J. 1980. Ph. D. thesis, University of Leicester, UK) suggested and Imhoff (37) proposed a separate family, the Ectothiorhodospiraceae (Table 7). This decision was fully supported by 16S-rRNA data (16, 81).

TABLE 7
SPECIES OF THE ECTOTHIORHODOSPIRACEAE
After Imhoff (37)
T: Type species

Ectothiorhodospira	*abdelmalekii*
	halochloris
	halophila
	mobilis (T)
	shaposhnikovii
	vacuolata

The family comprises anaerobic phototrophic sulfur bacteria of the following typical properties: cells spiral, vibrio or rod shaped, with intracytoplasmic membranes that are continuous with the cytoplasmic membrane and form lamellar stacks; reaction center and antenna bacteriochlorophyll *a* or *b*; carotenoids. motile by means of polar flagella. Sulfide is oxidized to elemental sulfur that is deposited as globules outside the cells and may be further oxidized to sulfate. Dependent on saline and alkaline growth conditions.

A 16S-rRNA study of 5 species (7 strains) showed that the family (genus) forms two subgroups, one containing the extremely halophilic *E. halophila* (bacteriochlorophyll *a*), *E. halochloris, E. abdelmalekii* (both: bacteriochlorophyll *b*), the other the moderately halophilic *E. mobilis* and *E. shaposhnikovii*. The branching between the two subgroups occurs at an S_{AB} of 0.52 (81). DNA-DNA and rRNA-DNA hybridizations by Ivanova et al. (42, 43) gave comparable though not congruent results, especially pointing out high homologies between *E. mobilis, E. shaposhnikovii* and *E. vacuolata*.

In addition to 16S-rRNA data and salinity requirements, the two subgroups of *Ectothiorhodospira* differ further in their flagellar type, i.e., the extreme halophiles possess bipolar sheathed flagella, whereas the moderate halophiles have unipolar flagella. In view of the elevation of *Ectothiorhodospira* to family level a proposal of a second genus for the three extremely halophilic species would be justifyable and senseful. As a name pointing to their natural environment, soda lakes, «*Soda spira*» or «*Alcalispira*» would seem appropriate.

Heliobacterium chlorum and related phototrophic bacteria

The isolation of the strictly anaerobic phototrophic eubacterium *Heliobacterium chlorum* by Gest and Favinger (20) was a sensation, because this organism could not be placed with any of the then existing taxonomic groups. Although its Gram stain reaction is clearly negative, Stackebrandt et al. (83) found that, based on 16S-rRNA analyses, like the Gram stain negative nonphototrophic genera *Sporomusa, Selenomonas* and *Megasphaera*, this new phototrophic eubacterium shows a distinct, although remote relationship to Gram positive eubacteria of the *Clostridium* sudivision of Division 2 (Table 2).

The most striking difference from the families dealt with above is that *Heliobacterium chlorum* contains a new type of bacteriochlorophyll, called *g* (6) and that it is devoid of intra-cytoplasmic membranes as well as of chlorosomes.

Meanwhile two more new phototrophic eubacteria with bacteriochlorophyll *g* have been announced: *Heliospirillum gestii* (J. Ormerod and T. Nesbakken, in press 1987, pers. communication) and *Heliobacillus mobilis* (3). Thus the existence of a whole family, the «Heliobacteriaceae» is being postulated (3) (Table 8). A formal description of such a family is lacking so far.

Bacteriochlorophyll *g* is particularly closely related to chlorophyll *a* which may be of evolutionary significance.

TABLE 8
SPECIES OF THE «HELIOBACTERIACEAE»
T: Type species for references cf. text

Heliobacterium	*chlorum* (T)
Heliospirillum	*gestii* (T)
Heliobacillus	*mobilis* (T)

Aerobic bacteria possessing bacteriochlorophyll

Since 1978, several obviously chemoorganoheterotrophic bacteria have been reported to contain bacteriochlorophyll *a*, although they were incapable of anaerobic phototrophic growth (35, 53, 70, 76). Only one new genus and species, however, has been described emphasizing these properties as physiologically and taxonomically important features: *Erythrobacter longus* (75). *E. longus* and similar bacteria were shown to occur widely distributed in aerobic marine environments (76). Two different clusters of strains were recognized besides *E. longus*, probably representing two more species. Harashima et al. (34) found vesicular intracytoplasmic membranes, reversible photo-oxidation of cytochromes, reversible photo-bleaching of bacteriochlorophyll, light inhibited O_2-uptake by cells of *Erythrobacter* species. Okamura et al. (55) reported the presence of cytochrome c-551. Biosynthesis of bacteriochlorophyll *a* (with phytanyl side chain) and carotenoids was found to be stimulated by O_2 (33). Shiba (74) showed that *Erythrobacter* species strain OCH 114 utilizes light under aerobic conditions: In comparison with dark controls he found 1) increased intracellular ATP levels in the light, 2) strongly increased survival periods in the light in the absence of organic energy sources, 3) enhancement of CO_2 incorporation by light as well as by oxygen. Meanwhile, detailed studies on bacteriochlorophyll-protein complexes (77), aerobic photosynthetic electron transport (55), photophosphorylation and oxidative phosphorylation in intact cells and chromatophores (56) have been reported, so that there remains absolutely no doubt that *Erythrobacter* is an aerobic phototrophic bacterium using photophosphorylation as an additional energy source for an organoheterotrophic type of metabolism. Such organisms, although strictly aerobic, cannot be grouped with the Oxyphotobacteria (Table 1) because they do not evolve oxygen during photosynthesis. Following Table 1 they would have to be placed with the Rhodospirillaceae (Anoxyphotobacteria), but the family description would have to be emended in order to accomodate anoxygenic photosynthesis under aerobic conditions. Fortunately, the 16S-rRNA data of *E. longus* (100) allowed to assign this organism to the subdivision of Division 9 (Table 2). Within this subdivision, however, it branches off rather early and its closest phototrophic relative appears to be *Rhodobacter sphaeroides* (100). *Erythrobacter* could be a phylogenetic transition phase between originally anaerobic photoorganotrophic and chemoorganoheterotrophic bacteria (100).

Sato (70) reported the occurrence of bacteriochlorophyll *a* in the facultatively methylotrophic aerobic bacteria «*Protaminobacter ruber*» and «*Pseudomonas* AM-1» —both neither listed in the Approved Lists of Names (78) (therefore put in quotation marks here). Sato and Shimizu (72) showed that in «*P. ruber*» bacteriochlorophyll *a*

formation is stimulated by light during early growth stages; also the presence of oxygen was needed. Continuous illumination, however, prevented pigment formation. A more detailed study (71) showed that a change from light to darkness in early growth stages stimulated pigment synthesis. Although the cells possessed vesicular intracytoplasmic membrane structures, the specific pigment protein complexes typical for anaerobic phototrophic bacteria (e.g., *Rhodobacter sphaeroides*) were lacking. Recently, however, photophosphorylation in membrane preparations (88) and the occurrence of cytochrome c-554 (87) in «*P. ruber*» was reported.

Nishimura et al. (53) found bacteriochlorophyll formation in the radiation-resistant *Pseudomonas radiora* (41), stated, however, that *P. radiora* is not identical with «*Pseudomonas* AM-1».

Several recent taxonomic studies (including chemotaxonomy as well as numerical taxonomy) on methylotrophic bacteria (5, 30, 31, 36, 45, 94, 95, 96, 103) have brought considerable progress to clarify the complicated taxonomic situation in this group.

It is obviously clear now that the pink-pigmented group of facultatively methylotrophic bacteria (PPFMs) possesses bacteriochlorophyll and carotenoids (96), and that «*Pseudomonas extorquens*», «*Mycoplana rubra*», «*Protaminobacter ruber*», *Pseudomonas rhodos*, «*Pseudomonas rosea*», *Pseudomonas mesophilica*, *Pseudomonas radiora*, «*Thiobacillus rubellus*» besides numerous unnamed strains belong into this group. Urakami and Komagata (96) introduced the genus name *Protomonas* for these bacteria with *P. extorquens* as the type species. They disregarded, however, that Green and Bousfield (30, 31) had already legitimately and validly classified these bacteria in the genus *Methylobacterium*. Thus, the PPFMs by priority belong to the genus *Methylobacterium* with the species *M. organophilum*, *M. rhodium* (formerly *Pseudomonas rhodos*), *Methylobacterium radiotolerans* (formerly *Pseudomonas radiora*), *Methylobacterium* with the species *M. organophilum*, *M. rhodinum* (formerly *Pseudomonas* (formerly «*Pseudomonas extorquens*» and including «*Protaminobacter ruber*»).

The taxonomic view of Green and Bousfield (5, 31) is fully supported by nucleic acid hybridization studies (36, 103) and chemotaxonomic data (45, 96). From the view point of bacteriologists interested in phototrophic bacteria, detailed studies on the importance of photometabolism in the recognized *Methylobacterium* species and other PPFMs remain highly desirable.

Also the phylogenetic position of *Methylobacterium* species, based upon 16S-rRNA data should be studied with respect to their relationship with anaerobic phototrophic bacteria. One could almost predict a position in Division 9, α-subdivision.

TABLE 9
AEROBIC HETEROTROPHIC BACTERIA CONTAINING BACTERIOCHLOROPHYLL
T: Type species
(For references cf. text)

Erythrobacter	*longus* (T)
Methylobacterium	*extorquens*
	mesophilum *
	organophilum (T)
	radiotolerans
	rhodinum

* For reasons of Latin orthography the usually applied epithet mesophilicum has to be corrected to *mesophilum*.

Phototrophic archaebacteria

Although not in the scope of this survey, it should be mentioned that an entirely different type of phototrophy exists in archaebacteria. Utilization of light energy for ATP synthesis occurs in several «facultatively phototrophic» species of the family Halobacteriaceae. These possess specialized areas (patches) in their cytoplasmic membrane that are called «purple membrane». The light-active pigments located there are not chlorophyllls but rhodopsins; bacteriorhodopsin and halorhodopsin function as light-driven ion pumps. A similar pigment, called slow rhodopsin functions as a signal transducer in phototaxis of these bacteria. For review cf. Stoeckenius and Bogomolni (86) and Lanyi (48).

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MINIREVIEW

Relationships between physico-chemical parameters and microbial groups in Manchego and Burgos cheeses studied by principal component analysis

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Summary

Principal component analysis was used to examine the correlations between two sets of variables, one representing physicochemical characteristics (pH, a_w and NaCl, moisture and fat content) of Manchego (36 samples) and Burgos (36 samples) cheeses, and the other representing counts of several microbial groups (mesophiles, psychrotrophs, lactic acid bacteria, coliforms, enterococci, staphylococci and molds and yeasts). Thermonuclease content was also included. In addition to the expected relationships (NaCl content, moisture, a_w, etc.), significant correlations between some compositional characteristics and levels of certain microorganisms were found. These correlations were dependent on the type of cheese. Thermonuclease content was positively related to enterococci and ripening (only in Manchego cheese). In contrast to former observations, no relationships were observed between coliforms and enterococci counts.

Key words: Cheese, principal components analysis, microbial counts.

Resumen

Se ha utilizado el análisis de componentes principales para investigar las posibles relaciones existentes entre dos grupos de variables: uno de ellos constituido por las propiedades físico-químicas (pH, a_w, contenido en ClNa, humedad y grasa) de 72 quesos (36 Manchego y 36 de Burgos) y el otro por los recuentos de los diversos grupos microbianos investigados (mesófilos, psicrotrofos, bacterias acidolácticas, coliformes, enterococos, estafilococos y mohos y levaduras). En el estudio se incluyó también el contenido en termonucleasa. Los resultados obtenidos pusieron de manifiesto que, además de las relaciones esperadas (contenido en NaCl, humedad, a_w, etc.), existían relaciones significativas entre algunos de los parámetros físico-químicos y los niveles de ciertos grupos microbia-

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nos que variaban según el tipo de queso. El contenido en termonucleasa se correlacionaba (positivamente) con los recuentos de enterococos y con el grado de maduración (sólo en queso Manchego). No se observó ningún tipo de relación entre los recuentos de coliformes totales y de enterococos.

Introduction

Manchego and Burgos cheeses are two of the most popular Spanish cheeses (15, 16). Both types are made total or partially from ewe's milk. Manchego is a hard cheese ripened for more than 60 d, whereas Burgos is an unripened soft cheese consumed whithin a short time after manufacture (48 h).

Although a great deal of information on cheese is available and several papers on Manchego and Burgos cheeses have been already published (8, 23, 27), there are very few works attempting to establish relationships between compositional and microbiological characteristics.

In recent years, there is a trend to consider food microbiology in the context of ecology. According to Board (6), the concepts of ecology aid interpretation of data derived from studies in food microbiology.

Principal component analysis is one of the best ways of studying multivariate variation. Application of this technique to research in ecology is useful and relatively frequent (17, 24) but publications using this method in food microbiology have not been found.

Data on composition and microbiological quality (range, mean, etc.) have been previously reported (10). The aim of this work was to examine the correlations between two sets of variables, one representing the physico-chemical characteristics of Manchego and Burgos cheeses, and the other representing counts of several microbial groups.

Material and methods

Cheeses

During a year, 72 samples (36 of Manchego cheese and 36 of Burgos cheese) were obtained at random from retail outlets in the city of León. Sampling was performed according to I.C.M.S.F. (13). All samples were manufactured at the Castilla-León area and their labels indicated «made with pasteurized ewe's milk».

Physicochemical variables

Fat content (7), moisture content and salt content (1), pH value (26) and water activity (20) were determined and used as data.

Microbiological variables

The following counts were performed: mesophiles and psychrotrophs (2), coliforms and yeasts and molds (14), enterococci (in Kanamycin Aesculin Azide broth —Oxoid—

24 h at 37°C, with confirmation of positive tubes on Kanamycin Aesculin Azide agar), lactic acid bacteria (on MRS agar —Oxoid— at 37°C for 3 d) and staphylococci (on Baird Parker medium —Oxoid— 48 h at 35°C).

Thermonuclease content

Thermonuclease extraction was carried out according to the method of Cords and Tatini (9). For quantitative determination, the Toluidine Blue DNA agar of Ibrahim (11) was used.

Statistical analysis

The principal component analysis (19, 21) was used. The analysis was performed on an IBM S/34 computer at the Business School, University of León, with the ACOPRI program (Basic extended) devised by Mallo (19). Principal component analysis is probably the best known of the multivariate models. In essence, it involves the extraction of the eigenvalues and eigenvectors of the matrix of correlation coefficients of the original variables. The resulting eigenvalues and eigenvectors define the components of the total variability described by the original variables as linear functions of these variables with coefficients so chosen that the functions are mathematically independent, or orthogonal, to each other.

Results and discussion

The calculated correlation matrices for Manchego cheese, Burgos cheese and the pooled samples showed that there were considerable interrelations between some of the

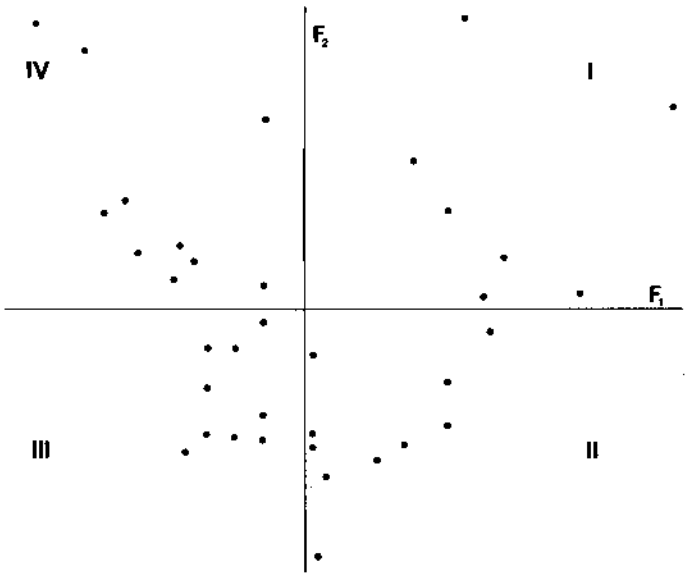

Fig. 1. Distribution of 36 samples of Manchego cheese on the plane represented by the components first (stage of ripening) and second (salting).

variables analyzed, the highest coefficients corresponding to total samples of Burgos cheese. As might be expected, salt content, water activity and moisture were all intercorrelated. Likewise, the latter two variables were negatively correlated with fat content.

For pooled samples, correlation coefficients higher than 0.5 are given in Table 1. Counts of coliforms and psychrotrophs showed a good correlation between them ($r = 0.64$) and with the compositional variables investigated (positive with moisture and a_w, and negative with fat and salt content). This finding suggests that counts of these organisms are more related to the stage of ripening that to other factors (i.e., pH). It must be also noted that both groups are considered a reliable index of post-pasteurization contamination (22). In contrast, significant relationships between coliforms and enterococci counts were not observed.

TABLE 1

CORRELATION COEFFICIENTS (HIGHER THAN 0.5) BETWEEN SEVERAL MICROBIAL GROUPS AND PHYSICOCHEMICAL PARAMETERS OF THE TOTAL SAMPLES STUDIED (MANCHEGO AND BURGOS CHEESES)

	Fat	NaCl	Moisture	a_w	pH
pH	−0.70	−0.51	0.65	0.55	
Fat		0.63			
Coliforms	−0.67	−0.55	0.74	0.65	0.57
Psychrotrophs	−0.60	−0.53	0.74	0.63	0.57

	Coliforms	Psychrotrophs	Molds & Yeasts	Staphylococci
Mesophiles	0.51	0.52	0.51	0.50
Psychrotrophs	0.64			0.50

In Burgos cheese, significant relationships were observed between pH and counts of enterococci ($r = -0.78$) and lactic acid bacteria ($r = -0.62$). A negative correlation was also obtained between a_w and psychrotrophs. Coliforms ($r = 0.67$), staphylococci ($r = 0.53$) and molds and yeasts ($r = 0.52$) numbers were positively correlated with total counts. Finally, significant correlations were found between levels of lactic acid bacteria and counts of staphylococci ($r = 0.53$) and enterococci ($r = 0.57$).

In Manchego cheese, apart from the expected relationships, only two correlation coefficients were higher than 0.5 (enterococci and yeasts and molds counts, and moisture and thermonuclease). The latter variable showed a certain degree of correlation with enterococci counts ($r = 0.33$) and a_w ($r = -0.37$).

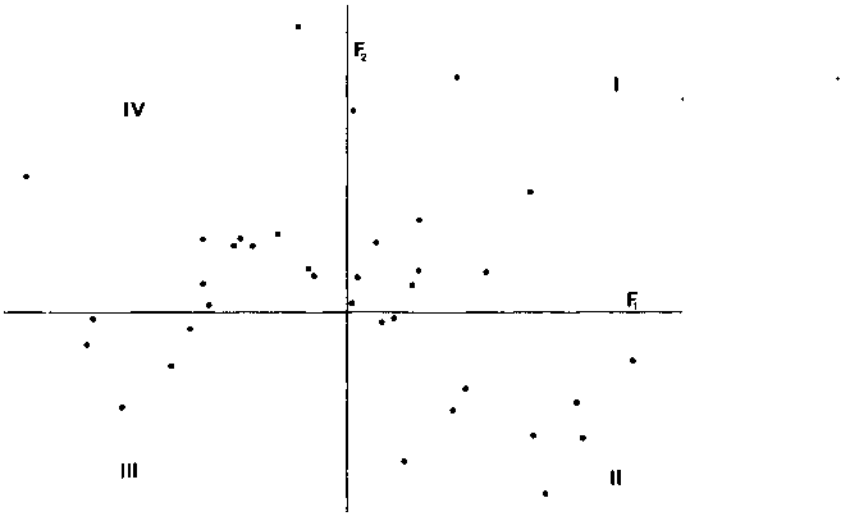

Fig. 2. Distribution of 36 samples of Burgos cheese on the plane represented by the components first (microbiological quality) and second (water activity).

The numbers of factors chosen was the number of eigenvalues larger than 1 (19, 21). The first four components were selected in Manchego cheese, the first five components in Burgos cheese and the first three components in pooled samples. The above components accounted for 66.7 %, 80.9 % and 69.6 % of the total variability, respectively.

The eigenvectors for the first components are shown in Tables 2 (Manchego cheese), 3 (Burgos cheese) and 4 (pooled samples). The first component in Table 2, considered a measure of the «stage of ripening» was positively correlated with fat content and counts of enterococci and molds and yeasts, and negatively correlated with moisture and water activity. The second component, considered as an index of «salting», was related to salt content (positively) and to water activity and counts of mesophiles, psychrotrophs and staphylococci (negatively). The third component, negatively related to coliforms counts, could not be interpreted and the fourth was considered as a measure of the pH. Distribution of Manchego cheeses according to components F_1 and F_2 (Fig. 1), revealed a great variability in the age of cheeses when analyzed. It must be also noted that 9 samples (25 %) showed short ripening period and low salt content (Fig. 1, quadrant III).

TABLE 2

EIGENVECTORS OF THE FIRST FOUR COMPONENTS OF THE MANCHEGO CHEESE VARIABLES

Variables	Components			
	1	2	3	4
pH	1.69	−1.55	11.36	50.77
Fat	48.41	−0.77	−3.84	−2.06
NaCl	11.76	46.10	20.89	−7.83
Moisture	−66.29	−10.44	0.15	−4.59
Thermonuclease	27.77	13.78	−6.15	1.06
a_w	−40.50	−42.65	−10.00	1.06
Coliforms	1.09	3.89	−55.80	−10.46
Enterococci	54.67	−10.88	−0.06	−0.06
Lactic acid bacteria	18.22	−5.23	−2.33	19.58
Mesophiles	12.23	−46.84	−6.39	0.55
Psychrotrophs	0.19	−39.63	13.54	−10.07
Molds & Yeasts	34.02	−18.11	−0.11	−3.62
Staphylococci	12.24	−30.57	18.58	−7.62
Proportion of variability	25.32	20.80	11.48	9.18

From data in Table 3 we can interpret the meaning of the components obtained for Burgos cheese. Taking into account that the first component was positively correlated with the microbial counts and fat content and negatively correlated with pH and water activity, it was considered an index of «microbial quality». The second component measures «water activity». The remaining components are measures of the «thermonuclease» (third and fifth) and «moisture» content (fourth). Perhaps the most important observation from Fig. 2 (distribution of Burgos cheeses according to the first two components) is the great variability in microbiological quality. In addition to heavy contamination and low water activity, samples in quadrant II showed high pH values. Since water activity for all 36 samples was quite similar, it is reasonable to suspect that microbial levels were more dependent on pH.

TABLE 3

EIGENVECTORS OF THE FIRST FIVE COMPONENTS OF THE BURGOS CHEESE VARIABLES

Variables	Components				
	1	2	3	4	5
pH	−31.12	−41.86	4.49	−7.21	3.70
Fat	49.86	−1.84	−11.09	−22.04	−2.31
NaCl	27.75	−51.78	−0.62	12.61	−0.10
Moisture	−21.43	16.70	12.30	41.78	0.43
Thermonuclease	0.65	1.08	−42.62	−0.006	35.75
a_w	−32.21	52.96	0.17	−10.45	0.54
Coliforms	32.17	0.36	26.60	−0.45	22.60
Enterococci	35.80	25.65	−13.91	3.09	−9.63
Lactic acid bacteria	59.60	6.02	−2.10	2.01	0.009
Mesophiles	47.97	2.18	29.35	−0.35	1.91
Psychrotrophs	43.91	−3.40	0.84	9.96	−0.03
Molds & Yeasts	20.99	1.21	23.40	−11.24	−12.69
Staphylococci	46.48	4.87	0.07	−1.36	12.85
Proportion of variability	34.62	16.15	12.90	9.43	7.89

The analysis of Table 4 (pooled samples of Manchego and Burgos cheeses) indicates that the first component, accounting for the 42.1% of the variability, is an index of the «stage of ripening». As shown in the above table, this component was positively correlated with fat and salt contents and negatively correlated with pH, moisture, a_w and levels of coliforms and psychrotrophs. The second component, correlated with microbial groups, was considered as an index of «microbiological quality». The third component was a measure of «thermonuclease» content.

TABLE 4

EIGENVECTORS OF THE FIRST THREE COMPONENTS OF THE TOTAL CHEESES VARIABLES

Variables	Components		
	1	2	3
pH	−53.79	−5.66	0.97
Fat	72.64	9.17	−1.97
NaCl	66.28	0.93	−0.20
Moisture	−85.40	−3.83	0.23
Thermonuclease	1.32	4.45	79.44
a_w	−77.71	−2.97	−0.01
Coliforms	−65.54	0.44	3.68
Enterococci	1.12	66.83	2.93
Lactic acid bacteria	−0.16	38.55	4.60
Mesophiles	−35.38	30.67	−5.20
Psychrotrophs	−68.08	3.78	−0.19
Molds & Yeasts	−7.16	37.34	−16.19
Staphylococci	−13.30	37.24	−0.11
Proportion of variability	42.14	18.61	8.87

Fig. 3 shows the distribution of the 72 samples (Manchego and Burgos cheeses) on the plane represented by the components first and second. This diagram clearly shows the separation between Manchego (ripened) in quadrants I and II, and Burgos (fresh) in quadrants III and IV. The only anomalous position was that of a Manchego cheese sample which had characteristics of Burgos cheese (low salt content and high a_w). Although the numbers of samples showing faulty microbiological quality was higher in Burgos (quadrant IV) than in Manchego cheeses (quadrant I), no important differences could be detected.

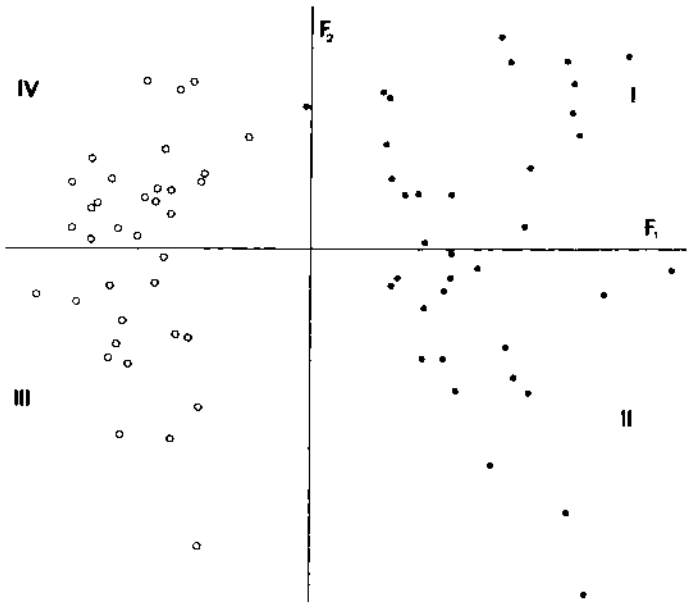

Fig. 3. Distribution of pooled samples on the plane represented by the components first (stage of ripening) and second (microbiological quality). •, Manchego cheese; ○, Burgos cheese.

Analysis of Fig. 4, which shows the distribution of cheeses on the plane of the components first and third, reveals that, in Manchego cheese, thermonuclease content is positively related to ripening. In general, results from other workers demonstrated that important staphylococcal thermonuclease inactivation or no changes occurred during cheese ripening (12, 18). Although data from several studies have also shown a high relationship between staphylococci counts and thermonuclease content in cheese and other dairy products (4, 9, 12), in this work no correlation between these two variables was observed. This fact and the correlation coefficient ($r = 0.33$) between enterococci levels and thermonuclease concentration suggest that perhaps some of the samples contained thermonuclease of non-staphylococcal origin. Production of thermonuclease by enterococci has been reported by several authors (3, 5, 25).

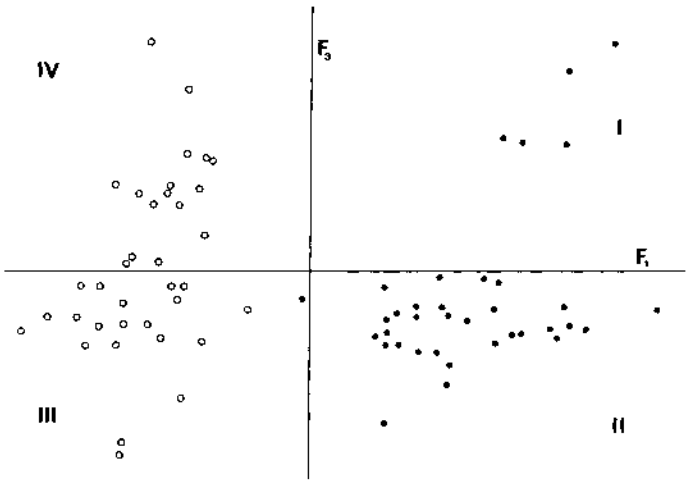

Fig. 4. Distribution of pooled samples on the plane represented by the components first (stage of ripening) and third (thermonuclease). •, Manchego cheese: , Burgos cheese.

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Inhibition by α factor of the glucose-induced activation of regulatory trehalase in *Saccharomyces cerevisiae*

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Summary

The enzyme activity of the regulatory trehalase (α, α-trehalose glycohydrolase; EC 3.2.1.28) in stationary-phase cells of *Saccharomyces cerevisiae* increased upon addition of glucose to cell suspensions. Such increase was temporarily retarded in the presence of α factor in the medium. The transient inhibition required the joined action of the pheromone-like factor and the protease inhibitor N-α-p-tosyl-L-lysine chloromethyl ketone. The inhibition of the glucose-induced activation of trehalase by α factor lends support to the involvement of adenosine 3',5'-cyclic monophosphate in the enzyme activation *in vivo*.

Key words: Trehalase, α factor, Saccharomyces cerevisiae.

Resumen

La actividad enzimática de la trehalasa regulatoria (α, α-trehalosa glicohidrolasa; EC 3.2.1.28) en células de *Saccharomyces cerevisiae* en fase estacionaria resulta incrementada tras la adición de glucosa a la suspensión celular. Tal incremento se retrasa temporalmente en presencia del factor α en el medio. La inhibición transitoria requiere la acción conjunta del factor sexual y del inhibidor de proteasas N-α-p-tosil-L-lisina clorometil cetona. La inhibición mediante el factor α de la activación de la trehalasa inducida por glucosa apoya la participación de adenosina 3',5'-monofosfato cíclico en el mecanismo de activación enzimática *en vivo*.

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Introduction

The enzyme activity of the regulatory trehalase in yeast cells appears to be controlled by an adenosine 3',5'-cyclic monophosphate (cAMP)-dependent phosphorylation of the enzyme protein (1, 2, 12, 16, 18). The addition of glucose to resting, stationary-phase cells produces a rapid rise in the activity of trehalase (15, 19) which is preceded by a transient increase in the level of the intracellular cAMP content (17). This change in the cAMP concentration might trigger the phosphorylation cascade that causes the enzyme activation.

On the other hand, the treatment of *a* mating type yeast cells with the pheromone-like α factor promotes an inhibitory effect on the membrane-bound adenylate cyclase that is considered as the first biochemical event for inducing the mating program in these cells (7, 8, 14). Because glucose and α factor are apparently able to develop opposite signals on the intracellular concentration of cAMP in *a* cells, we investigated the effect of both compounds on the activity of the regulatory trehalase to analyse the *in vivo* mechanism of trehalase activation in yeast cells.

Materials and methods

The standard haploid wild-type strain of *Saccharomyces cerevisiae* X2180-1A (Mat *a*) was a gift of Prof. L. Rodriguez (University of La Laguna, Spain) and was originally obtained from the Yeast Genetics Stock Center, Donner Laboratory, Berkeley, California, USA. Cells were grown in a minimal medium containing 0.5% (w/v) glucose and 0.67% (w/v) yeast nitrogen base without aminoacids (Difco). The cultures were incubated at 30°C in a gyratory shaker at 200 r.p.m.

Log-phase cells were directly treated in growing cultures (OD of 0.2-0.3 at 600 nm) with α factor added to a final concentration of 1.6 μg per ml. Stationary-phase cells were harvested from non-proliferating cultures (OD of 3.8-4.0 at 600 nm), washed twice and resuspended in distilled water at a cell density of 2.0×10^7 cells per ml. N-α-p-tosyl-L-lysine chloromethyl ketone (TLCK) and α factor were added to the stationary cell suspensions at the concentrations indicated in each particular experiment.

Trehalase activation *in vivo* by glucose was performed, in the presence or absence of TLCK and α factor, essentially as described previously (2) except that cycloheximide, NaF and PMSF were omitted. The suspensions containing the stationary-phase cells for enzyme activation were incubated with 1% (w/v) glucose at 30°C in a gyratory shaker at 200 r.p.m. Glucose was always the last component added to the cell suspensions. Samples (usually 4 ml) were removed at different times, the cells collected by centrifugation, washed twice and finally resuspended in a small volume (0.5-1.0 ml) of chilled 0.1 M sodium acetate buffer pH 5.6. Permeabilised cells were obtained as described elsewhere (1). Cell number was determined in a Thoma haemocytometer slide.

The trehalase assays contained 50 μmol trehalose, 50 μmol sodium acetate buffer pH 5.6 and 2-4.0 $\times 10^7$ toluenized cells in a final volume of 0.5 ml. Acetate buffer was used in these assays to avoid the interference of the non-regulatory type of trehalase which is also present in yeast cells (11). The mixtures were maintained at 30°C and the

enzyme reaction stopped by heating in a boiling water bath for 3 min. Glucose was measured in the supernatants of the enzyme assays after low speed centrifugation according to the glucose oxidase-peroxidase method (10). Control assays to correct for potencial autohydrolysis were also carried out in parallel. A unit of trehalase activity released one nmol of glucose per min under the stated conditions.

Synthetic α factor, glucose oxidase (EC 1.1.3.4) type V, horseradish peroxidase (EC 1.11.1.7) and o-dianisidine were from Sigma. TLCK was obtained from Fluka AG, Buchs, Switzerland. All other reagents used were of analytical grade.

Results and discussion

The presence of α factor in early exponential-phase cultures of yeast cells caused a transient arrest of cell division (Fig. 1a) whose temporal length was dependent on the concentration of glucose present in the culture medium and the concentration of added α factor (13). Trehalase activity in the cells treated by α factor remained stabilized during the arrest period and showed values close to the enzyme activity of the control growing cells (Fig. 1b). The addition of glucose to exponentially growing cells, either treated or untreated by α factor, did not substantially change the level of trehalase activity (not shown).

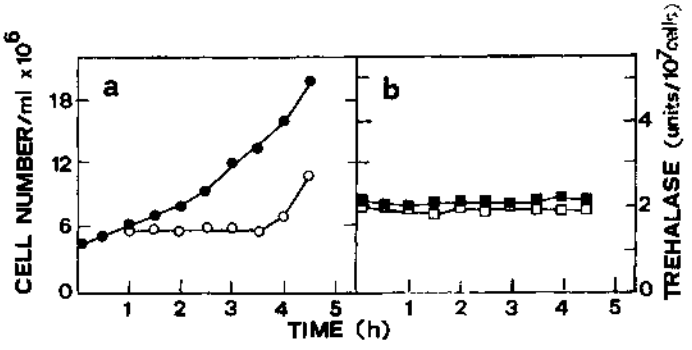

Fig. 1. Effect of α factor on the cell growth (A) and trehalase activity (B) of exponentially growing Mat a cells. Yeast cells of the a mating type were treated with α factor as described in the text and samples were taken for determination of cell number and trehalase activity. Cell number (●) and trehalase activity (■) of control cultures untreated with α factor; cell number ( ) and trehalase activity (□) of cultures treated with α factor.

In contrast, stationary-phase cells removed from non-proliferating cultures showed a comparatively lower trehalase activity (about 0.4 units per 10^7 cells) and the addition of glucose to these cells maintained under resting conditions resulted in a marked increase in the level of trehalase activity (Fig. 2a). The increase appears to be a postranslational event since it was not inhibited by the presence of inhibitors of protein synthesis such as

cycloheximide. The above result confirmed previous observations obtained with this and other yeast species (1, 15, 17, 19). This glucose-induced trehalase activation was subsequently lost and the enzyme activity returned to base values unless a nitrogen source was added to the cells (15).

The inclusion of α factor in the assays for glucose-induced trehalase activation with resting cells did not prevent the enzyme activation (Fig. 2a). These cells responded poorly to α factor, as indicated by the fact that practically none of the cells exhibited the aberrant, pear-like («shmoo») morphology that is characteristically induced by action of the pheromone.

The lack of response of *a* cells to α factor, as well as the recovery of the cells from the treatment by this factor, probably depends on the inactivation of the pheromone, which seems to be due its proteolytic digestion to biologically inactive fragments (4-6). Derepressed, stationary-phase cells show in fact an increased capacity to inactivate the pheromone, presumably by action of surface-bound endopeptidases (5, 9, 13).

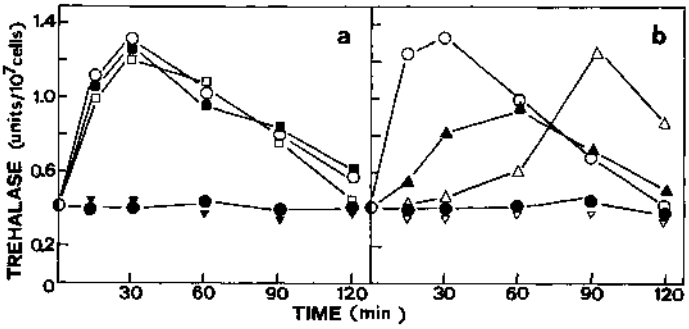

Fig. 2. Influence of addition of glucose, α factor and TLCK on the activation of trehalase in stationary-phase *a* cells. A stationary culture was divided into several aliquots and the cells treated as indicated in the text with the additions shown. Panel A: ●, control cells with no additions; ○, 1% glucose added; ■, 1% glucose and α factor (3.3 $\mu g.mL^{-1}$) added; □, 1% glucose and 1mM TLCK added; ▼, α factor (3.3 $\mu g.mL^{-1}$) added. Panel B: ●, control cells with no addition; ○, 1% glucose added; ▲, 1% glucose, α factor (1.6 $\mu g.mL^{-1}$) and 1 mM TLCK added; △, 1% glucose, α factor (3.3 $\mu g.mL^{-1}$) and 1 mM TLCK added; ▽, 1 mM TLCK added.

The protease inhibitor TLCK has been reported to inhibit the enzyme(s) involved in the proteolysis of α factor and thus to potentiate the effect of the pheromone (5, 13). We therefore checked the effect of α factor on the glucose-induced trehalase activation of stationary-phase cells under the presence or absence of TLCK assuming a different rate in the degradation of the pheromone by the cells. According to such expectation, when both TLCK and α factor were present in the cell suspensions the activation of the trehalase by addition of glucose was significantly delayed, the greatest activity being reached at a later time whose length was somehow dependent on the concentration of added α factor (Fig. 2b). Moreover, the retarded increase in enzyme activity was temporarily related to the inactivation of α factor because, at the point of maximum activity, the supernatants of the cell suspensions were no longer able to arrest the division

of exponentially growing cells from control cultures in reconstituted medium. Neither the pheromone nor the TLCK, acting separately from each other, were able to transiently inhibit the activation of trehalase by glucose and the delayed increase in the level of enzyme activity always required the copresence of the mating factor and the protease inhibitor (Fig. 2a, b). Under these conditions, morphological changes elicited by α factor were detected in the cells and after two hours of treatment by α factor and TLCK at least 30% of the cells exhibited the abnormal, shmoo morphology.

The inhibition by α factor of the trehalase activation by glucose in stationary-phase cells does not merely imply a limited supply of the sugar to intracellular targets since treated and untreated cells show a similar uptake of radiolabelled D-glucose (9). Rather, the results presented here may be interpreted as revealing an opposite, competitive effect between glucose and active α factor on the levels of a common effector involved in the enzyme activation. In spite of some controversial results (3), it has been shown that α factor decreases the intracellular concentration of cAMP (14) and that glucose increases the content of such metabolite in resting cells (17, 19). Consequently, the above observations may well be considered as an additional, indirect evidence for the *in vivo* occurrence of a cAMP-dependent phosphorylation during the activation of the regulatory trehalase in yeast cells (12, 18).

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O_2-Dependent nitrogenase switch-off in *Rhodobacter capsulatus* E1F1

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Summary

Nitrogenase of *Rhodobacter capsulatus* E1F1 (formerly known as *Rhodopseudomonas capsulata* E1F1) was partially resistant to O_2 inactivation *in vivo*. This inactivation was reversed by restoring anaerobic conditions, was independent from *de novo* protein synthesis and its extent was decreased upon preincubation of the cells with dioxygen at low pressures and also in the presence of H_2. Illuminated cells exhibited a low rate of O_2 uptake which was enhanced in the presence of H_2, particularly in cells preincubated with O_2. These results indicate that *R. capsulatus* E1F1 can develop for nitrogenase a protective system against dioxygen which, at least, consists of an uptake hydrogenase and an inducible electron transport system linked to a respiratory chain.

Key words: Nitrogenase, switch-off, dioxygen, hydrogenase, phototrophic bacteria.

Resumen

La nitrogenasa de *Rhodobacter capsulatus* E1F1 (antes *Rhodopseudomonas capsulata* E1F1) era parcialmente resistente *in vivo* a la inactivación por O_2. Esta inactivación era reversible e independiente de la síntesis *de novo* de proteínas y su grado era menor si las células se preincubaban en presencia de O_2 a baja presión o se situaban en presencia de H_2, sobre todo si habían sido preincubadas con O_2. Estos resultados indican que *R. capsulatus* E1F1 puede desarrollar para su nitrogenasa un sistema protector frente al oxígeno que consiste, como mínimo, en una actividad hidrogenasa acoplada a una cadena de transporte electrónico con dioxígeno como aceptor final.

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Introduction

In phototrophic bacteria, as well as in other non-photosynthetic nitrogen fixers, nitrogenase activity is subjected to a reversible metabolic interconversion triggered by environmental factors (ammonia, light-dark transitions, O_2) or uncouplers and redox agents (6, 10, 12).

Dioxygen inhibits nitrogen fixation at the levels of nitrogenase synthesis and activity, activity being inhibited through reversible and/or irreversible inactivation mechanisms (9). The reversible inactivation has been named switch-off/switch-on because the nitrogenase activity can be completely restored *in vivo*, either lowering or eliminating the O_2 pressure (4). By contrast, irreversible inactivation of the enzyme is due to an oxidative damage of the FeS clusters of the nitrogenase components, particularly those included in the subunits of Fe-protein, which present high reactivity for O_2 radical derivatives (1).

In this paper we show that, like other *Rhodospirillaceae* (4), the nitrogenase activity of *R. capsulatus* E1F1 is partially resistant to the *in vivo* inactivation by O_2. Besides, we present evidence that H_2 prevents this reversible inactivation process, probably by a rapid lowering of O_2 concentration through an inducible system catalyzing the Knallgas reaction:

$$H_2 + {}^1/_2\, O_2 \rightarrow H_2O$$

Material and Methods

Organism and growth conditions

Rhodobacter capsulatus E1F1 (a gift from Prof. Dr. W. G. Zumft, University of Karlsruhe, West Germany) was cultured anaerobically at 30°C under continuous illumination with tungsten lamps (4 W/m^2), in the RCV medium (11). L-glutamate (1 g/l) was used as source of nitrogen. The cells were harvested at the mid-logarithmic phase of growth, washed with 50 mM Tris-HCl (pH 7.5) and resuspended in the same culture medium without carbon and nitrogen sources.

Nitrogenase assay.

Nitrogenase activity was assayed gas chromatographically using a PORAPAK column. Washed cells, resuspended in culture media lacking carbon and nitrogen sources, were placed in 15 ml flasks and then supplied with 20 mM sodium pyruvate. Anaerobiosis was achieved by closing up the vessels with rubber serum stoppers which adjust hermetically and degassing ten times the flasks with highly purified argon. The reaction was started by injecting acetylene up to a partial pressure of 10%, and then the vessels were placed in an illuminated Warburg bath with continuous shaking. The ethylene formed was measured at the indicated times in 0.1 ml aliquots which were injected in the gas chromatograph with air-tight syringes.

One unit of enzyme activity is defined as the amount of enzyme which catalyzes the reduction of 1 μmol acetylene per minute.

O_2-uptake activity

Uptake of O_2 was determined in a Clark electrode with an illuminated chamber coupled to an XY recorder. When necessary, H_2 was injected into the electrode chamber which contains 1 ml cell suspension prepared under anaerobic conditions.

Analytical determinations

Protein was estimated according to the Lowry procedure (7), using bovine serum albumin as standard.

Results and Discussion

In vivo, nitrogenase was partially resistant to O_2 inactivation. Total inactivation was attained only at O_2 pressures corresponding to 0.17 mM of O_2 dissolved at 25°C (Fig. 1). Values of dissolved O_2 in cultures of some aerotolerant diazotrophs ranging from 1 μM *(Rhizobia)* to 8 μM *(Azospirillum)* had been previously reported (9), whereas in *R. rubrum, R. capsulata* B10 and *C. vinosum* (4) nitrogenase activity was only 50% inhibited by 0.73, 0.32 and 0.26 μM of dissolved O_2, respectively.

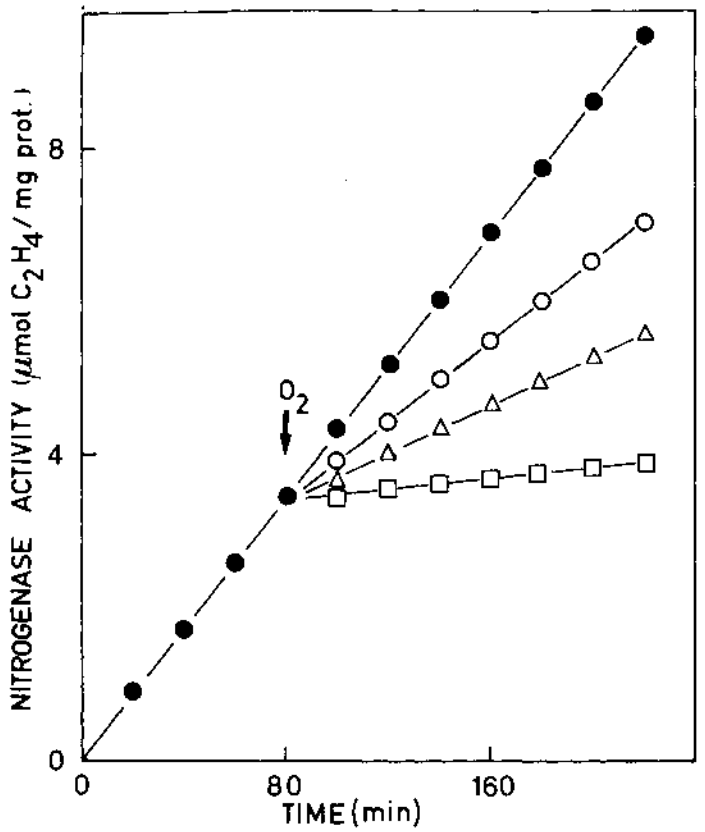

Fig. 1. O_2-dependent nitrogenase switch-off in *R. capsulatus* E1F1. Nitrogenase activity was measured in anaerobic vessels which, at the time indicated by the arrow, were injected with 2 ml Ar (•), 0.5 ml O_2 (○), 1 ml O_2 (△) and 2 ml O_2 (□).

Unlike *R. rubrum* and *C. vinosum* (4) the inhibition of nitrogenase activity was completely suppressed in *R. capsulatus* E1F1 when anaerobic conditions were reestablished (Fig. 2). This recovery was independent from *de novo* protein synthesis (Fig. 2).

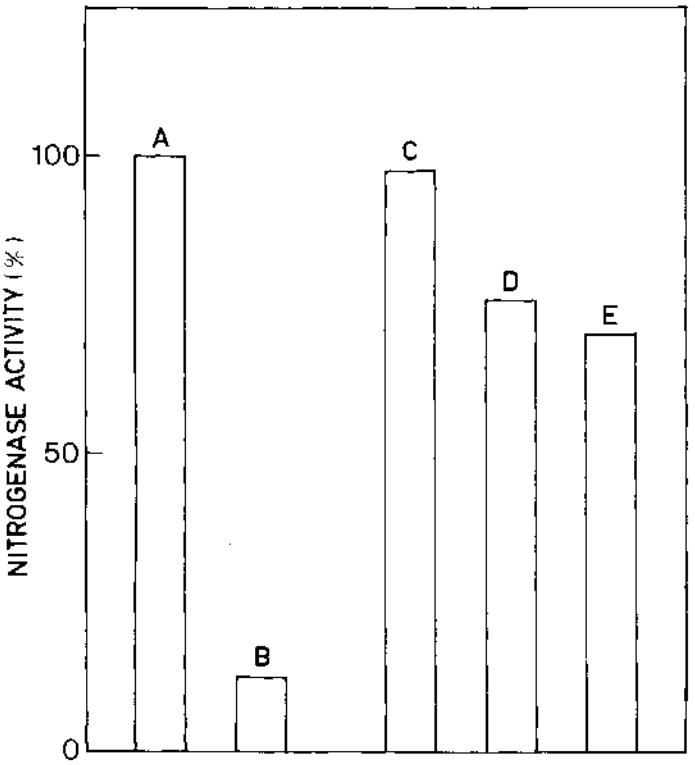

Fig. 2. Effect of rifampicin on nitrogenase switch-off in *R. capsulatus* E1F1. Cells grown phototrophically with glutamate were incubated for 90 min in the absence (A) or in the presence (B) of 82 μmol O_2, and nitrogenase activity was followed as described in Material and Methods. After anaerobic conditions were reestablished by degassing the vessels and refilling them with Ar, nitrogenase was measured in the absence (C) or in the presence (E) of 50 μg rifampicin. (D): control of inhibition of nitrogenase by rifampicin in O_2 non-treated cells. 100% activity corresponded to 50 mU/mg protein.

Damage of nitrogenase by O_2 was significantly prevented when H_2 was injected previously into the reaction vessel (Fig. 3), particularly if the cells were preincubated with O_2 (Fig. 3B). This effect can be explained by assuming the occurrence of a Knallgas reaction which lowered significantly the O_2 pressure. In fact, *R. capsulatus* possesses a membrane-bound uptake hydrogenase which is responsible for driving reducing equivalents through the respiratory chain to O_2 with the concomitant phosphorylation of ADP (8). As proposed in cells depleted of organic substrates (5), this uptake hydrogenase would act by recycling the H_2 evolved during nitrogen fixation reaction. It has been proposed that O_2 switch-off in phototrophic bacteria can be explained in terms of a

competition for electrons between nitrogenase and the respiratory chain (4), although recently the reversible inhibition of nitrogenase by O_2 has been ascribed to the same regulatory mechanism which operates in ammonia or dark shocks (6, 10).

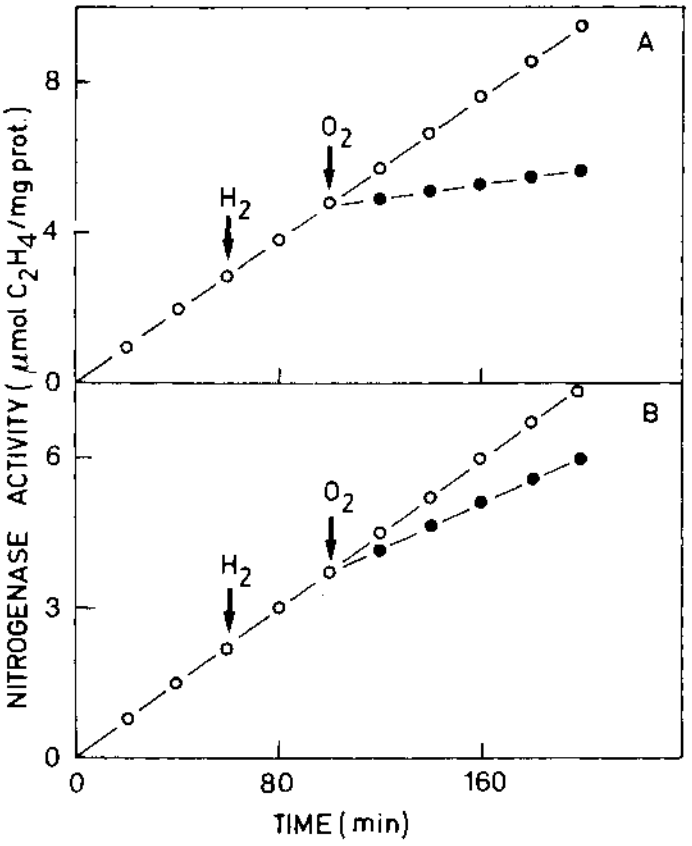

Fig. 3. Effect of O_2 preincubation on nitrogenase switch-off in *R. capsulatus* E1F1. Cells kept under anaerobic conditions (A) or preincubated with 82 μmol O_2 (B) were assayed for nitrogenase activity. Once anaerobic conditions were reestablished (zero time), the reaction was started by injecting acetylene, as described in Material and Methods. Nitrogenase activity was measured in the presence of 82 μmol of H_2 (injected at the time indicated by the first arrow) and either in the absence ( ) or in the presence (•) of 82 μmol O_2 (injected at the time indicated by the second arrow).

The O_2-dependent switch-off of nitrogenase was partially prevented in cells previously incubated under low O_2 pressure (up to 16%). After 90 min preincubation, nitrogenase became significantly insensitive to O_2 inactivation *in vivo* (Table 1). This protecting effect depended on the O_2 concentration during preincubation. In cells preincubated with O_2 (up to 16% pressure) for 90 minutes and allowed to be reactivated, nitrogenase was only 50% inhibited when the reactivated cells were reexposed the same O_2 concentration (Table 1). This indicates that the protective system has been induced

and/or activated in the presence of O_2. The results shown in Table 2 corroborate this conclusion. In the presence of H_2, the O_2-dependent nitrogenase switch-off was partially prevented (Fig. 2A) and this protecting effect was enhanced in cells preincubated with O_2 (Fig. 3B; Table 2), particularly at high O_2 pressures. On the other hand, in the light and in the presence of H_2, *R. capsulatus* E1F1 exhibited rates of O_2 uptake (24 μmol O_2/min mg protein) which were considerably higher in cells previously exposed to O_2 (110 μmol O_2/min mg). Therefore, we conclude that the effect of H_2 on the reversible inhibition of nitrogenase by O_2 can be attributed to a dioxygen consuming system, which minimally consists of an uptake hydrogenase and an electron transport system linked to a respiratory chain. The latter, under our conditions, was induced, at least partially, by preincubation of the cells under O_2. These results are not in agreement with the previously reported repression of hydrogenase by O_2 and organic compounds in *R. capsulata* B10 (3).

TABLE 1

EFFECT OF O_2 PREINCUBATION ON NITROGENASE SWITCH-OFF IN *R. CAPSULATUS* E1F1

O_2 added (ml)	Nitrogenase (mU/mg)		% Inhibition
	$-O_2$	$+O_2$	
0	44	3	93
0.5	37	5	85
1.0	28	8	72
2.0	22	11	50

Cells grown phototrophically with glutamate as nitrogen source were placed in 4 anaerobic vessels each containing 3 ml cell suspension and 12 ml gas phase. Dioxygen was injected at the indicated amounts into the vessels which, after 90 min incubation, were degassed to eliminate excess O_2. The cells were maintained under an inert atmosphere and illumination for 90 min, after which nitrogenase activity was totally or partially restored ($-O_2$). At the end of this reactivation treatment, 2 ml O_2 were injected into each vessel ($+O_2$), and then nitrogenase activity was monitored gas chromatographically. Presented results are the means of three independent experiments.

From an evolutionary point of view, photoproduction of H_2 linked to nitrogen fixation could be a protective mechanism to prevent both switch-off and/or irreversible damage of nitrogenase by dioxygen (2). In fact, even in the absence of exogenous H_2, nitrogenase activity was less sensitive to O_2 switch-off in *R. capsulatus* E1F1 cells preincubated with O_2 probably because of a rapid elimination of dioxygen through the Knallgas reaction with the H_2 photoproduced in the nitrogen fixation reaction (Table 2).

TABLE 2

EFFECT OF DIHYDROGEN AND O_2 PREINCUBATION ON NITROGENASE SWITCH-OFF IN *R. CAPSULATUS* E1F1

O_2 added (ml)	Nitrogenase activity (%)			
	Without preincubation		Preincubated with 2 ml O_2	
	+ Ar	+ H_2	+ Ar	+ H_2
0	100	100	100	100
0.5	53	76	88	93
1.0	30	48	72	87
2.0	8	25	50	67

Cells grown on glutamate as nitrogen source were placed in 16 vessels made anaerobic and containing each 3 ml cell suspension. Eight vessels were kept anaerobic under argon and to the others 2 ml O_2 were injected. After 90 min preincubation O_2 was eliminated by degassing the vessels ten times and refilling with argon. After 90 min under inert atmosphere and illumination, 2 ml H_2 or 2 ml Ar and the amounts of O_2 indicated in the Table were injected into the corresponding vessels. Nitrogenase activity was followed for 90 min as indicated in Material and Methods. One hundred percent activity corresponded to 63 mU/mg. Presented results are means of three independent experiments.

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Evolución de algunas actividades enzimáticas de *Aureobasidium pullulans* durante la transición de levadura a micelio inducida por etanol

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Summary

Some key enzyme activities from the energy metabolism of *A. pullulans* have been studied during the ethanol-induced yeast-to-mycelium transition. Both the mycelial and yeast-like forms showed greater glucose-6-phosphate dehydrogenase activity than phosphofructokinase. During the morphological transition, the most outstanding variations occurred in large cells (3 days), especially for citrate synthase, malate dehydrogenase and isocitrate lyase activities. However, similar variations were detected in cultures without glucose or ethanol, which showed no morphological transition. Therefore, the observed changes in the enzymatic activities may be attributed to the absence of glucose. As this is not sufficient to induce the morphological transition, we conclude that there is no evidence of a clear-cut relationship between morphology and the activity of the enzymes studied.

Key words: Aureobasidium pullulans, *dimorfism, ethanol, energetic metabolism.*

Resumen

Se ha estudiado la actividad de algunas enzimas características del metabolismo energético de *A. pullulans*, a lo largo de la transición de levadura a micelio inducida por etanol. Tanto en la fase micelial como en la levaduriforme la glucosa-6-fosfato deshidrogenasa mostró mayor actividad que la fosfofructoquinasa. Durante la transición de levadura a micelio, las variaciones más acusadas se observaron en las células gruesas de tres días, que mostraron un considerable aumento de las actividades citrato sintetasa, malato deshidrogenasa e isocitrato liasa, respecto a las levaduras del inóculo. Por otra parte, se observaron variaciones similares en cultivos que no experimentaban transición morfológica, en los que tanto la glucosa como el etanol estaban ausentes. Estos cambios en las

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actividades enzimáticas estudiadas podrían atribuirse a la ausencia de glucosa, condición que no es suficiente para inducir la transición morfológica; se concluye por tanto que la morfología de *A. pullulans* no está directamente determinada por la actividad de las rutas metabólicas representadas por las enzimas estudiadas.

Introducción

Aureobasidium pullulans es una levadura negra ampliamente distribuida en la naturaleza, a la que se ha prestado cierta atención en los últimos años por su frecuente implicación en el bio-deterioro de superficies pintadas y otros materiales, así como por su capacidad para producir exopolisacárido, el pululano, de gran interés comercial. Es, además, una levadura dimórfica cuya morfología está controlada por los nutrientes y/o por la densidad de población (3, 5, 18, 22, 24).

Frecuentemente se ha relacionado el diformismo en hongos con la abundancia de fuente de energía en el medio, con la disponibilidad de poder reductor en las células, o con otros factores directa o indirectamente relacionados con el metabolismo energético. En general se puede decir que las condiciones que favorecen el metabolismo fermentativo inducen el desarrollo levaduriforme, mientras que aquellas que favorecen el metabolismo oxidativo promueven el crecimiento en forma miceliar. Por otra parte, es preciso tener en cuenta que una de las diferencias más notables entre las formas miceliar y levaduriforme de los hongos difórmicos reside en las distintas proporciones de quitina, glucano y manano que forman parte de sus paredes celulares respectivas (28). Todo ello sugiere que las actividades enzimáticas responsables de la degradación y síntesis de carbohidratos, así como de la obtención de energía, controlan de alguna manera el diformismo fúngico.

En el presente trabajo se han estudiado las actividades de algunas enzimas del metabolismo energético de *A. pullulans* a lo largo de la transición de levadura a micelio inducida por etanol (25), con el fin de esclarecer la supuesta relación existente entre morfología y metabolismo energético.

Materiales y métodos

Microorganismo y condiciones de cultivo

La cepa de *A. pullulans* utilizada (CECT 2660, ATCC 48433) así como los métodos de mantenimiento y crecimiento, han sido descritos anteriormente (25). El inóculo consistió en 10^5 levaduras/ml, crecidas durante 24 h en medio basal mineral completado con $SO_4(NH_4)_2$ 0,15% (p/v) y glucosa 0,1% (p/v). La inducción de la transición morfológica de levadura a micelio se llevó a cabo en el medio basal mineral completado con $SO_4(NH_4)_2$ 0,15% (p/v), etanol 2,7% (v/v) y Tween 80 0,7% (v/v); el cuarto día de cultivo se añadió glucosa 0,1% (p/v). Las células gruesas se recogieron a los 3 días de cultivo, los tubos germinales a los 5 días y los micelios maduros a los 5,6 días. Los culti-

vos control que no experimentaban transición morfológica se llevaron a cabo en el medio basal mineral con $SO_4(NH_4)_2$ 0,15% (p/v) al que se añadió glucosa 0,1% (p/v) el cuarto día.

Obtención de los extractos acelulares

Las células levaduriformes del inóculo y las células gruesas se recogieron por centrifugación a 1500xg, 10 min a 4°C, lavándose a continuación dos veces con agua destilada a 4°C. Los tubos germinales y micelios maduros se separaron mediante filtración a través de placa de vidrio filtrante y fueron después resuspendidos en agua y centrifugados de la misma manera que las células gruesas y levaduriformes. Las células así obtenidas se liofilizaron y conservaron en desecador a −20°C. Los extractos acelulares se obtuvieron mediante rotura mecánica en homogeneizador de perlas de vidrio; para ello, las células liofilizadas fueron suspendidas en tampón Tris-ClH 0,05M pH 7,1, mezclados con un volumen igual de perlas de vidrio de 0,45-0,50 mm de diámetro y sometidas a rotura en el homogeneizador durante cuatro períodos de 15 segundos, separados por pausas de 30 segundos. Todo el proceso se llevó a cabo bajo refrigeración mediante gas carbónico. El grado de rotura fue comprobado microscópicamente. Las células enteras se retiraron por centrifugación y los extractos se conservaron a 4°C hasta su utilización dentro de las 8 horas siguientes.

Ensayos enzimáticos

Se determinaron las siguientes actividades enzimáticas en los extractos acelulares: fosfofructoquinasa (FFK) según el método de Sols y Salas (27); glucosa-6-fosfato deshidrogenasa (G6FDH) y 6-fosfoglucónico deshidrogenasa (6FGDH) según el método de Kuby y Noltmann (14); citrato sintetasa (CS) según el método de Parvin (19); isocitrato deshidrogenasa (ICDH) según el método de King (12); succinato deshidrogenasa (SDH) según el método de Veeger et al. (29); malato deshidrogenasa (MDH) según Kitto (13); isocitrato liasa (ICL) según Dixon y Kornberg (7) y malato sintetasa (MS) según Simon et al. (26).

Medida de proteínas

Se empleó el método de Lowry et al. (15) utilizando seroalbúmina bovina como patrón.

Medida de etanol

Se llevó a cabo enzimáticamente según el método de Bonnichsen y Theorell (2).

Resultados y discusión

Se estudiaron las enzimas características de las vías de degradación de hexosas, con los resultados que se muestran en la Tabla I. La enzima G6FDH mostró en todos los tipos celulares una actividad sensiblemente mayor que la FFK. Esta observación concuerda con los datos descritos previamente por Clark y Wallace para el mismo hongo (4), que se asemeja por tanto a otras levaduras no fermentativas, aerobias estrictas, que utilizan preferentemente la vía de las pentosas fosfato para la degradación de glucosa (1). La variación que sufrieron estas actividades enzimáticas a lo largo de la transformación morfológica fue la esperada dada la ausencia de glucosa durante los cuatro primeros días. Así, la FFK mostró mayor actividad en las células gruesas de tres días, en las que por la ausencia de glucosa externa los niveles intracelulares de ATP debían de ser bajos. Por el contrario la actividad de la G6FDH experimentó un sensible descenso en dichas células, presumiblemente como consecuencia de la falta de glucosa. La adición de glucosa el cuarto día, sin embargo, no restableció inmediatamente la situación inicial, y tanto en los tubos germinales de 5 días, como en los micelios maduros, la FFK era aún unas tres veces más activa que en las células del inóculo; de esta forma la relación G6PDH/FFK se mantuvo muy baja, a pesar de que la actividad de la G6PDH en los micelios maduros adquiría valores próximos a los encontrados en las levaduras del inóculo.

TABLA I

ACTIVIDAD DE ALGUNAS ENZIMAS CARACTERISTICAS DEL CATABOLISMO DE LA GLUCOSA, EN EXTRACTOS ACELULARES DE LOS DISTINTOS TIPOS MORFOLOGICOS DE *A. PULLULANS*

	Actividad específica (Unidades/mg proteína)[a]			
Tipos morfológicos	FFK (10^3)	G6FDH	6FGDH (10^3)	G6FDH/FFK
Levaduras de inóculo (O d)	21 ± 6	1.37 ± 0.09	31 ± 11	64.8
Células gruesas (3 d)	51 ± 16	0.55 ± 0.04*	81 ± 7*	10.8
Tubos germinales (5 d)	75 ± 23*	0.58 ± 0.04*	42 ± 4	7.9
Micelios (5,6 d)	66 ± 20*	1.01 ± 0.08*	98 ± 6*	15.3

[a] media más desviación standard de tres experimentos independientes.
* diferencias significativas con las levaduras del inóculo, para $p < 0.05$ (test t de Student)

La vía de las pentosas fosfato parece jugar un papel importante en cultivos de hongos que sufren diferenciación; consecuentemente, numerosos investigadores han buscado alteraciones en la actividad de dicha ruta metabólica y su relación con los procesos dimórficos, con resultados diversos (6, 10, 11, 16, 21, 23). Algunos de ellos (10, 16, 21) han observado que no existen variaciones significativas en la actividad de esta vía de utilización de hexosas, o que si existen no se pueden relacionar con el cambio morfológico. En nuestro caso las diferencias se podrían explicar por la ausencia de glucosa y, dado que esta condición no es suficiente para inducir la transición morfológica (25), pensamos que en *A. pullulans* la actuación de una u otra ruta metabólica no es determinante para dicho proceso.

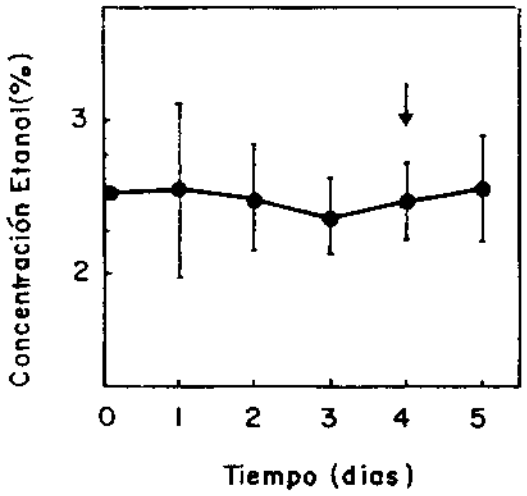

Fig. 1. Variación de la concentración de etanol en medio basal con $SO_4(NH_4)_2$ 0,15% (p/v); etanol 2,7% (v/v) y Tween 80 0,7% (v/v).

La utilización de compuestos de dos átomos de carbono como única fuente de carbono y energía está ligada generalmente al funcionamiento anaplerótico del ciclo del glioxilato. Pese a que en nuestro estudio no se detectó variación en la concentración de etanol (Fig. 1), es posible que hubiera habido consumo suficiente com para modificar la actividad de las enzimas implicadas en su utilización, aunque no para producir cambios apreciables en dicha concentración. En consecuencia, creímos interesante estudiar las enzimas caracteristicas del ciclo del glioxilato y del de los ácidos tricarboxílicos en los distintos tipos morfológicos. En la Tabla 2 se puede apreciar cómo en las células de tres días se produce un aumento de casi todas las actividades enzimáticas determinadas, respecto a las células levaduriformes del inóculo. Tras la adición de glucosa el cuarto día, al menos tres de las enzimas estudiadas —MDH, ICL y CS— mostraron un descenso en su actividad, registrándose valores próximos a los encontrados en las levaduras del inóculo. Estas variaciones pueden ser debidas a i) desrepresión de las enzimas del ciclo de Krebs y del glioxilato por ausencia de glucosa, o ii) inducción de dichas rutas metabólicas por la presencia de etanol y posterior represión debida a la acción de glucosa. En efecto, estos datos son comparables con los obtenidos por Duntze et al. (8) para *Saccharomyces cerevisiae* en medio sin glucosa, por Gosling y Duggan (9) durante la adaptación de la levadura de panadería a la utilización de acetato, y por O'Connell y Paznokas (17) relativos a la utilización de acetato como única fuente de carbono por *Mucor racemosus*. Con el fin de establecer el papel del etanol en las variaciones enzimáticas de *A. pullulans*, detectadas en este estudio, se midió la actividad de las enzimas MDH, CS e ICL, en células levaduriformes de 3, 5 y 5,6 días, procedentes de los cultivos control; la elección de estas tres enzimas se debió al hecho de ser las que más claramente respondían a la presencia de etanol primero y glucosa después. Como puede observarse en la Figura 2, en la que se comparan los datos procedentes del medio control y del medio de in-

TABLA 2

ACTIVIDAD DE ALGUNAS ENZIMAS DE LOS CICLOS DE LOS ACIDOS TRICARBOXILICOS Y DEL GLIOXILATO EN EXTRACTOS ACELULARES DE DISTINTOS TIPOS MORFOLOGICOS DE *A. PULLULANS*

Tipos morfológicos	Actividad específica (Unidades/mg proteína)[a]					
	CS	ICDH (10^3)	SDH	MDH	ICL (10^3)	MS
Levaduras de inóculo (O d)	,55 ± 0,06	55 ± 21	3,6 ± 0,4	9,9 ± 1,3	5 ± 0,1	—
Células gruesas (3 d)	0,75 ± 0,01*	53 ± 5	9,3 ± 1,9*	27,8 ± 5,8*	12 ± 0,3*	—
Tubos germinales (5 d)	0,39 ± 0,00*	129 ± 3*	10,0 ± 1,9*	13,6 ± 3,0	7 ± 0,0*	2,05 ± 0,56
Micelios (5,6 d)	0,49 ± 0,04	39 ± 1,8*	12,5 ± 1,8*	9,0 ± 1,6	5 ± 0,1	0,13 ± 0,10

[a] media más desviación standard de tres experimentos independientes.

* diferencias significativas con las levaduras del inóculo, para $p < 0,05$ (test t de Student).

ducción de la transición morfológica, también en este caso las actividades enzimáticas mencionadas fueron más altas a los tres días de cultivo, encontrándose variaciones incluso más acusadas en ausencia de etanol que en presencia de este compuesto.

Todo ello nos lleva a insistir en la conclusión de que aun cuando existan diferencias en el metabolismo energético de una y otra morfología, ambos hechos no tienen una relación de causa y efecto. Como ya sugeríamos en anteriores trabajos (25), quizá el efecto del etanol se produce a nivel superficial, alterando las características físico-químicas de la membrana plasmática, lo que a su vez puede provocar otras modificaciones que culminan en el cambio morfológico. Recientemente Pollack y Hashimoto (20) han estudiado la inducción por etanol de tubos germinales en *Candida albicans*, concluyendo que en ese caso el efecto del etanol se ejerce a través de su metabolismo. De nuestros datos en *A. pullulans*, sin embargo, se desprende que el etanol no es metabolizado ni ejerce un efecto singular sobre las enzimas estudiadas, por lo que podemos hablar de una inducción inespecífica que, en nuestra opinión, puede estar muy relacionada con la membrana celular, hipótesis que también han apuntado otros investigadores (23, 28).

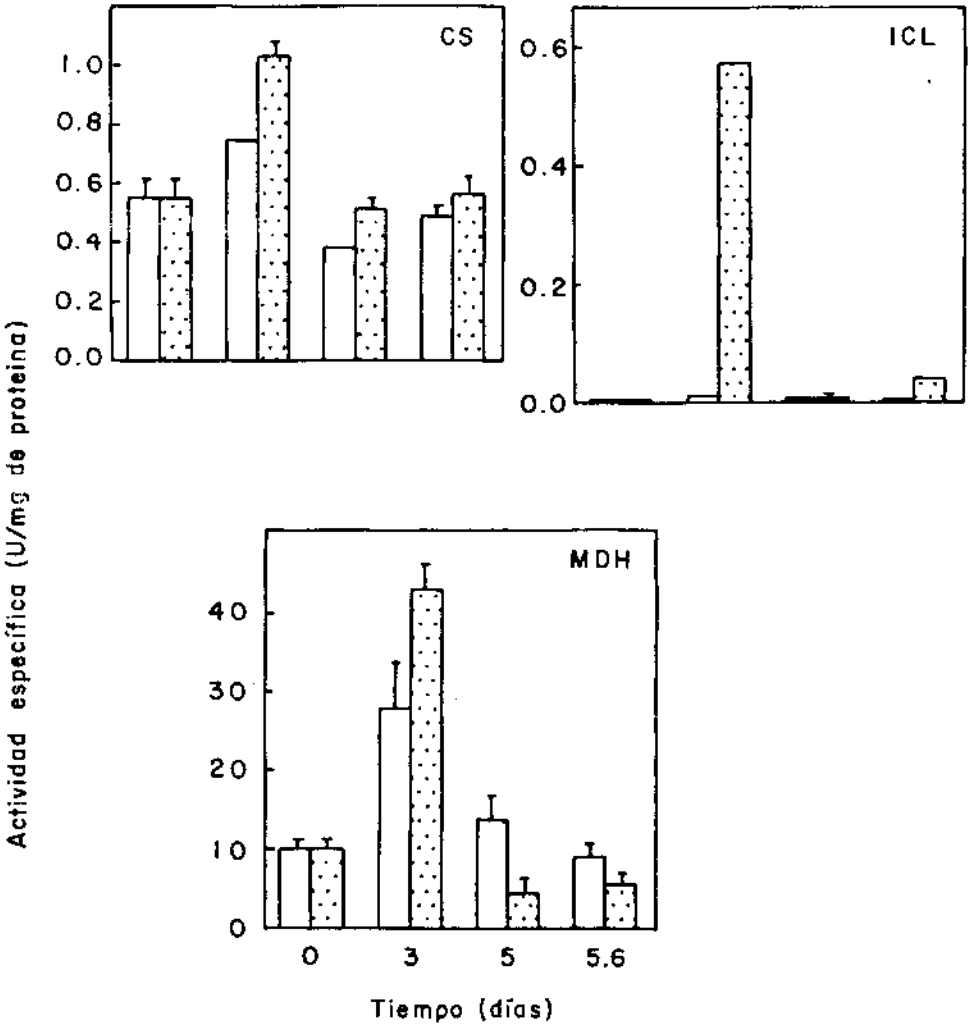

Fig. 2. Actividad especifica de las enzimas citrato sintetasa (CS), malato deshidrogenasa (MDH) e isocitrato liasa (ICL) en medio con etanol ⊡ y en medio control sin etanol □.

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Taxonomía numérica de algunos patovares de *Xanthomonas campestris*

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Summary

Numerical methods were used in order to study the relationships between 26 bacterial strains of 18 pathovars of *Xanthomonas campestris* according to 115 morphological, cultural, physiological and pathogenic characteristics. Similarity for each pair of cultures was calculated by simple matching coefficient. Using single linkage method of association, the strains fall into a major group formed by pathovars *undulosa, translucens, secalis, hordei, pelargonii, hederae, pruni, cucurbitae, zinniae, citri, holcicola, vesicatoria, juglandis, campestris* and *begoniae*, clustered at a similarity level of 76.5%. *Xanthomonas campestris* pv. *gummisudans, X. campestris* pv. *papavericola* and *X. campestris* pv. *ricini* appeared in separate branches at similarity levels of 74, 66, and 62%, respectively.

The results demonstrated that *Xanthomonas campestris* pathovars formed a close group with high similarity of morphological and physiological characteristics, being only distinguished by their host range. In cross-inoculations, the strains showed many relationships but this «pathogenicity» may not of itself constitute a differencial character.

Key words: Numerical taxonomy, phytopathogenic bacteria, Xanthomonas campestris *pathovars.*

Resumen

Se realizó un análisis numérico de 26 cepas pertenecientes a 18 patovares de *Xanthomonas campestris*, considerando 116 características morfológicas, culturales, fisiológicas y patogénicas de las mismas. Los valores de semejanza para cada par posible de cepas se calcularon mediante el coeficiente de apareamiento simple.

A partir del dendrograma obtenido mediante la técnica de acoplamiento sencillo, a un nivel de semejanza del 76,5% se diferencia un gran grupo de cepas formado por los

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patovares: *undulosa, translucens, secalis, hordei, pelargonii, hederae, pruni, cucurbitae, zinniae, citri, holcicola, vesicatoria, juglandis, campestris y begoniae*. De forma aislada, se integran tres cepas: *X. campestris* pv. *gummisudans*, *X. campestris* pv. *papavericola* y *X. campestris* pv. *ricini*, a un nivel de semejanza del 74, 66 y 62%, respectivamente.

Los resultados muestran que los patovares de *X. campestris* analizados forman un grupo muy homogéneo, con gran similitud en cuanto a características morfológicas y fisiológicas, siendo sólo diferenciables por su variación en el rango de hospedadores naturales, mientras que en inoculaciones cruzadas demuestran una mayor interespecificidad, no pudiendo considerarse a esta «patogenicidad» como un carácter diferencial.

Introducción

Todas las especies del género *Xanthomonas* son fitopatógenas y se encuentran asociadas con vegetales superiores. El género se ubica en la familia *Pseudomonadaceae* y agrupa 5 especies: *Xanthomonas campestris, X. albilineans, X. ampelina, X. fragariae* y *X. axonopodis* (1).

Dentro de la especie *Xanthomonas campestris* existen 125 patovares (1) que difieren entre sí por su capacidad patógena, mientras que sus variaciones morfológicas y fisiológicas hacen que su separación por las técnicas bacteriológicas de rutina sea prácticamente imposible (5).

El término patovar fue creado por Young *et al.* (24) para nominar a las bacterias de acuerdo con su patogenicidad distintiva sobre uno o más hospedadores. La ventaja del empleo de dicho epíteto es que no afecta el nombre del taxón superior ni interfiere con el proceso formal de una clasificación binomial.

El presente trabajo, tiene como punto de partida los conceptos establecidos por estos autores (24) y, sobre la base de los mismos, el estudio de los patovares de *X. campestris*, de los cuales se seleccionaron 26 cepas para determinar sus características morfológicas, culturales, fisiológicas y patogénicas sobre hospedadores específicos y sobre otros no específicos por medio de inoculaciones cruzadas.

Con los resultados de estos ensayos, se efectuó un análisis numérico para determinar el grado de semejanza y las relaciones existentes entre los patovares de este grupo.

Materiales y métodos

Cepas bacterianas

Se seleccionaron 26 cepas bacterianas pertenecientes a 18 patovares de *Xanthomonas campestris* que ocasionan enfermedades de importancia económica. Once de las cuales, se aislaron de material naturalmente infectado, en este laboratorio y el resto provino de distintas colecciones internacionales (Tabla 1).

Los aislamientos se efectuaron cortando trozos de material con lesiones típicas de la enfermedad y se colocaron en tubos con 5 ml de agua destilada estéril (un trozo por tubo). Se dejaron difundir durante 30 min, al cabo de los cuales se sembró una asada de la suspensión, previa agitación durante 30 seg, en placas de Petri con agar sacarosa peptona (SPA) (12). Las placas se incubaron a 25-28°C hasta la aparición de crecimiento bacteriano. Todas las colonias individuales aisladas se examinaron considerando su forma, tinción de Gram (9), tinción de flagelos (9), tinción de ácido-alcohol resistencia (9), producción de catalasa (9), tinción de esporas (11), producción de indol (8), producción de amoníaco (5), hidrólisis de gelatina (8), desarrollo en medio D_5 específico para *Xanthomonas* (13), utilización del citrato de Simmons (11), utilización de asparragina como única fuente de carbono, nitrógeno y energía (5), metabolismo oxidativo-fermentativo sobre la glucosa (8) y producción de alcalinidad a partir de ácidos orgánicos (8). Todas estas pruebas son las recomendadas para la identificación del género (1, 5).

TABLA 1

ORIGEN DE LAS CEPAS BACTERIANAS EMPLEADAS EN EL ANALISIS NUMERICO DE 18 PATOVARES DE *XANTHOMONAS CAMPESTRIS*

Xanthomonas campestris pv.	Sigla	Procedencia	Hospedador
begoniae	BE	PDDCC 184	*Begonia* sp.
campestris	CA	Aislamiento de hojas	*Brassica oleracea* var. *capitata* (repollo)
citri	CI (ER)	Aislamiento de hojas	*Citrus paradisi* (pomelo)
citri	CI (CO)	Aislamiento de hojas	*C. paradisi*
citri	CI (CA)	Aislamiento de hojas	*Citrus sinensis* (naranjo)
citri	CI (SP)	Aislamiento de hojas	*C. sinensis*
cucurbitae	CU	NCPPB 2026	*Cucurbita maxima* (calabaza)
gummisudans	GU	PDDCC 5780	*Gladiolus communis* (gladiolo)
hederae	HE	NCPPB 939	*Hedera helix* (hiedra)
holcicola	HOL	PDDCC 3130	*Sorghum bicolor* (sorgo)
hordei	HOR	PDDCC 5735	*Hordeum vulgare* (cebada forrajera)
juglandis	JU (A)	Aislamiento de hojas	*Juglans regia* (nogal europeo)
juglandis	JU (UK)	NCPPB 411	*J. regia*
papavericola	PA	ATCC 14179	*Papaver* sp. (amapola)
pelargonii	PE	PDDCC 4321	*Pelargonium peltatum* (pelargonio)
pruni	PR	Aislamiento de ramas	*Prunus salicina* (ciruelo japonés)
ricini	RI	ATCC 19317	*Ricinus communis* (tártago)
secalis	SE	PDDCC 5749	*Secale cereale* (centeno)
translucens	TR	ATCC 19319	*Hordeum vulgare* (cebada forrajera)
undulosa	U (NZ)	PDDCC 5765	*Triticum turgidum* (trigo)
undulosa	U (A)	Aislamiento de glumas	*Triticum aestivum* (trigo pan)
vesicatoria	VE (J)	Aislamiento de hojas	*Capsicum annuum* (pimiento)
vesicatoria	VE (M)	Aislamiento de hojas	*C. annuum*
vesicatoria	VE (UK)	NCPPB 422 —cultivo tipo—	*Lycopersicon esculentum* (tomate)
zinniae	Z (LP)	Aislamiento de hojas	*Zinnia elegans* (zinnia)
zinniae	Z (NZ)	PDDCC 5762	*Z. elegans*

Pruebas fenotípicas y de patogenicidad

Sobre las 26 cepas obtenidas e identificadas como pertenecientes al género *Xanthomonas* (Tabla 1), se determinaron las siguientes características morfológicas, culturales, fisiológicas y patogénicas: tamaño celular en cultivos de 24 h, incubados a 25°C y sembrados en agar nutritivo (AN). Características de las colonias: forma, color, borde, consistencia y coloración del medio en agar de patata glucosado (APG), agar nutritivo (AN), agar extracto de malta y levadura (AML), agar extracto de levadura con carbonato de calcio (YDC) (5), agar SX (17) y agar avena (15), desarrollo en cilindros de patata y en caldo nutritivo (CN), acción sobre leche descremada y leche descremada más azul de metileno, desarrollo en solución de Cohn (22) y de Fermi (22) e hidrólisis de almidón (8).

Para los tests de fermentación de hidratos de carbono se utilizó el medio C de Dye (5) con púrpura de bromocresol como indicador. Se ensayaron los siguientes carbohidratos: almidón, L (+)-arabinosa, D(+)-celobiosa, dulcitol, D(+)-galactosa, glicerol, D-glucosa, inulina, lactosa, D-levulosa, maltosa, manitol, pectina, rafinosa, D-sacarosa, D-salicina, D-sorbitol, trehalosa y xilosa.

Para determinar la hidrólisis del Tween 80 se empleó el medio de Sierra (18). Las temperaturas letales se comprobaron de acuerdo con la técnica de Stapp (22).

Reacción de hipersensibilidad (RH). Se inyectó una suspensión bacteriana de 1×10^9 cel/ml en los espacios intercelulares de hojas de tabaco *(Nicotiana tabacum* var. *turkish)*, por el método de inyección-infiltración en las nervaduras, de acuerdo con la técnica de Klement (14). El colapso completo del tejido después de 24 h se considera positivo (17).

Inoculaciones cruzadas. Los distintos patovares de *Xanthomonas campestris* analizados en este estudio (Tabla 1), se inocularon por el método de inyección-infiltración sobre hospedadores no específicos pero susceptibles al ataque de otros patovares en forma espontánea en la naturaleza. La concentración de inóculo empleada fue de 1×10^5 células/ml. Se estudiaron los síntomas de la inoculación en los siguientes hospedadores: begonia, repollo, citrus, calabaza, gladiolo, hiedra, sorgo, cebada, nogal, amapola, pelargonio, ciruelo, tártago, centeno, trigo, tomate, pimiento y zinnia.

Infectividad natural. Se estudió la sintomatología sobre los mismos hospedadores utilizados en las inoculaciones cruzadas.

Análisis numérico

Para llevar a cabo el estudio de taxonomía numérica, se utilizaron 115 características diferenciales entre las cepas estudiadas, que se codificaron como 1 y 0, presente y ausente, respectivamente. Los datos fueron analizados mediante el coeficiente de apareamiento simple (S_{SM}) (20) y la técnica de agrupación de acoplamiento sencillo (19). Para este análisis se confeccionó un programa en lenguaje BASIC que fue procesado en un microcomputador Commodore 128, en modo 128.

A partir de los porcentajes de semejanza calculados, se confeccionó una matriz de semejanza y el dendrograma correspondiente. Para medir la distorsión respecto a la matriz original, se construyó una matriz cofenética y se calculó el coeficiente de correlación cofenética (4, 21).

Resultados y discusión

Las 26 cepas analizadas resultaron ser bacilos Gram negativos, móviles por medio de un flagelo polar, no ácido-alcohol resistentes y no esporulados. Eran catalasa positivos y no produjeron indol; produjeron amoníaco, hidrolizaron la gelatina, se desarrollaron en medio D_5, no utilizaron citrato de Simmons, no utilizaron asparragina como unica fuente de carbono, nitrógeno y energia, mostraron un metabolismo oxidativo de la glucosa, produjeron alcalinidad a partir de acetato y fueron halladas en asociación con vegetales superiores. Todas estas caracteristicas corresponden al género *Xanthomonas* (1).

TABLA 2

RESULTADOS DE LAS INOCULACIONES CRUZADAS DE 26 CEPAS DE *XANTHOMONAS CAMPESTRIS* SOBRE 18 HOSPEDADORES POR EL METODO DE INYECCION-INFILTRACION

	Xanthomonas campestris patovar:																		
	begoniae	*campestris*	*citri* (4 cepas)	*cucurbitae*	*gummisudans*	*hederae*	*holcicola*	*hordei*	*juglandis* (2 cepas)	*papavericola*	*pelargonii*	*pruni*	*ricini*	*vesalis*	*translucens*	*andulosa* (2 cepas)	*vesicatoria* (3 cepas)	*zinniae* (2 cepas)	
HOSPEDADOR																			
begonia	+	+	+	+	+	−	−	−	−	−	+	+	−	+	−	−	+	+	
repollo	+	+	+	+	+	−	−	+	+	+	+	+	+	−	+	+	+	+	
citrus	+	+	+	+	+	−	−	−	−	+	−	+	+	−	−	+	+		
calabaza	+	+	+	+	−	−	−	−	+	−	−	+	−	−	+	+	−	+	
gladiolo	+	+	−	+	+	−	−	+	+	+	−	+	−	−	+	+	+	+	
hiedra	−	+	−	+	−	+	−	−	+	−	+	+	−	+	−	−	+	−	
sorgo	−	−	+	+	−	−	+	−	+	−	+	+	+	−	−	+	+	−	
cebada	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	
nogal	+	+	+	−	+	−	−	−	+	−	+	+	+	+	+	−	+	+	
amapola	+	+	−	+	+	−	+	−	−	+	−	−	+	−	−	−	+	+	
pelargonio	+	+	+	+	+	−	−	−	+	−	+	+	+	−	+	−	+	+	
ciruelo	+	+	−	+	−	+	+	−	−	−	+	+	−	−	−	+	+	+	
tártago	+	+	+	+	−	−	+	+	+	−	+	−	+	−	−	−	+	+	
centeno	+	+	−	+	−	−	−	+	−	−	+	+	+	+	+	+	−	−	
trigo	+	+	−	−	−	−	−	+	−	−	+	−	−	+	+	+	−	−	
tomate	+	+	+	+	+	−	−	+	+	−	+	+	+	+	−	+	+	+	
pimiento	+	+	−	−	−	+	−	+	+	+	+	+	+	+	−	+	+	+	
zinnia	+	+	+	+	+	+	−	+	+	−	+	+	+	+	−	−	+	+	

El análisis del dendrograma obtenido mediante el coeficiente de apareamiento simple (S_{SM}) y la técnica de acoplamiento sencillo (Fig. 1) muestra a 23 de las cepas estudiadas agrupadas en un sólo fenón a un nivel de semejanza del 76,5% y, los 3 patovares restantes, aparecen como unidades aisladas: *Xanthomonas campestris* pv. *gummisudans* integrada en un nivel de semejanza del 74%, *X. campestris* pv. *papavericola* en un 66% y, por último, el patovar *ricini* integrado en un 62%.

El coeficiente de correlación cofenética entre la matriz de similitud y la matriz cofenética obtenido fue de 0,9 lo que indica una buena representación de la matriz de semejanza por parte del dendrograma.

Dentro del fenón se distingue un subconjunto, formado por 16 cepas, que se agrupa a un nivel de semejanza del 77,3%. Un primer subgrupo está formado por las cepas aisladas de gramíneas, cuya patogenicidad y características fisiológicas y culturales son muy semejantes. Dicho grupo se consideraba antiguamente como formas especiales de *Xanthomonas translucens* (10), las cuales sólo diferían entre sí por su rango de hospedantes naturales y su infectividad en inoculaciones cruzadas. En el presente trabajo, las diferencias entre los patovares del primer grupo, correspondientes a los aislados de gramíneas y a los patovares *pelargonii* y *hederae* se deben a ligeras variaciones culturales y fisiológicas, rango de hospedadores e inoculaciones cruzadas (Tabla 2).

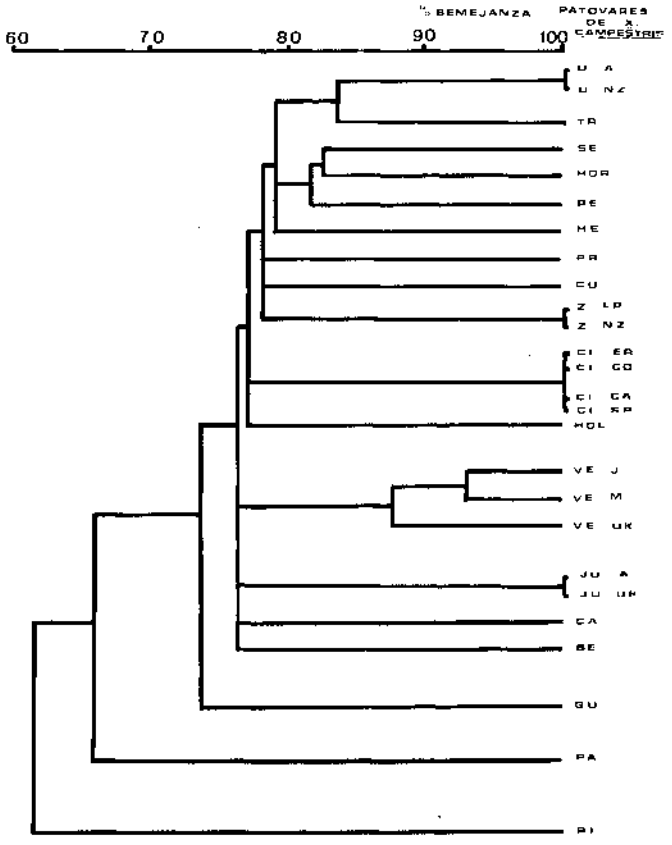

Fig. 1. Dendrograma que muestra el agrupamiento de las 26 cepas pertenecientes a 18 patovares de *Xanthomonas campestris*, obtenido mediante el coeficiente de semejanza S_{SM} y la técnica de agrupación de acoplamiento sencillo.

Los patovares *pruni*, *cucurbitae* y *zinniae*, forman el segundo subgrupo, con un 78% de semejanza; dentro del mismo, las dos cepas de *X. campestris* pv. *zinniae* comparten todas las características analizadas.

Dentro del tercer subgrupo, las cepas de *X. campestris* pv. *citri* aisladas de pomelo y naranjo comparten el 100% de similitud y se relacionan con *X. campestris* pv. *holcicola* en sus características culturales, en la mayoría de las reacciones de fermentación de azúcares y en que no producen reacción de hipersensibilidad en tabaco.

En el segundo subconjunto, formado por 7 cepas, las cepas de *X. campestris* pv. *vesicatoria* forman un pequeño grupo con valor de semejanza del 89%, los cultivos aislados de pimiento difieren de los de tomate en su capacidad de hidrolizar el almidón y presentar leves diferencias culturales. La distinta capacidad para hidrolizar el almidón ya había sido observada por Burkholder y Li (2) en cultivos de *X. campestris* pv. *vesicatoria*, sólo las cepas aisladas de tomate daban reaccion positiva, mientras que las aisladas de pimiento no eran capaces de hidrolizar el almidón. Por su parte, Yano *et al.* (23) hallaron que las cepas de *X. campestris* pv. *vesicatoria* aisladas de tomate y pimiento formaban un grupo serológico muy homogéneo.

En el segundo subgrupo, los dos cultivos del patovar *juglandis* se comportaron de la misma forma frente a todas las pruebas y los patovares *campestris* y *begoniae* presentaron el mayor grado de infectividad en las inoculaciones cruzadas (Tabla 3).

X. campestris pv. *gummisudans* se diferencia del grupo principal en las pruebas de inoculaciones cruzadas y en que no produce reacción de hipersensibilidad en tabaco ni hidroliza el almidón. Elrod y Braun (6), al estudiar serologicamente a 36 especies de *Xanthomonas* hallaron que *X. campestris* pv. *gummisudans* no se relacionaba serologicamente con ninguna. Los resultados aquí expuestos también relacionan a *X. campestris* pv. *gummisudans* a un nivel mas bajo que el gran grupo principal.

Otros estudios serológicos (7) relacionaron estrechamente a las cepas aisladas de gramíneas, con excepción de *X. campestris* pv. *holcicola*; Colwell *et al.* (3) analizaron numéricamente un grupo de *Xanthomonas*, basándose en reacciones con bacteriofagos y obtuvieron también un grupo muy homogéneo de cepas de gramíneas. En este último estudio, el grupo *translucens* se integró en un 79% de semejanza, alejado de *X. campestris* pv. *holcicola* pero relacionado con los patovares *pelargonii* y *hederae*.

Xanthomonas campestris pv. *papavericola* y *X. campestris* pv. *ricini* se hallan completamente aisladas del resto, a niveles de semejanza del 66 y 62%, respectivamente. La primera difiere en sus características culturales, por dar reacciones negativas en los tests de fermentación de azúcares y en las pruebas de inoculaciones cruzadas (Tablas 2 y 3). El patovar *ricini* se integra al resto en un porcentaje tan bajo porque se trata de una cepa albina y cromógena correspondiente a la «raza atípica» descrita por Sabet (16), razón por la cual, las diferencias culturales son notorias (Tabla 3).

Habida cuenta de los resultados expuestos, puede concluirse que los patovares de *X. campestris* analizados forman un grupo muy homogéneo, con gran similitud en cuanto a características morfológicas, culturales y fisiológicas, siendo sólo diferenciables entre si por su variación en el rango de hospedadores naturales, mientras que en inoculaciones cruzadas demostraron una mayor interrelación, no pudiendo considerarse a esta «patogenicidad» como un carácter diferencial. Por otra parte, la especificidad patógena también es cuestionable debido a que virulencia y patogenicidad no son necesariamente características estables.

TABLA 3

CARACTERES FENOTIPICOS DIFERENCIALES DE LAS 26 CEPAS PERTENECIENTES A 18 PATOVARES DE *XANTHOMONAS CAMPESTRIS*

Caracteristicas	Grupo general (23 cepas)	*X. campestris* pv. *gummisudans*	*X. campestris* pv. *papavericola*	*X. campestris* pv. *ricini*
Color de colonias en APG:				
amarillo claro	91[a]	+[b]	–	–
amarillo oscuro	9	–	–	–
blanco	0	–	+	+
Color de colonias en AN:				
amarillo claro	100	+	+	–
amarillo oscuro	0	–	–	+
blanco	0	–	+	+
Color de colonias en YDC:				
amarillo claro	100	+	+	–
amarillo oscuro	0	–	–	+
blanco	0	–	–	+
Produccion de pigmento difusible	0	–	–	+
Desarrollo abundante en cilindros de patata	100	+	+	+
Reaccion de hipersensibilidad en tabaco	70	+	+	–
Reduccion de la leche descremada al azul de metileno	100	+	+	+
Hidrolisis del almidon	61	+	+	–
Produccion de acidos de:				
D-salicina	0	–	–	+
D sacarosa	0	–	+	+
Temperatura letal mayor de 50°C	80	+	+	+

[a] Los numeros corresponden al porcentaje de cepas que dan positiva la prueba correspondiente.
[b] +, reaccion positiva; –, reaccion negativa.

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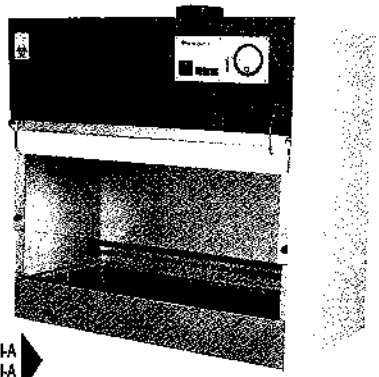

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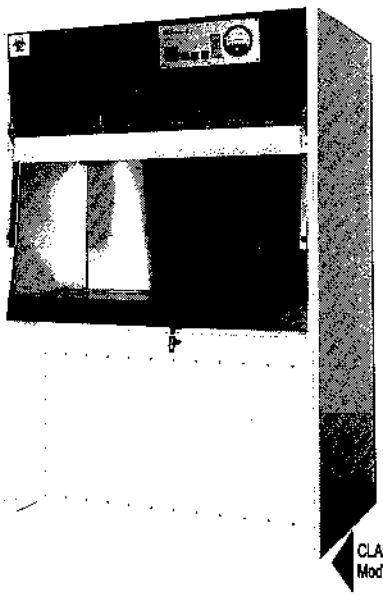

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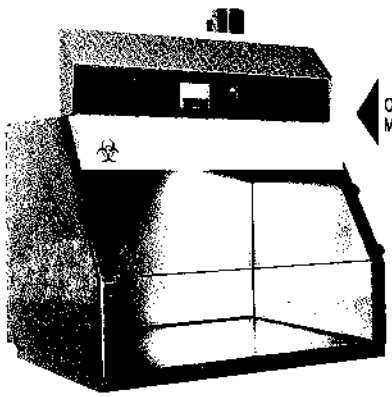

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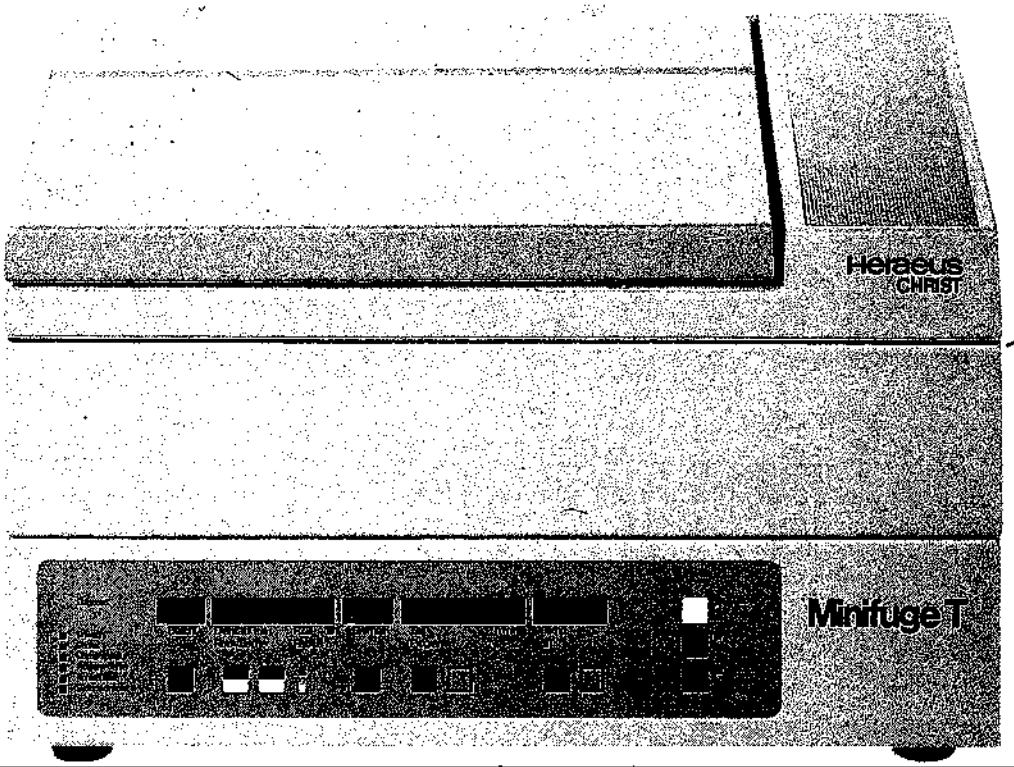

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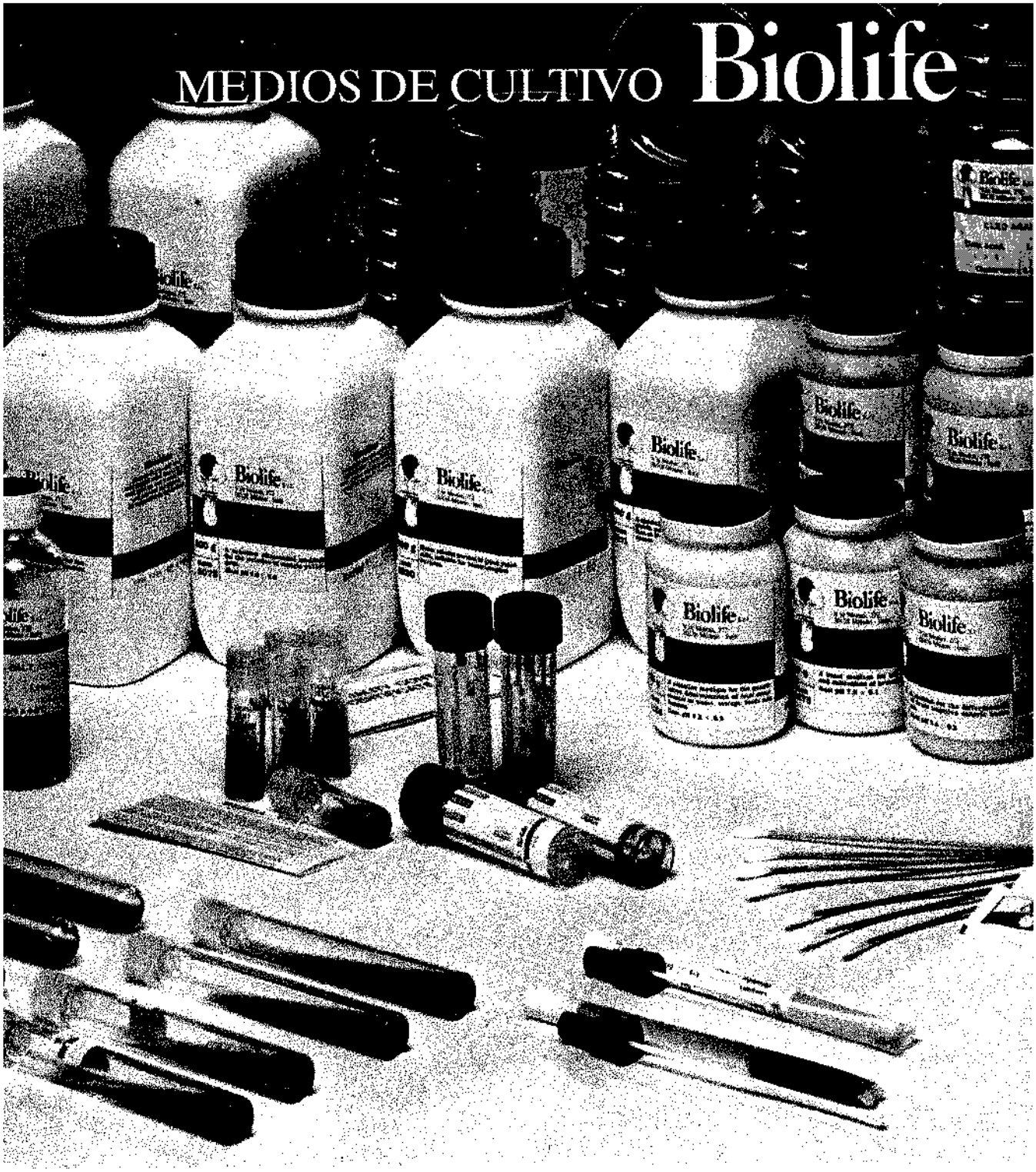

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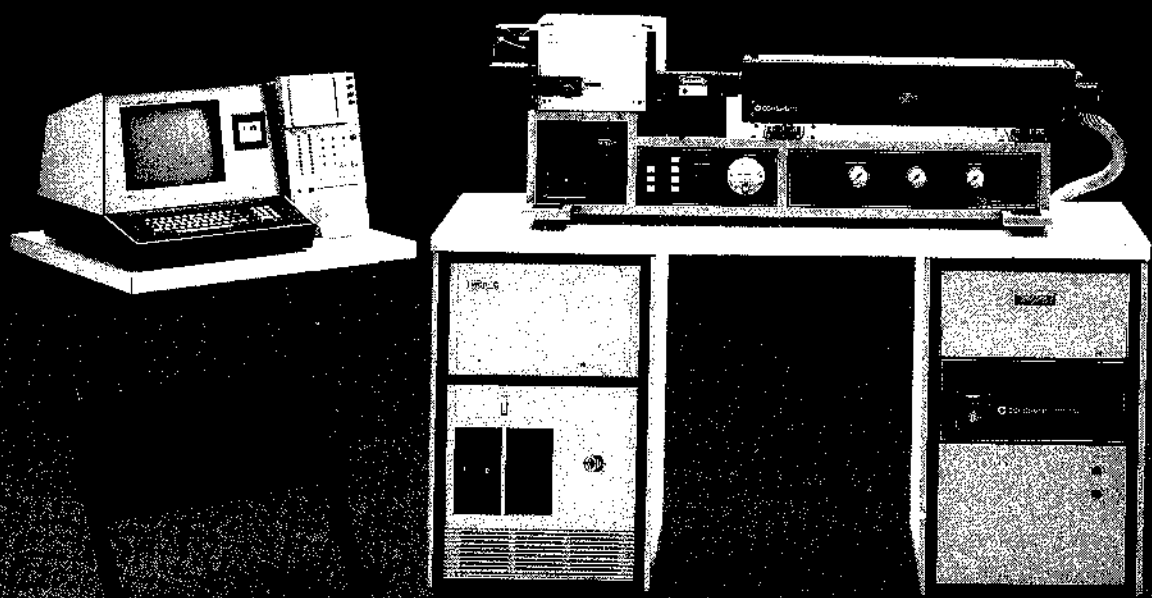

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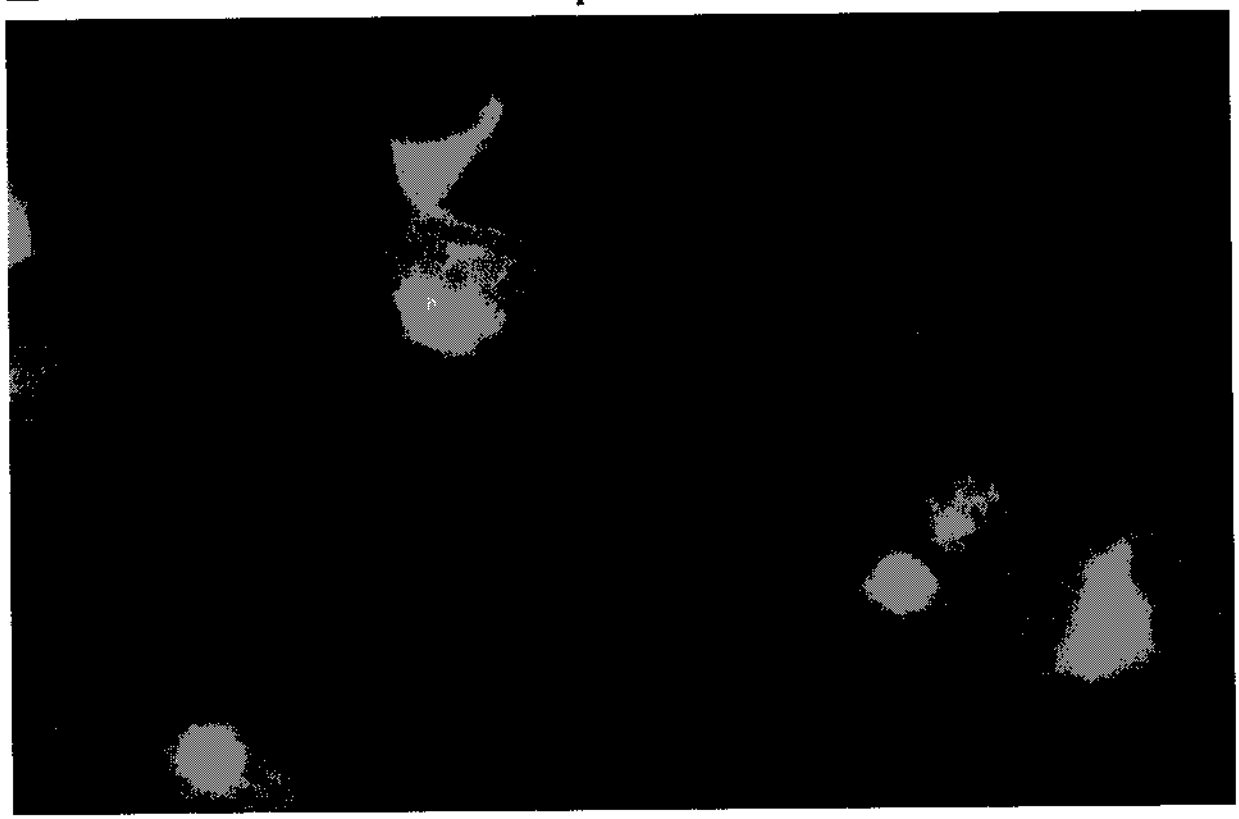

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