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Taxonomic Study of Non-alkaliphilic Halococci

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Ninety-six extremely halophilic, non-alkaliphilic cocci were isolated from several salterns in different geographical areas of Spain. These strains, together with seven reference strains of the genus *Halococcus*, were characterized by means of 114 phenotypic features, the results being analysed by numerical techniques using the simple matching (S_{SM}) coefficient and the unweighted pair group clustering (UPGMA) algorithm. At the 70% similarity level, four phenons were obtained. Phenon A contained 87 strains, including all the reference strains, and was considered to comprise members of the only named species of the genus *Halococcus*, *H. morrhuae*. Phenons B and C, which included five and seven strains respectively, showed greater metabolic versatility than phenon A. The four strains belonging to phenon D were significantly different from the other phenons in that they produced acid from glucose and were able to use most of the organic compounds tested. The results indicate that there is phenotypic diversity among the members of the genus *Halococcus* and that phenon D may constitute a new taxon.

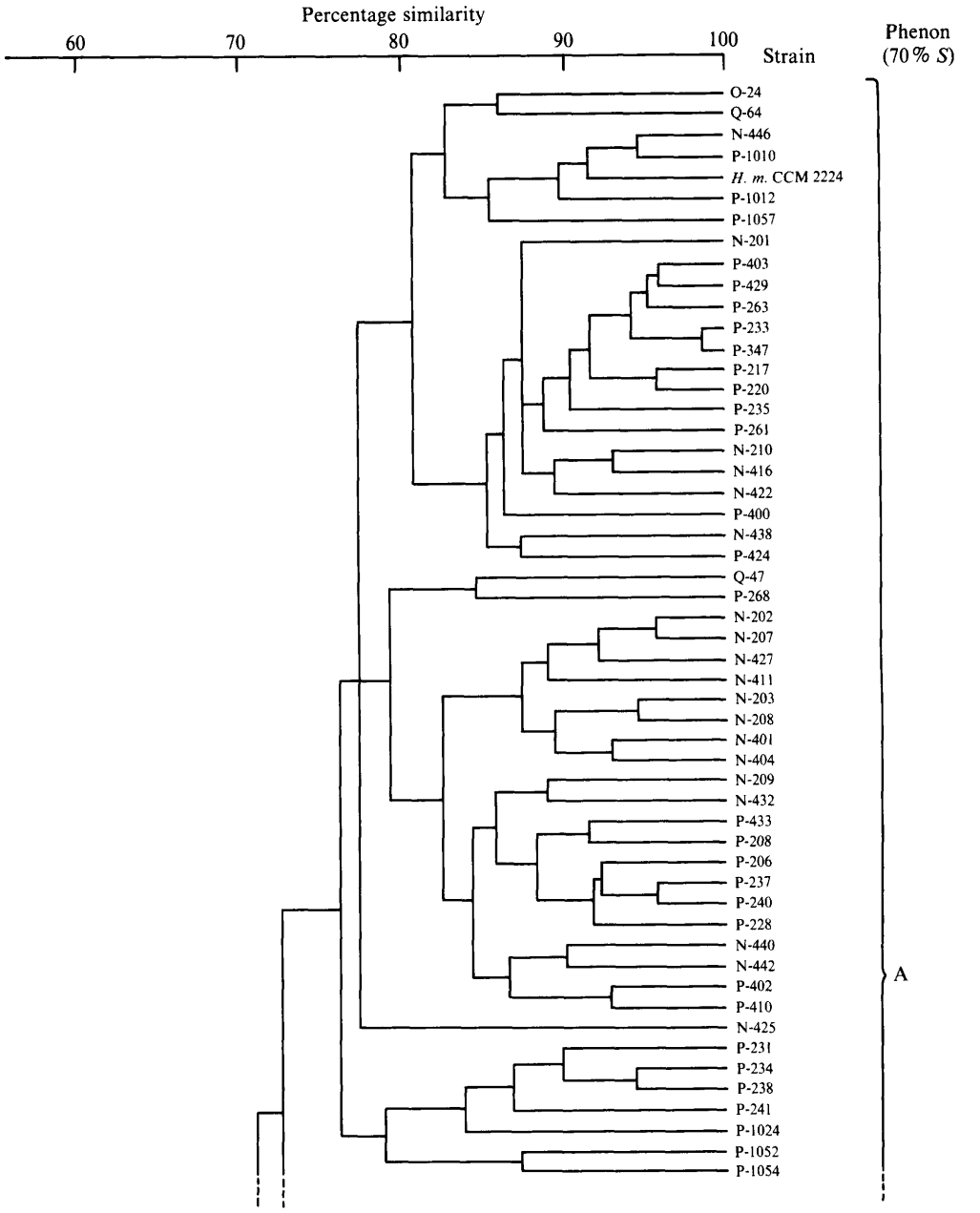
INTRODUCTION

The family Halobacteriaceae has traditionally been defined as a homogeneous group of extremely halophilic bacteria comprising only two genera: *Halobacterium* and *Halococcus* (Larsen, 1984). However, the genus *Halobacterium* includes species which are very different, both phenotypically and genotypically (Oren, 1983; Rodríguez-Valera *et al.*, 1983; Ross & Grant, 1985; Tindall & Trüper, 1986; Torreblanca *et al.*, 1986) and recently, the new genera *Natronobacterium*, *Natronococcus*, *Haloarcula* and *Haloferax* have been proposed in order to accommodate other examples of extremely halophilic archaebacteria (Tindall *et al.*, 1984; Torreblanca *et al.*, 1986).

That this natural diversity remained unexplored for so long can be attributed to the use of a very restricted variety of culture media and the lack of any systematic isolation of these kinds of organisms from diverse natural saline habitats. The genus *Halococcus* has received even less attention, and only one species, *H. morrhuae*, is recognized (Kocur & Hodgkiss, 1973; Larsen, 1984). However, relatively few strains have been studied, because the isolation of halophilic cocci from hypersaline environments is usually more difficult than that of the extremely halophilic rods, due to their low proportion in such natural habitats (Rodríguez-Valera *et al.*, 1985; Márquez *et al.*, 1987). The purpose of this work was to carry out a taxonomic study of a large number of extremely halophilic cocci isolated from several hypersaline habitats and to compare these with appropriate reference strains.

METHODS

Isolation of strains. The micro-organisms were isolated from ponds of salterns located in Cadiz, Majorca and near Alicante (Spain). Samples were taken at regular intervals from September 1984 to November 1985 (24 samplings from 11 different sites). The Cl^- content of the samples (determined using $AgNO_3$) was in the range 12·7–34·7%. For the isolation of the extremely halophilic cocci two methods were used: (i) direct plating on several isolation media; (ii) large water samples (about 5 litres) were centrifuged at 27000 *g* for 10 min, resuspended in distilled water for 24 h, and then plated on media, since halophilic cocci survive in distilled water (Rodríguez-



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Valera *et al.*, 1982) whereas *Halobacterium* spp. lyse under these conditions (Brown & Cho, 1970). Several isolation media were used: HM medium (Ventosa *et al.*, 1982) contained (%, w/v): yeast extract (Difco), 1; Proteose-peptone no. 3 (Difco), 0·5; glucose, 0·1; Bacto-agar (Difco), 2. This medium was supplemented with a balanced mixture of sea salts giving a final concentration of 25% (w/v) (Rodríguez-Valera *et al.*, 1980), and with 500 IU sodium penicillin G ml^{-1} in order to inhibit the growth of moderately halophilic and halotolerant eubacteria. Other media used included those previously described by Bertulo (1960–1961) and Eimjhellem (1965), and a defined minimal medium containing 25% (w/v) final salts concentration (Rodríguez-Valera *et al.*, 1980) and 0·5% (w/v) NH_4Cl, supplemented with 1% (w/v) of different substrates (glucose, glutamic acid, sodium acetate, mannitol or xylose). In a second set of experiments, these minimal media were supplemented with 0·1% (w/v)

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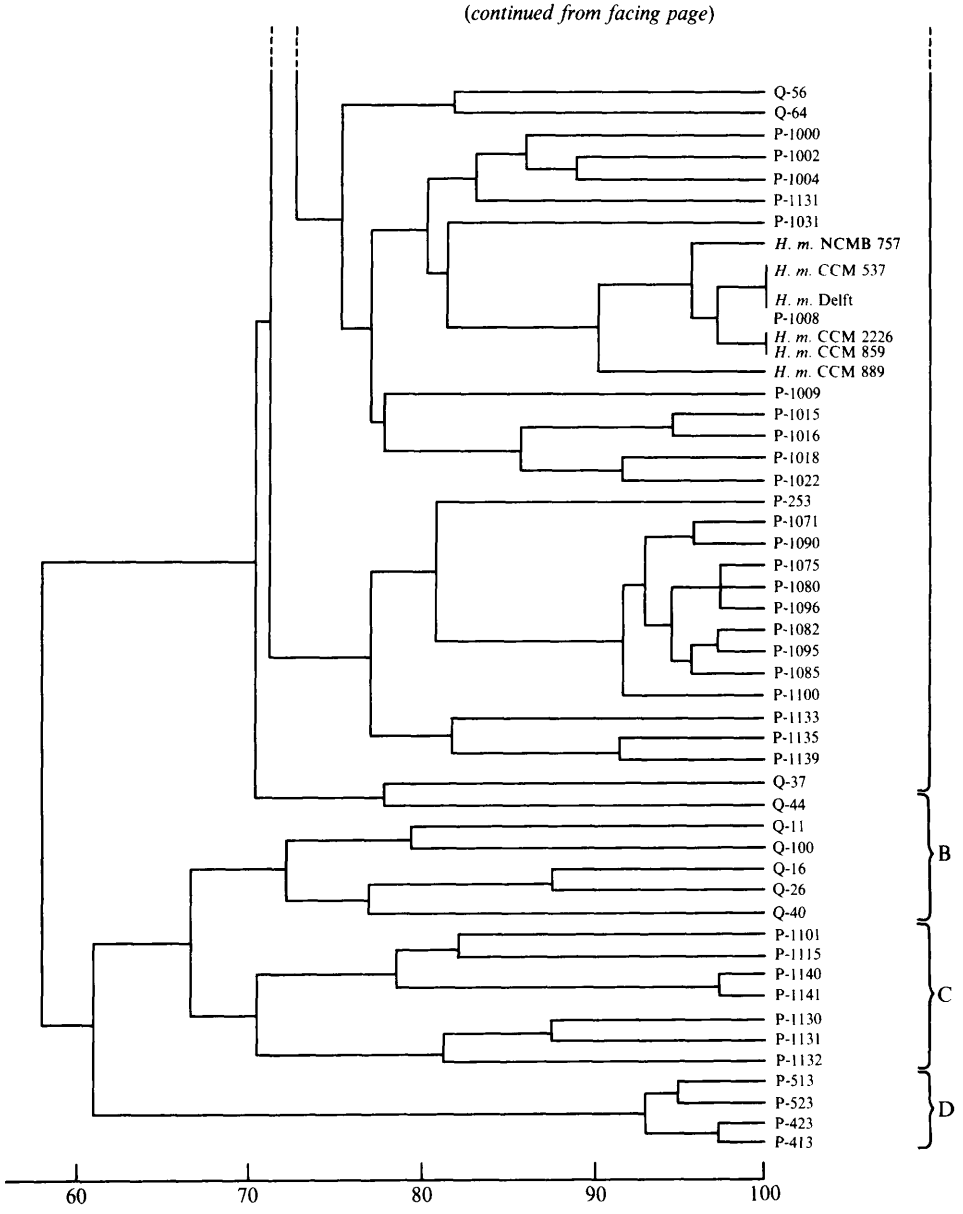

Fig. 1. Dendrogram showing the clustering of the strains based on the S_{SM} coefficient and UPGMA clustering, for 96 extremely halophilic non-alkaliphilic cocci from solar salterns and seven reference strains of *Halococcus morrhuae* (*H. m.*).

Casamino acids (Difco) or with 0·01 % (w/v) of the following amino acids: arginine, methionine, valine, isoleucine, leucine, lysine, glutamine and asparagine (Dundas *et al.*, 1963; Onishi *et al.*, 1965). All media were adjusted to pH 7·2 with 1 M-NaOH.

After incubation for 30 d at 37 °C, and subculturing on the same isolation medium to ensure purity, a total of 96 isolates were selected on the basis of their spherical morphology and extremely halophilic response.

Reference strains. The following extremely halophilic cocci were also included in this study: *Halococcus morrhuae* strains CCM 537^T, CCM 859, CCM 889, CCM 2224, CCM 2226, NCMB 757 and Delft.

Maintenance medium. The strains were maintained on agar slants of HM medium with 25% (w/v) salts. The pH was adjusted to 7·2 with 1 M-NaOH.

Table 1. *Frequencies of positive characters found in the four phenons, expressed as a percentage of the total scored to each group for the given test*

The differential characteristics of the four phenons are marked with asterisks. Of the 114 characters, 42 turned out to be constant over the 103 strains studied, and these are described in the footnote to this table.

Characteristic	Phenon A (87 strains)	Phenon B (5 strains)	Phenon C (7 strains)	Phenon D (4 strains)
Salts, growth at % (w/v):				
10	1	0	0	0
15*	62	100	86	0
20	95	100	100	100
Acid production from:				
D-Glucose*	3	0	0	100
Lactose	1	0	0	0
D-Mannitol	1	0	0	0
Indole production	1	0	0	0
Methyl red	0	0	0	50
Gelatin hydrolysis	92	100	100	50
Starch hydrolysis*	23	100	0	0
Tween 80 hydrolysis	76	100	86	0
Casein hydrolysis	5	0	0	0
Phosphatase	41	20	71	50
DNAase*	12	0	71	50
NO_3^- reduction to NO_2^-*	86	20	100	100
NO_2^- reduction to gaseous compounds	46	20	71	50
Koser's citrate	98	100	71	100
H_2S production from:				
Thiosulphate	16	60	14	0
Cysteine*	61	80	100	100
Utilization of organic compounds as sole source of carbon and energy:				
Carbohydrates:				
Aesculin	47	40	71	0
L-Arabinose*	18	80	71	0
Amygdalin*	3	60	43	100
D-Cellobiose	53	80	86	50
D-Fructose	67	80	100	100
D-Galactose	48	100	100	100
D-Glucose	79	100	100	100
Lactose*	9	20	14	100
Maltose	41	80	57	100
D-Mannose	34	40	57	100
D-Ribose	0	40	0	0
D-Salicin*	5	80	14	100
Starch	61	80	100	100
Sucrose	41	100	100	0
D-Xylose*	14	100	100	0
Alcohols:				
Adonitol*	2	100	43	100
Dulcitol	2	60	57	0
Ethanol	7	100	43	100
Erythritol	2	60	57	100
DL-Glycerol*	15	100	0	100
meso-Inositol	11	100	71	100
D-Mannitol	14	100	71	100
Propanol	5	100	71	100
D-Sorbitol	15	100	100	100
Carboxylic acids:				
Acetate	44	100	86	100
cis-Aconitate*	0	0	0	100
δ-Aminovalerate	23	100	57	100
Fumarate	41	100	80	100
D-Gluconate*	1	0	29	100
D-Glucuronate	3	80	57	100

Table 1. (*continued*)

Characteristic	Phenon A (87 strains)	Phenon B (5 strains)	Phenon C (7 strains)	Phenon D (4 strains)
Hippurate*	5	0	0	100
DL-Lactate	0	20	29	100
DL-Malate	20	20	100	100
Pyruvate	66	80	100	100
Propionate*	0	0	29	100
Quinate	1	20	86	100
D-Saccharate	2	40	100	100
Succinate	32	40	100	100
Utilization of amino acids as sole source of carbon, nitrogen and energy:				
L-Alanine	14	20	29	100
L-Arginine	24	0	14	100
L-Asparagine	71	60	71	100
L-Aspartic acid	28	80	71	0
L-Glutamic acid*	6	40	100	100
L-Isoleucine	48	20	71	100
L-Leucine	82	80	100	100
L-Lysine	56	40	100	100
L-Methionine*	1	0	0	100
L-Ornithine	89	80	100	100
DL-Phenylalanine	16	20	43	100
Sarcosine	6	20	43	0
L-Serine	89	100	100	100
L-Threonine	37	40	71	100
L-Tryptophan*	37	0	100	100

All strains were Gram-negative non-motile cocci, occurring in pairs, tetrads, sarcinae or irregular clusters, formed catalase, and were strict aerobes and oxidase positive. They grew at 25 and 30% (w/v) total salts. None of the strains grew at 0, 0·5, 3 or 5% (w/v) total salts. All were pigmented pink to brick-red. None produced acid from sucrose, or grew on D-galactosamine, D-glucosamine, inulin, D-melibiose, D-raffinose, L-rhamnose, α-aminobutyrate, benzoate, caprylate, *p*-hydroxybenzoate, malonate, oxalate, salicylate, suberate or D-tartrate as sole source of carbon and energy. None grew on alantoin, creatinine, ethionine, glycine or putrescine as sole source of carbon, nitrogen and energy. All grew on trehalose, *N*-acetylglucosamine or butyrate as sole source of carbon and energy. All grew on L-glutamine, L-histidine, L-proline or L-valine as sole source of carbon, nitrogen and energy.

Characterization of isolates. For each strain 114 phenotypic characteristics (see Table 1) were determined. The tests have been described previously (Kocur & Hodgkiss, 1973; Rodríguez-Valera *et al.*, 1983; Torreblanca *et al.*, 1986). Unless otherwise indicated, all media contained 25% (w/v) marine salts and their pH was adjusted to 7·2 with 1 M-NaOH. Incubation was at 37 °C for up to 30 d in sealed containers.

Numerical analysis. Taxonomic characters were coded in binary form of the presence/absence type. Strain similarities were estimated with both simple matching (S_{SM}) (Sokal & Michener, 1958) and Jaccard (S_J) (Jaccard, 1908) coefficients, and cluster analysis was carried out using the unweighted pair group method of association (UPGMA) (Sneath & Sokal, 1973). Test error was estimated in eight replicate strains (Sneath & Johnson, 1972). Cophenetic correlation was also evaluated (Sneath & Sokal, 1973). These computations were done using the MINT program of Dr F. J. Rolf (Department of Ecology and Evolution, State University of New York at Stony Brook, NY, USA), on a Univac 1108 computer in the Computer Centre of the University of Sevilla, Sevilla, Spain.

RESULTS

All the strains of extremely halophilic cocci were isolated by the distilled water selection method. Direct plating without selection did not yield any isolates, presumably because the numbers of organisms were too small compared with those of halobacteria. All solid media gave similar counts and the 96 isolates were picked to try and get the maximum variety with regard to the isolation medium and geographical origin.

The result of the numerical study by means of the S_{SM} coefficient and UPGMA clustering method is shown in the dendrogram (Fig. 1). The cophenetic value was 0·816 and the estimated test error was less than 3%. The cluster composition was not markedly affected by using the S_J

coefficient. At the 70% similarity (*S*) level, the strains were grouped into four phenons (A, B, C and D). The phenotypic characteristics of the four phenons are listed in Table 1; their differential characters are marked with asterisks.

Phenon A. This group included 87 strains clustered at 71% *S*. It included all the reference strains, except *H. morrhuae* CCM 2224, as a subphenon at 90% *S*. The strains were non-motile cocci, strict aerobes, able to hydrolyse gelatin and Tween 80, and reduced nitrate to nitrite. Acid was not generally produced from glucose or from other sugars. They used a very low number of carbohydrates, alcohols or carboxylic acids as the sole source of carbon and energy. Only some amino acids were utilized, particularly by some strains which formed a subphenon at 92% *S*. The isolates in this phenon derived from all the sites sampled and they appeared on all the media used.

Phenon B. The five strains of this phenon clustered at 72% *S*. They hydrolysed gelatin, starch and Tween 80, but not casein; DNAase was not produced. Acid was not produced from sugars. They were able to use more compounds than strains in phenon A as the sole source of carbon and energy, particularly with respect to alcohols. The five representatives of this phenon were isolated from Alicante saltern and on HM medium.

Phenon C. This phenon comprised seven strains clustered at 71% *S*. They hydrolysed gelatin, reduced nitrate to nitrite and produced H_2S from cysteine; starch and casein were not hydrolysed. They showed a higher nutritional versatility than strains in phenon A. Their main difference from strains of phenon B was their lower capacity to use alcohols, but higher capacity to use other tested compounds. These strains came from the Cádiz saltern; five were isolated on HM medium, one on minimal medium plus xylose and Casamino acids, and one on minimal medium plus sodium acetate and Casamino acids.

Phenon D. The four strains of this phenon clustered at 93% *S*. They were able to produce acid from glucose, but did not hydrolyse starch or Tween 80. They reduced nitrate to nitrite and produced H_2S from cysteine but not from thiosulphate. One of the most noticeable features of the strains of this phenon was their high nutritional versatility: they were able to use a large number of carbohydrates, alcohols and organic acids as sole source of carbon and energy, and amino acids as sole source of carbon, nitrogen and energy (see Table 1), being the only group able to use *cis*-aconitate as the sole source of carbon and energy. These strains were isolated from the Cádiz saltern on HM medium.

DISCUSSION

The scarce information available on non-alkaliphilic halococci suggests that they are a very homogeneous group and comprise a single species, *Halococcus morrhuae* (Larsen, 1984). However, because of the difficulties of isolating extremely halophilic cocci, only a few strains are available. To increase the number of isolates we have used an enrichment method based upon the higher resistance of the halococci to low salt concentrations (Rodríguez-Valera *et al.*, 1982). Most of these new isolates of extremely halophilic cocci grouped in phenon A and were identified as members of the species *H. morrhuae*. Phenons B and C, because of their higher metabolic versatility, could be considered as *Halococcus* sp., closely related to *H. morrhuae*.

There have been several previous studies with strains of the genus *Halococcus* (Kocur & Hodgkiss, 1973; Colwell *et al.*, 1979; Larsen, 1984; Javor, 1984) and we find some differences when these are compared with our results. In the study by Kocur & Hodgkiss (1973), most of the results resemble those obtained with our isolates in phenons A, B and C, but we obtained different results in regard to the reduction of nitrate to gaseous compounds and the hydrolysis of gelatin, starch and casein. The utilization of a variety of compounds as the only source of carbon and energy, or of carbon, nitrogen and energy, has not been previously reported. We have shown that the nutritional abilities of strains of halophilic, non-alkaliphilic cocci are very heterogeneous, and there are differences between the 'typical' strains of phenon A and those included in phenons B or C.

Javor (1984) described three strains with characteristics similar to those of phenons B and D. However, our strains used D-mannitol and D-sorbitol as sole source of carbon and energy, in contrast with the results obtained by Javor (1984). Our results for D-glucose, D-fructose, D-galactose, sucrose, D-ribose, pyruvate, acetate and lactate are in agreement with those of Javor (1984).

Phenon D constitutes a group of strains able to produce acid from glucose, which do not hydrolyse starch or Tween 80, but which reduce nitrate to nitrite and produce H_2S from cysteine. The polar lipid pattern of a representative of this phenon is also different from those of representatives of phenons A, B and C, which are identical (unpublished results). There are no previous reports, to our knowledge, of acid being produced from sugars by members of the genus *Halococcus*, and our findings also differ from previous taxonomic descriptions in demonstrating a remarkably wide nutritional versatility for halophilic, non-alkaliphilic cocci (Larsen, 1984). Phenon D could thus constitute a new taxon. More extensive chemotaxonomic studies are under way to characterize and describe this possible new group of halophilic archaebacterial cocci more fully.

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