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Survey of lipids of a new group of extremely halophilic bacteria from salt ponds in Spain

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The lipids of eight strains of a new group of extreme halophiles isolated from Spanish salt ponds have been studied in comparison with those of *Halobacterium cutirubrum*, *H. halobium*, *H. salinarium*, *H. marismortui*, *H. saccharovorum*, and *H. vallismortis*. All strains showed the presence of C_{30} isoprenoids (squalene, dihydrosqualene, and tetrahydrosqualene), C_{40} carotenes (β-carotene), C_{50} pigments (bacterioruberins), and vitamin MK-8. All polar lipids were derivatives of 2,3-di-*O*-phytanyl-*sn*-glycerol. Diphytanyl glycerol ether analogues of phosphatidyglycerol and phosphatidylglycerophosphate were also present in all the strains but sulfated glycolipids were found in only six strains: two had a sulfated tetraglycosyl diether, a sulfated triglycosyl diether, and possibly a sulfated diglycosyl diether, whereas the other four had only sulfated diglycosyl diether. All strains contained diglycosyl diphytanyl diether. Significantly large amounts of the diphytanyl glycerol ether analogue of phosphatidylglycerosulfate were present in four strains. Strain R-4 exhibited marked changes in contents of individual pigments, but not in total lipids, when grown in different salt concentrations. Thus, in 15% salt it produced 18- to 19-fold more bacterioruberin than when grown in 25 or 35% salt. Similar reductions in the amounts of other C_{50} pigments and β-carotene were also observed. No significant changes in the contents of squalenes and vitamin MK-8 were observed. In contrast, *H. cutirubrum* produced C_{50} pigments and β-carotene only when grown in salt concentrations greater than 15%. Possible ecological consequences of the changes in pigmentation with salt concentration are discussed.

KUSHWAHA, S. C., G. JUEZ-PÉREZ, F. RODRIGUEZ-VALERA, M. KATES et D. J. KUSHNER. 1982. Survey of lipids of a new group of extremely halophilic bacteria from salt ponds in Spain. Can. J. Microbiol. **28**: 1365–1372.

Les lipides de huit souches bactériennes fortement halophiles, isolées d'étangs salins en Espagne, ont été étudiés sur une base comparative avec *Halobacterium cutirubrum*, *H. halobium*, *H. salinarium*, *H. saccharovorum* et *H. vallismortis*. Toutes les souches ont démontré la présence d'isoprenoïdes C_{30} (squalène, dihydrosqualène et tétrahydrosqualène), carotènes C_{40} (β-carotène), pigments C_{50} (bactériorubérines) et la vitamine MK-8. Tous les lipides polaires étaient des dérivés du 2,3-di-*O*-phytanyl-*sn*-glycérol. Toutes les souches contenaient des analogues d'éther de diphytanyl glycérol du phosphatidylglycérol et du phosphatidylglycérophosphate mais seulement six souches révélèrent la présence de glycolipides sulfates: deux de ces souches possédaient un diéther tétraglycosyl sulfaté, un diéther triglycosyl sulfaté et vraisemblablement un diéther diglycosyl sulfaté, tandis que les quatre autres avaient seulement le diéther diglycosyl sulfaté. Toutes les souches contenaient du diéther diglycosyl diphytanyl. Des quantités relativement importantes d'analogue de l'éther de diphytanyl glycérol du phosphatidylglycérosulfate furent décelées dans quatre souches. La souche R-4 a démontré des changements prononcés dans le contenu des pigments individuels, mais pas dans le nombre total des lipides, lorsqu'elle était cultivée dans diverses solutions salines. Ainsi, dans une solution saline de 15%, elle a produit de 18 à 19 fois plus de bactériorubérine que lorsqu'elle était cultivée dans une solution saline de 25 ou 35%. Nous avons également observé des réductions semblables dans les quantités d'autres pigments C_{50} et de β-carotène. Nous n'avons cependant observé aucun changement d'importance dans les teneurs de squalènes et de vitamine MK-8. Par contre, le *H. cutirubrum* a produit des pigments C_{50} et de la β-carotène uniquement lorsque cultivé dans des solutions salines de plus de 15%. Nous discutons des conséquences écologiques possibles de la réaction de la pigmentation à des concentrations salines.

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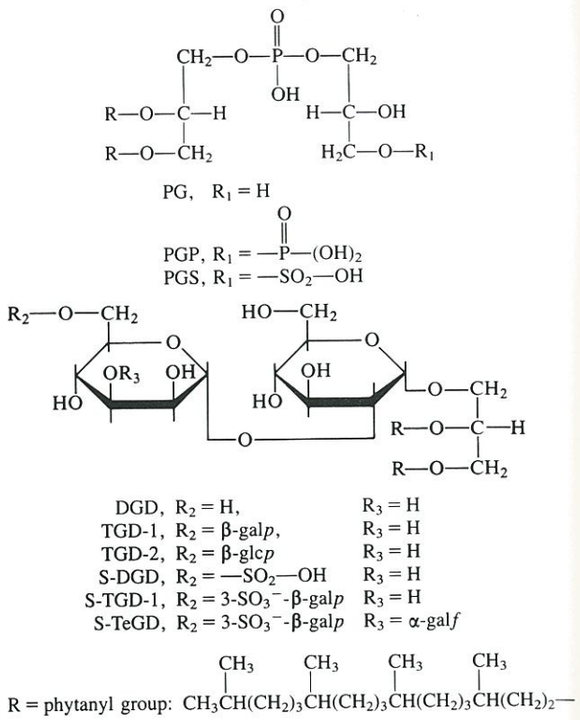
Introduction

Detailed studies of the lipids of extremely halophilic bacteria (which belong to the Archaebacteria) have been carried out mainly on *Halobacterium cutirubrum* (Kates 1978; Smallbone and Kates 1981), *H. halobium* (Kates 1978), and *H. marismortui* (Evans *et al.* 1980). These organisms contain unusual lipids (Kates 1978) which are exclusively derived from 2,3-di-*O*-phytanylglycerol ether. The polar lipid components in *H. cutirubrum* and *H. halobium* consist mostly of a sulfated triglycosyl diether (S-TGD-1) (galactosyl sulfate-mannosylglucosyl diphytanyl ether) and the diphytanyl glycerol ether analogues of phosphatidylglycerol (PG), phosphatidylglycerophosphate (PGP), and phosphatidylglycerosulfate (PGS) (Scheme 1). *Halobacterium marismortui*, which inhabits the Dead Sea, contains mainly the diphytanyl glycerol ether analogues of phosphatidylglycerol, phosphatidylglycerophosphate, phosphatidylglycerosulfate, and a triglycosyl diether (TGD-2) (glucosyl-mannosyl-glucosyl diphytanyl glycerol ether) but no glycolipid sulfate (Evans *et al.* 1980). Recently, a sulfated tetraglycosyl diether (S-TeGD) (galactopyranosyl sulfate-mannopyranosyl(←galactofuranosyl)glucopyranosyl diphytanylglycerol ether) has been identified in *H. cutirubrum* (Smallbone and Kates 1981). The occurrence of phytanyl diether lipids has also been reported in another group of Archaebacteria, the methane-producing bacteria (Makula and Singer 1978; Tornabene and Langworthy 1979; Kushwaha *et al.* 1981).

Recently, several new strains of extremely halophilic bacteria were isolated from seawater evaporation salt ponds near Alicante in Spain using enrichments in which carbohydrates were the main carbon and energy source (Rodriguez-Valera *et al.* 1980*a*, 1980*b*). They showed wide physiological and biochemical variability (Rodriguez-Valera *et al.* 1980*a*; G. Juez-Pérez, F. Rodriguez-Valera, and D. J. Kushner, in preparation). The polar lipids of one of these strains (R-4) were investigated (Kushwaha *et al.* 1982) and were found to contain the same phospholipids as in the well-known extreme halophiles, but the sulfated glycolipids (S-TGD and S-TeGD) were replaced by a single novel glycolipid sulfate, mannosyl sulfate-glucosyl-diphytanyl ether (S-DGD).

In view of these findings, it was of interest to survey the lipids of the other strains of Spanish halophiles, and seven representative strains were chosen for this study. Two recently identified extreme halophiles, *H. saccharovorum* (Tomlinson and Hochstein 1976) and *H. vallismortis* (Gonzalez *et al.* 1978), were also included in this survey, since no data are available on their lipid composition. We have also investigated the effect of salt concentration on the lipids of one of the Spanish strains (R-4) and of *H. cutirubrum*.



SCHEME 1. Structures of phospholipids and glycolipids in extremely halophilic bacteria. PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; PGS, phosphatidylglycerosulfate; DGD, diglycosyl diether; TGD-1 and TGD-2, triglycosyl diether; S-DGD, sulfated diglycosyl diether; S-TGD-1, sulfated triglycosyl diether; S-TeGD, sulfated tetraglycosyl diether.

Materials and methods

Materials

Authentic samples of C_{40} carotenes were prepared from tomatoes or carrot oil (Kushwaha *et al.* 1972); C_{50} carotenoids and vitamin MK-8 were prepared from *H. cutirubrum* (Kushwaha *et al.* 1975). All solvents were distilled before use. Petroleum ether refers to a fraction boiling between 40–60°C.

Organisms and growth conditions

Several new strains of extremely halophilic bacteria were isolated from seawater evaporation ponds near Alicante, Spain, using enrichment with carbohydrates as energy source, as described previously (Rodriguez-Valera *et al.* 1980*a*, 1980*b*). These strains were grouped according to physiological criteria, and representative strains (R-4, Gla-2.2, Y-27, Gaa-3, Gaa-6, Gca-19, M-2.5, and Ma-2.38) were chosen to study their lipid composition in comparison with that of previously studied extreme halophiles obtained from the National Research Council of Canada culture collection. All cultures were grown at 38°C in a medium (Rodriguez-Valera *et al.* 1980*a*, 1980*b*) composed of (grams per 100 mL) inorganic salts NaCl (19.4), $MgCl_2$ (1.6), $MgSO_4$ (2.4), $CaCl_2$ (0.1), KCl (0.5), $NaHCO_3$ (0.02), NaBr (0.05), and yeast extract (0.5), pH 7.3, in 1-L batches in 4-L Erlenmeyer flasks as described elsewhere (Rodriguez-Valera *et al.* 1980*a*, 1980*b*). When large amounts of cells were required, each culture was grown in 10-L batches in a 15-L cylindrical glass flask, which was magnetically stirred and aerated by a stream of compressed air through a sparger. The cultures were grown for 4 days (stationary phase), harvested by centrifugation, and washed twice with 25% NaCl solution. Harvested cells were then extracted for lipids as described below. Before extracting lipids, cell protein determinations were carried out by the method of Lowry *et al.* (1951).

Extraction of lipids

Total lipids were extracted from harvested cells by the method of Bligh and Dyer (1959) as modified for extremely halophilic bacteria (Kates 1972). However, the chloroform used in these extractions was washed in turn with water, 1% sodium bicarbonate, and water prior to use. Nonpolar lipids were separated from polar lipids by precipitation with ice-cold acetone (Tornabene *et al.* 1969; Kates 1972). Individual polar and nonpolar lipids were then purified by thin-layer chromatography (TLC) as described below.

Chromatography

TLC of polar lipids was performed on precoated silica gel G plates (Sil G-25, 0.25 mm silica gel without gypsum, Brinkmann Instruments) in solvent A, $CHCl_3$ – MeOH – CH_3COOH – H_2O (85:22.5:10:4, by volume), for the separation of phospholipids and glycolipids. TLC of nonpolar lipids was performed on silica gel H plates in the following solvents: B, petroleum ether – diethyl ether – acetic acid (60:40:1, by volume) for the separation of phytanylglycerol diether; C, $CHCl_3$ – MeOH (93:7, v/v) for bacterioruberin and monoanhydrobacterioruberin; D, petroleum ether – diethyl ether (99:1, v/v) for vitamin MK-8, lycopene, and β-carotene; E, petroleum ether – diethyl ether (99.5:0.5, v/v) for squalene, dihydrosqualene, tetrahydrosqualene, β-carotene, and lycopene.

Lipids were detected by the following spray reagents (Kates 1972): $(NH_4)_2MoO_4$ – $HClO_4$ for phosphatides; 0.5% α-naphthol – H_2SO_4 for glycolipids; H_2SO_4 – ethanol (1:1, v/v) followed by charring and 0.005% aqueous rhodamine 6G followed by visualization under ultraviolet light for detection of all lipids. Colored isoprenoids were also detected by their visual colors, whereas colorless nonpolar components were detected by iodine vapor (Kushwaha *et al.* 1974).

Quantitative determination of purified pigments and other components

Purified carotenoids were eluted from TLC plates, identified by their visible spectra, and quantitated spectrophotometrically as described previously (Kushwaha *et al.* 1974; Kushwaha *et al.* 1975). Other nonpolar and polar lipids were quantitated by weighing the material eluted from the TLC plate with acetone or chloroform–methanol–water (1:1:0.1, by volume), respectively (Kushwaha *et al.* 1974; Evans *et al.* 1980). Analyses were carried out at least twice and results are given as averages.

Acid methanolysis

Total polar lipids of all species were methanolyzed in 2.5% HCl (gas) in anhydrous methanol as described elsewhere (Kates 1972). The petroleum ether soluble material was identified as diphytanyl glycerol ether by its mobility in solvent B (Kushwaha *et al.* 1981). No biphytanyl diglycerol tetraether was detected (Kushwaha *et al.* 1981).

Results and discussion

The C_{30} isoprenoids, squalene, dihydrosqualene, and tetrahydrosqualene, were present in all strains (Table 1). However, the relative proportions of the above squalenes in strains R-4, Gla-2.2, M-2.5, Ma-2.38, and *H. vallismortis* were in reverse order to that observed in the well-known extreme halophiles. Similarly, all strains had much lower amounts of C_{40} carotenoids than the C_{30} isoprenoids. β-Carotene was detected in all strains (Table 1); however, strains R-4, Gla-2.2, M-2.5, and Ma-2.38 contained smaller amounts of β-carotene which could only be detected when the TLC plates were overloaded. The presence of lycopene was detected only in overloaded plates in a few strains of Spanish halophiles, in contrast with the common halobacteria (Table 1). All strains examined showed the presence of C_{50} carotenoids, e.g., bacterioruberin, monoanhydrobacterioruberin, and bisanhydrobacterioruberin (Table 1). As discussed further below, strain R-4, under the normal growth conditions used for extreme halophiles (e.g., 4 *M* NaCl), had much lower concentrations of C_{50} carotenoids than the other strains; these pigments could just be detected visually on the plate.

In regard to the phospholipids, the diphytanyl glycerol ether analogues of phosphatidylglycerol and phosphatidylglycerophosphate were present as major components in all strains (Fig. 1; Table 1). Strains R-4 and Gla-2.2 contained much higher proportions of the phosphatidylglycerol than in *H. cutirubrum*, *H. halobium*, or *H. salinarium* (Fig. 1; Table 1). Significantly high proportions of phosphatidylglycerosulfate were found in strains Y-27, Gaa-3, Gaa-6, *H. vallismortis*, and *H. marismortui*. In contrast, considerable variability was observed in the occurrence and distribution of glycolipid and sulfated glycolipid components. Only *H. saccharovorum* and strain Gca-19 contained the sulfated tetraglycosyl diether (S-TeGD) (Smallbone and Kates 1981) and the sulfated triglycosyl diether (S-TDG-1) previously found in *H. cutirubrum* (Kates and Deroo 1973) and in *H. halobium* and *H. salinarium* (Kates 1978) (Fig. 1; Table 1). Strains R-4, Gla-2.2, M-2.5, Ma-2.38, and possibly Gca-19 contain a new sulfated diglycosyl diether (S-DGD) whose structure (see Scheme 1) has recently been established (Kushwaha *et al.* 1982). The remaining four strains (i.e., strains Y-27, Gaa-3, Gaa-6, and *H. vallismortis*) do not contain any sulfated glycolipid, but contain instead a triglycosyl diether with an R_f value similar to that of TGD-2, which has recently been identified in *H. marismortui* (Evans *et al.* 1980; see structure in Scheme 1). Furthermore, a

TABLE 1. Distribution of both polar and nonpolar lipid components

Strains	Polar lipids								
	PG	PGP	PGS	DGD	TGD-1	TGD-2	S-DGD	S-TGD-1	S-TeGD
R-4	++++(44)	++++(30)	−	+	−	−	++++(25)	−	−
Gla-2.2	+++	++++	−	+	−	−	+++	−	−
Y-27	++	+++	++	++	−	+	−	−	−
Gaa-3	++	+++	++	+	−	+	−	−	−
Gaa-6	++	+++	++	+	−	+	−	−	−
Gca-19	+	+	?	+	+	−	?	+	+
M-2.5	++	+++	−	+	−	−	++	−	−
Ma-2.38	++	+++	−	+	−	−	++	−	−
H. vallismortis	++	+++	++	+	−	+	−	−	−
H. saccharovorum	+	++++	?	−	?	−	?	+	+
H. marismortui	+(11)	++++(62)	++(17)	+	−	+(11)	−	−	−
H. salinarium	+	++++	?	+	+	−	−	+++	++
H. halobium	+	++++	?	−	+	−	−	+++	++
H. cutirubrum	+(4)	++++(70)	+(4)	+	+	−	−	++(21)	+

NOTE: Data were obtained from TLC plates of lipids run in solvent A for polar lipids and solvents C to E for nonpolar lipids (see Materials and methods) (see Fig. 1 independently and − indicates absence of lipids; (+) indicates detectable only on overloaded TLC plates. Values in parentheses represent polar lipid composition DGD, glycodiosyl diether; TGD-2, glycotriosyl diether (diether-glucose-mannose-glucose); TGD-1, diether-glucose-mannose-galactose; S-DGD, sulfated R_1, R_2, bacterioruberin; R_3, monanhydrobacterioruberin; R_4, an unknown C_{50} pigment; R_5, bisanhydrobacterioruberin.

diglycosyl diether (DGD), recently identified in strain R-4 (Kushwaha *et al.* 1982), is present in all strains of the Spanish extreme halophiles studied here (Fig. 1; Table 1). As in *H. cutirubrum*, all polar lipids of these strains are derived from 2,3-di-*O*-phytanyl-*sn*-glycerol moiety and not from the C_{40} tetraether as shown by TLC of the products of acid methanolysis of total polar lipids in solvent B (see Fig. 3 in Kushwaha *et al.* 1981).

The above results on the polar and nonpolar lipids of the new extreme halophiles from Spain indicate that the strains examined can be divided into three main groups on the basis of their glycolipid components. The lipids of the first group (Y-27, Gaa-3, Gaa-6) resemble, in general, those found in *H. marismortui*, whereas the lipids of the second group (Gca-19, *H. saccharovorum*) are similar to those in *H. cutirubrum*, *H. halobium*, and *H. salinarium*. A third group (strain R-4, Gla-2.2, M-2.5, Ma-2.38) does not contain the main sulfated glycolipids (S-TDG-1, S-TeDG) and the glycolipid (TGD-2) found in members of the other two groups but contains the new sulfated glycolipid (S-DGD). Other quantitative differences in phospholipid components between these groups also exist, e.g., the relative proportions of phosphatidylglycerol and phosphatidylglycerophosphate (Table 1; Fig. 1).

The strains of Spanish extreme halophiles thus appear to be similar to other extreme halobacteria with respect to the presence of isoprenoid compounds and pigments but vary widely in their polar lipid compositions. These differences could have important taxonomic implications.

Effect of salt concentration on lipids of strain R-4

Growth of strain R-4 and its total, polar, and nonpolar lipid contents were approximately the same in media containing 15, 20, and 25% total salt (Table 2). However, decreases in growth and in content of total lipids were observed at 35% salt concentration. A gradual reduction in the pigmentation of the cells was noticed with increase in salt concentration of the growth medium from 15 to 35%; thus, a 14- to 19-fold reduction occurred in the concentration of bacterioruberins in cells grown in 25 or 35% salt as compared with those grown in 15% salt, and similar reductions in the contents of the other C_{50} pigments were also observed (Table 3). In contrast, *H. cutirubrum*, in 15% salt, did not form any C_{40} or C_{50} carotenoid pigments, but high concentrations of these pigments appeared in cells grown in 20 to 35% salt (Table 3).

Growth of strain R-4 in media of increasing salt concentration (15 to 30%) did not significantly affect the concentration of total squalenes (Table 3) but strongly affected the relative proportions of squalene to dihydrosqualene to tetrahydrosqualene (Fig. 2). Vitamin MK-8 did not show as pronounced a change in concentration with increasing salt concentration as the bacterioruberins, but its content in cells grown at 20 to 35% salt was significantly lower than that at 15% salt (Table 3).

in Spanish extreme halophiles and other extreme halobacteria

Nonpolar lipids								Vitamin MK-8
S	S_2	S_4	β-carotene	Lycopene	$R_1 + R_2$	$R_3 + R_4$	R_5	
+	+	++++	(+)	−	(+)	(+)	(+)	+
+	+	++++	(+)	−	+	+	+	+
++++	++	+	+	−	+	++	+	+
++++	++	+	+	(+)	++	++	++	+
++++	++	+	+	−	+	++	++	+
++++	+	+	+	(+)	++	++	++	+
+	+	++++	(+)	(+)	++	++	+	+
+	+	++++	(+)	(+)	++	++	+	+
+	++	++++	+	−	+	+	++	+
++++	++	+	+	−	+	+	−	+
++++	++	+	+	+	+	+	++	+
++++	++	+	+	+	+	+	+	+
++++	++	+	+	+	+	+	+	+
++++	++	+	+	+	+	+	+	+

for a representative TLC plate for polar lipids). The number of + signs indicates the relative proportions of components for the polar and nonpolar lipid classes taken as mole percentages of total polar lipids (Kushwaha *et al.* 1982). PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; PGS, phosphatidylglycerosulfate; glycodiosyl diether; S-TGD-1, sulfated glycotriosyl diether; S-TeGD, sulfated glycotetraosyl diether; S, squalene; S_2, dihydrosqualene; S_4, tetrahydrosqualene;

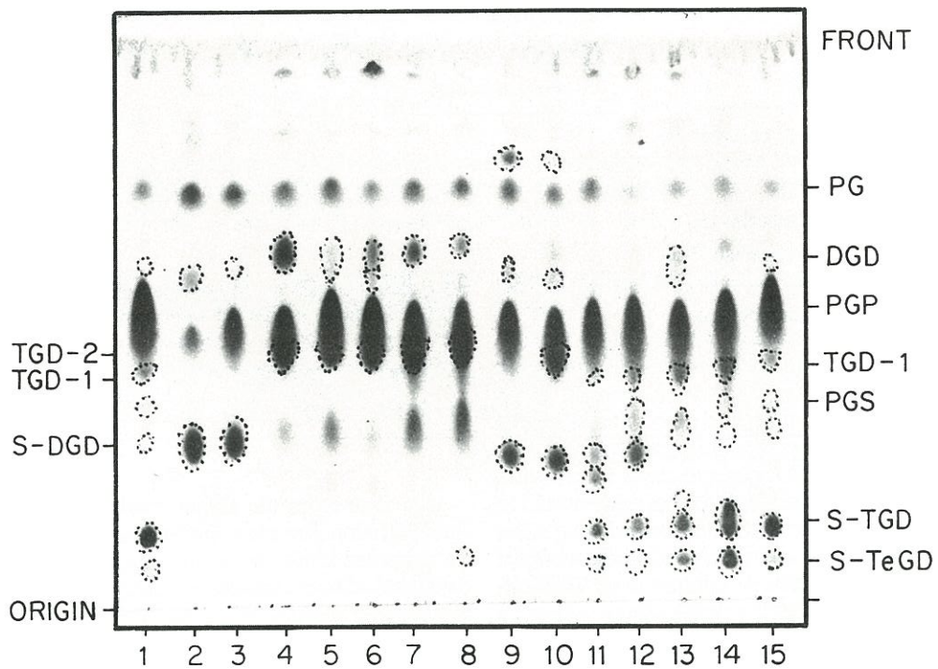


FIG. 1. Thin-layer chromatogram on precoated silica gel G of polar lipids from the following strains: 1, *H. cutirubrum*; 2, R-4; 3, Gla-2.2; 4, Y-27; 5, Gaa-3; 6, Gaa-6; 7, *H. vallismortis*; 8, *H. marismortui*; 9, M-2.5; 10, Ma-2.38; 11, *H. saccharovorum*; 12, Gca-19; 13, *H. salinarium*; 14, *H. halobium*; 15, *H. cutirubrum*. The solvent system was chloroform – methanol – acetic acid – water (85:22.5:10:4, by volume), double development. PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; PGS, phosphatidylglycerosulfate; DGD, diglycosyl diether; TGD-1, triglycosyl diether (galactosyl-mannosyl-glucosyl diether); TDG-2, triglycosyl diether (glucosyl-mannosyl-glucosyl diether); S-DGD, sulfated diglycosyl diether; S-TGD-1, sulfated triglycosyl diether; S-TeGD, sulfated tetraglycosyl diether; dotted spots are sugar positive.

TABLE 2. Effect of salt concentration on growth and lipid content of an extremely halophilic bacterium (strain R-4) from Spain and of *H. cutirubrum*

	Total salt concn., %							
	H. cutirubrum				Strain R-4			
	15	20	25	35	15	20	25	35
Growth of cells (g protein/L cell culture)	0.05	0.62	0.63	0.68	1.18	0.93	0.98	0.65
Lipid, % (on cellular protein basis)	9.3	7.1	10.2	11.3	5.1	4.6	3.3	3.3
Polar lipid (% of total lipid)	—	85.7	83.1	81.0	87.0	87.4	87.9	88.0
Nonpolar lipid (% of total lipid)	—	14.3	16.9	19.0	13.0	12.6	12.1	12.0

NOTE: Cells were grown under the conditions and in the medium described in Materials and methods, except that only the amount of NaCl was varied to give the total salt concentration indicated.

TABLE 3. Effect of salt concentration on production of carotenoids and other isoprenoids by an extremely halophilic bacterium (strain R-4) from Spain and by *H. cutirubrum*

	Total salt concn., %							
	H. cutirubrum				Strain R-4			
Compound, μg/g cell protein	15	20	25	35	15	20	25	35
β-Carotene	0	12	3	2	3	<0.5	0	0
Bacterioruberins ($R_1 + R_2$)	0	1560	1440	1410	420	80	22	30
Monoanhydrobacterioruberin ($R_3 + R_4$)	0	185	174	102	65	14	8	11
Bisanhydrobacterioruberin (R_5)	0	9	8	5	20	4	1	2
Total squalenes	—	—	—	6200	3700	2900	3900	4200
Vitamin MK-8	—	1500	1370	1110	1100	600	700	750

NOTE: Cells were grown as described in note to Table 2.

Although indole is not an isoprenoid compound and is not regarded as a lipid compound, it is extracted into the lipid fraction by the Bligh–Dyer procedure (Kushwaha *et al.* 1977). Its intracellular content was 100-fold greater in cells grown in 20% salt than those grown in 35% salt (data not shown).

Among the polar lipids, a gradual increase in the relative proportion of the sulfated diglycosyl diether (S-DGD) was observed as the total salt concentration was increased from 15 to 30% (Table 4). In contrast, the proportion of diphytanyl ether analogue of phospatidylglycerol was one-third as great in 30% salt as in 15% salt. The proportions of phosphatidylglycerophosphate were similar at 15 and 30% salt but a significantly lower value was found at 20% salt (Table 4).

It is clear from the above results that the strains of Spanish halophiles examined contain all the characteristic nonpolar lipids found in extreme halophiles, e.g., squalene, dihydrosqualene, tetrahydrosqualene, vitamin MK-8, and C_{50} pigments. These strains also show the presence of polar lipids derived from 2,3-di-*O*-phytanyl-*sn*-glycerol which are characteristic of the extremely halophilic bacteria studied previously (Kates 1978). However, a few strains (R-4, Gla-2.2, M-2.5, Ma-2.38) contain a novel sulfated diglycosyl diether (Kushwaha *et al.* 1982), which is not found in any of the well-known extreme halophiles.

One of the most interesting findings was that strain R-4 is much more strongly pigmented at lower than at higher salt concentrations, and this was also true of some

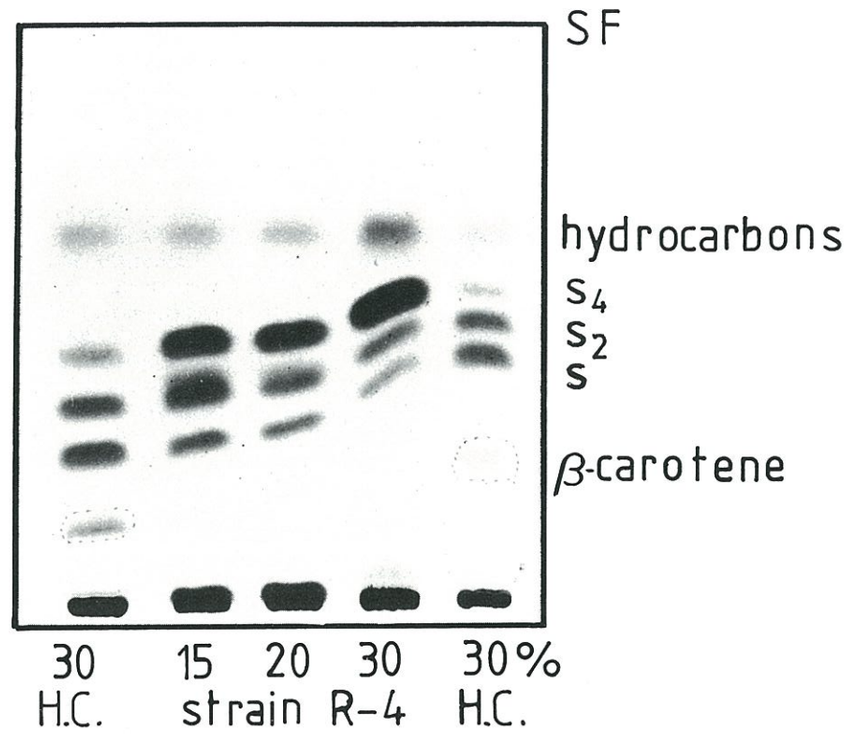


FIG. 2. Thin-layer chromatogram on silica gel H of squalenes from strain R-4 grown in media containing 15, 20, and 30% salt and from *H. cutirubrum* (H.C.) grown in 30% salt. The solvent system was petroleum ether – ethyl ether (99.7:0.3, v/v). S, squalene; S_2, dihydrosqualene; S_4, tetrahydrosqualene; SF, solvent front.

TABLE 4. Effect of salt concentration on the individual polar lipids of an extremely halophilic bacterium (strain R-4) from Spain

	Total salt concn., %		
Lipid, % weight of total polar lipids	15	20	30
PG	33	38	11
PGP	42	29	46
S-DGD	21	28	37
Other minor components	5	4	6

NOTE: Absolute contents of total polar lipids are given in Table 2. PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; S-DGD, sulfated glycodiosyl diether.

of the other Spanish halophiles (Rodríguez-Valera *et al.* 1980*a*, 1980*b*). In contrast, we found that *H. cutirubrum* is unpigmented in 15% salt but is strongly pigmented at salt concentrations of 20% and higher. *Halobacterium cutirubrum*, like other commonly studied halobacteria (e.g., *H. salinarium* and *H. halobium*), has a higher NaCl requirement for growth and more complex nutritional requirements (Kushner 1978) than the Spanish halophiles (Rodriguez-Valera *et al.* 1980*a*, 1980*b*).

Since pigmentation protects extreme halophiles from the harmful effects of visible light (Kushner 1978), we might expect that the differences in pigmentation response to salt could determine the progressive appearance of various halobacterial species in evaporating salterns. At approximately 15% salt, halobacteria such as R-4 would grow, producing large amounts of pigment. As the salt concentration is increased to 20%, halobacteria such as *H. cutirubrum* would begin to grow. With continued rise in salt concentration, pigmentation of the former halobacteria would decrease, and that of the latter would increase. Thus, the *H. cutirubrum* type of halophile might soon have a selective advantage at the higher salt concentrations under conditions of bright sunlight. We might also expect that in a saltern, less tolerant organisms would die as the salt concentration increased; this might supply amino acids and vitamins for the more nutritionally demanding halophiles of the *H. cutirubrum* type and further offset any advantage of those, such as R-4, which are able to grow on minimal media.

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Immunosuppression, nonspecific B-cell activation, and mitogenic activity associated with a high molecular weight component from *Listeria monocytogenes*

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OTOKUNEFOR, T. V., and S. B. GALSWORTHY. 1982. Immunosuppression, nonspecific B-cell activation, and mitogenic activity associated with a high molecular weight component from *Listeria monocytogenes*. Can. J. Microbiol. **28**: 1373–1381.

A high molecular weight component of a saline extract derived from *Listeria monocytogenes* contained amino acids, carbohydrates, and phosphorus. The same fraction was capable of promoting both the *in vitro* mitogenic and adjuvant activities and the *in vivo* immunosuppressive activity displayed by the crude extract. The material was mitogenic to B but not to T lymphocytes *in vitro*. Responses to sheep and horse erythrocytes as well as to lipopolysaccharide were suppressed. Immunosuppression was dose dependent and was present at 1, 2, or 3 days but absent 7 days after injection. Both primary and secondary responses to sheep erythrocytes were impaired.

OTOKUNEFOR, T. V., et S. B. GALSWORTHY. 1982. Immunosuppression, nonspecific B-cell activation, and mitogenic activity associated with a high molecular weight component from *Listeria monocytogenes*. Can. J. Microbiol. **28**: 1373–1381.

Un extrait en soluté salin de *Listeria monocytogenes* contient une substance de poids moléculaire élevé composée d'acides aminés, d'hydrates de carbone et de phosphore. Cette fraction possède des propriétés comparables à celles de l'extrait brut soit la capacité de stimuler *in vitro* l'activité mitogène et adjuvante et *in vivo* l'activité immunosuppressive. *In vitro* cette substance est mitogène pour les lymphocytes B mais pas pour les T. La réponse aux globules rouges de mouton et de cheval ainsi qu'au lipopolysaccharide est supprimée. L'immunosuppression dépend de la dose et se manifeste après 1, 2 ou 3 jours après l'injection, mais pas après 7 jours. La réponse primaire et secondaire aux globules rouges de mouton est diminuée.

[Traduit par le journal]

Introduction

Resistance to *Listeria monocytogenes* requires activation of macrophages by immunologically specific T cells (Mackaness 1969). The modulation of lymphocytes, macrophages, or both by surface components of *Listeria* may affect the ability of these cells to coordinate their activities for defense of the host. Cell walls of *Listeria* act as adjuvant and mitogen to lymphocytes *in vitro* (Campbell *et al*. 1975; Cohen *et al*. 1975). Killed cells (Jungi and McGregor 1977) and cell walls (Baker *et al*. 1977) are chemotaxigenic to macrophages, fix complement, and activate thioglycolate-induced macrophages *in vitro*.

A saline extractable fraction (SE) from lipid-depleted cells of *Listeria* possesses a monocytosis-producing activity (MPA) (Tadayon *et al*. 1970), has mitogenic and adjuvant properties in spleen cell cultures (Otokunefor *et al*. 1979), but paradoxically has immunosuppressive activity (ISA) when injected prior to antigen *in vivo* (Kim *et al*. 1976). ISA and MPA are separable by a two-step chromatographic procedure (Galsworthy *et al*. 1977). Purified material rich in MPA is devoid of the other activities (Otokunefor *et al*. 1979). Five fractions can be resolved from the crude extract by gel filtration. Of these, material in fraction A, estimated to have a molecular weight of approximately 150 000, emerges first as a discrete peak and induces the greatest immunosuppression. Materials in fractions B and C are less immunosuppressive and materials in fractions D and E are not suppressive (Galsworthy *et al*. 1977).

The purpose of these studies was to characterize the biological activities of the high molecular weight material (fraction A). Fraction A possessed the *in vitro* mitogenic and adjuvant activities described earlier (Otokunefor *et al*. 1979). Fraction A was mitogenic for B cells but not for T cells. Fraction A, when injected prior to antigen, suppressed the immune response *in vivo* to sheep erythrocytes (SRBC), horse erythrocytes (HRBC), and to lipopolysaccharide (LPS). The immunosuppression induced by SE and fraction A was dose dependent, was maximum 1, 2, or 3 days after injection of extract, but disappeared 7 days after injection of extract. Both the primary and secondary responses to SRBC were suppressed. The possible relationship between the *in vitro* mitogenic and adjuvant effects and *in vivo* immunosuppression is discussed.

Materials and methods

Bacteria

Listeria monocytogenes strain 42 (serotype 1) was obtained

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