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Biology of Moderately Halophilic Aerobic Bacteria

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INTRODUCTION

Compared to the extensive literature on the physiology, biochemistry, and ecology of the aerobic red halophilic archaea (family *Halobacteriaceae*), the aerobic halophilic bacteria have been relatively little studied. Research on the halophilic and halotolerant bacteria often seems to be less glamorous than the study of the archaea, with their unique adaptations, including a highly saline cytoplasm, specialized salt-requiring proteins, and the unique light-driven proton and chloride pumps bacteriorhodopsin and halorhodopsin (171). During early research on the microbiology of hypersaline environments, the halophilic bacteria were often neglected, even though they inhabit a wide range of habitats such as saline lakes, saltern ponds, desert and hypersaline soils, and salted foods, a range much less restricted than the habitats in which the halophilic archaea thrive (276, 284, 285).

However, Kushner (168) clearly states: "Though they are less exciting at first glance than the extreme halophiles the moderately halophilic bacteria, and solute-tolerant microorganisms in general, pose quite sufficiently interesting questions, especially those implied by their ability to grow over wide ranges of solute concentrations. Further work on these relatively little-studied microorganisms may be expected to bring dividends in the form of insight on the relation of internal and external solute concentrations, and on the state of cell-associated ions within the cytoplasm. If the last decade has been that of the extreme halophiles, we can hope that the next one will see their more modest, moderate cousins (in the spiritual sense only) take their proper place in the scientific canon." The moderately halophilic bacteria pose specific questions to the scientist, many of them related to their adaptability to a wide range of salinities. Thus, species such as *Salinivibrio costicola* and *Halomonas halodenitrificans* are able to grow over a range of water activities between 0.98 (close to freshwater) to 0.86 (close to saturated NaCl) (168). This in itself is a feat that may be much more difficult to achieve than the rigid, salt-requiring metabolism of the halophilic archaea, which lyse the moment the salt concentration in their environment drops below 10 to 15%.

The occurrence of nonpigmented halotolerant bacteria was probably first mentioned in 1919 by LeFevre and Round in their study of the microbiology of cucumber fermentation brines. One of the bacterial groups isolated grew in 0 to 15% NaCl, whereas other bacteria studied exhibited growth over the range of 5 to 25% (178). An early classic study of halophilic bacteria is the work of Hof (127), who inoculated salt mud from a solar salt facility on Java onto a variety of media of different salinities. In addition to red archaeal types, different types of white colonies were isolated, including endospore-containing *Bacillus* species able to grow at 24% NaCl. Using media containing between 12 and 18% salt, she isolated a *Pseudomonas*-type bacterium from salted beans preserved in brines varying in salt concentration from 6 to 29%. This organism, designated *Pseudomonas beijerinckii*, grew from 3 to 18% salt but not at 0.5%, showing its obligate halophilic character. To quote from Hof's paper: "it may be concluded that most of the important groups of bacteria are able to live in concentrations up to about 15% salt and that many groups are physiologically active even at much higher salt concentrations."

To describe microorganisms according to their behavior toward salt, different classification schemes have been devised. Although several classifications or categories have been proposed (274, 329, 351), the most widely used is that of Kushner, who defined moderate halophiles as organisms growing optimally between 0.5 and 2.5 M salt (168). Bacteria able to grow in the absence of salt as well as in the presence of relatively high salt concentrations (e.g., 8% in the case of *Staphylococcus aureus*) are designated halotolerant (or extremely halotolerant if growth extends above 2.5 M). A rare case of a bacterium that requires 2 M salt at least (optimal growth at 3.4 M), such as is exemplified by the actinomycete *Actinopolyspora halophila* (95), is considered a borderline extreme halophile (137, 168).

It should be pointed out that the salt requirement and tolerance of many species vary according to growth conditions such as temperature and medium composition. The growth temperature should be specified, especially for the definition of the lower salt range enabling growth. Thus, *Marinococcus halophilus* grows at NaCl concentrations as low as 0.01 M at 20°C but at least 0.5 M is required at 25°C (228). Similarly, *S. cos-*

TABLE 1. Main characteristics of moderately halophilic gram-negative members of the *Halomonadaceae*[a]

Feature	*H. elongata* (357)	*H. subglaciescola* (74)	*H. halodurans* (115)	*H. halmophila* (54)	*H. eurihalina* (200, 266)	*H. halophila* (269)
Morphology	Rods	Rods	Rods	Rods	Short rods	Rods
Size (μm)	ND	0.5–1.1 × 5–10	0.4–0.6 × 1.5–2.0	0.3–0.6 × 0.9–1.3	0.8–1.0 × 2.0–2.5	0.5–0.7 × 1.5–2.0
Pigmentation	None	Cream	None	Cream	Cream	Cream
Motility	+	+	+	+	−	+
Facultative anaerobe	+	−	−	−	−	−
Oxidase	+	+	+	+	−	+
NaCl range (%)	3.5–20	0.5–20	3.5–20	0.5–20	5–20	2–30
NaCl optimum (%)	3.5–8	ND	8	ND	7.5	7.5
Temp range (°C)	15–45	0–25	4–37	ND	15–45	15–45
pH range	5–9	5–9	5.5–8.5	ND	5–10	5–10
Acid production from:						
Arabinose	−	−	+	ND	−	−
Glucose	+	+	+	+	−	+
Lactose	−	+	−	−	−	−
Trehalose	ND	ND	ND	ND	−	ND
Mannitol	−	−	−	+	−	−
Hydrolysis of:						
Gelatin	d	−	−	−	+	−
Casein	−	d	ND	−	−	−
Starch	−	−	−	−	−	−
Esculin	−	−	+	−	+	+
Tween 80	−	−	ND	−	+	−
DNA	−	−	ND	+	+	−
H_2S production	−	−	−	ND	+	+
Nitrate reduction	+	+	−	−	+	+
Nitrite reduction	+	−	−	−	−	−
Phosphatase	ND	−	−	−	+	+
Type strain	ATCC 33173	UQM 2927	ATCC 29686	ATCC 19717	ATCC 49339	CCM 3662
Habitat	Salterns	Antarctic saline lakes	Estuarine water	Dead Sea	Saline soils, salterns	Saline soils
G+C content (mol%)	60.5	60.9–62.9	63.2	63	59.1–65.7	66.7
Phylogenetic branch	Gamma *Proteobacteria*	Gamma *Proteobacteria*	Gamma *Proteobacteria*	Gamma *Proteobacteria*	Gamma *Proteobacteria*	Gamma *Proteobacteria*

[a] +, positive; −, negative; d, differs among strains; ND, not determined.

ticola can grow between 0.5 and 4 M NaCl at 30°C but can grow down to 0.2 M at 20°C (168).

TAXONOMY AND PHYLOGENY

Moderately halophilic bacteria constitute a heterogeneous physiological group of microorganisms which belong to different genera. Our current knowledge of the taxonomic status of these bacteria contrasts with early studies, since in 1980 only six moderately halophilic species were included in the *Approved Lists of Bacterial Names* (312) and most strains used in physiological and biochemical studies were isolated from salted cured foods or unrefined salt or were even laboratory culture contaminants. These moderate halophiles were *Vibrio* (*Salinivibrio*) *costicola* (314), *Micrococcus* (*Nesterenkonia*) *halobius* (237), *Paracoccus* (*Halomonas*) *halodenitrificans* (159), *Flavobacterium* (*Halomonas*) *halmephilum* (60), *Planococcus* (*Marinococcus*) *halophilus* (228), and *Spirochaeta halophila* (103).

During the last decade, the extensive studies on hypersaline environments that have been carried out in many geographical areas have permitted the isolation and taxonomic characterization of a large number of moderately halophilic species. Thus, moderate halophiles are represented by several methanogenic archaea as well as strictly anaerobic bacteria that have been reviewed recently (182, 232) and are not included in this article. However, most species are gram-negative or gram-positive aerobic or facultatively anaerobic moderately halophilic bacteria (340, 341). Although some gram-negative species were considered members of different genera (*Halomonas*, *Deleya*, *Volcaniella*, *Flavobacterium*, *Paracoccus*, *Pseudomonas*, *Halovibrio*, or *Chromobacterium*), phenotypic and phylogenetic data support their close relationship, and they are currently included in the family *Halomonadaceae* as members of two genera: *Halomonas* and *Chromohalobacter* (53, 76). Table 1 shows the features that differentiate the validly published moderately halophilic species included in these two genera.

Besides members of the family *Halomonadaceae*, several other gram-negative strictly aerobic or facultatively anaerobic species have been described as moderate halophiles belonging to genera that include nonhalophilic species as well, such as *Pseudomonas*, *Flavobacterium*, or *Spirochaeta*, while others are placed in genera represented, at least until now, exclusively by halophilic species: *Salinivibrio*, *Arhodomonas*, or *Dichotomicrobium*. The differential features of these species are shown in Table 2.

Three organisms have been extensively used in physiological and biochemical studies dealing with the mechanisms of haloadaptation and adaptability: *Salinivibrio costicola*, *Halomonas elongata*, and *Halomonas israelensis.*

S. costicola was originally isolated from rib bones in a sample of Australian bacon (91, 314). Similar strains have been isolated from saltern ponds near Alicante (96) and other locations in Spain and the Canary Islands (91).

H. elongata was isolated from a solar salt facility on Bonaire, Netherlands Antilles. It is an extremely versatile organism, able to grow at a very wide range of salt concentrations. It can also grow anaerobically with nitrate as the electron acceptor,

TABLE 1—*Continued*

H. salina (53, 336)	*H. halodenitrificans* (53, 159)	*H. variablis* (53, 65)	*H. canadensis* (128)	*H. israelensis* (128)	*H. pantelleriense* (290)	*Chromohalobacter marismortui* (344)
Short rods	Short rods	Curved rods	Rods	Rods	Rods	Rods
0.7–0.8 × 2.0–2.5	0.5–0.9 × 0.9–1.2	0.5–0.8 × 1.0–3.0	ND	ND	0.4–0.7 × 1.4–2.6	0.6–1.0 × 1.5–4.0
Yellowish or cream	Cream	Cream	White	Cream	Cream-pink	Brown-yellow
−	−	−	+	ND	+	+
−	−	−	−	−	−	−
+	+	+	−	−	−	−
2.5–20	3–20	7–28	3–25	3.5–20	1.2–15	1–30
5	5–9	10	8	8	10	10
15–40	5–32	15–37	15–30	15–45	10–44	5–45
6–10	ND	6.5–8.4	5–9	5–9	7.5–11	5–10
−	ND	ND	ND	ND	ND	+
−	−	ND	ND	ND	ND	+
−	ND	ND	−	−	ND	+
−	ND	ND	ND	ND	ND	+
−	ND	ND	ND	ND	ND	ND
−	−	−	−	ND	−	−
−	ND	ND	−	ND	−	−
−	−	−	−	ND	−	−
−	−	+	ND	ND	ND	−
−	−	ND	ND	ND	ND	−
−	ND	ND	ND	ND	ND	−
+	−	−	−	−	ND	−
+	+	−	+	+	+	+
−	+	−	−	−	ND	−
−	−	ND	ND	ND	ND	−
ATCC 49509	ATCC 13511	DSM 3051	ATCC 43984	ATCC 43985	DSM 9661	ATCC 17056
Saline soils, salterns	Meat-curing brines	Great Salt Lake	Unknown (contaminant)	Dead Sea	Alkaline saline lake	Dead Sea, salterns
60.7–64.2	64–66	61	57	64	65	62.1–64.9
Gamma *Proteobacteria*	Gamma *Proteobacteria*	Gamma *Proteobacteria*	ND	ND	Gamma *Proteobacteria*	Gamma *Proteobacteria*

forming nitrite, and it has also been reported to grow fermentatively on glucose (357). However, glucose fermentation was not confirmed in later studies (83).

H. israelensis (previously designated strain Ba_1) was originally obtained from unrefined solar salt obtained from the Dead Sea (272, 273) and was only recently assigned a species name (128).

The gram-positive moderately halophilic aerobic bacteria, with the exception of two *Bacillus* species, belong to genera that include only species with halophilic requirements: the genera *Halobacillus*, *Marinococcus*, *Salinicoccus*, *Nesterenkonia*, and *Tetragenococcus*. The validly described species currently accepted as moderate halophiles and their characteristics are shown in Table 3. Finally, there are some moderately halophilic actinomycetes that have been recently isolated from different saline soil samples and have been characterized as species of the genera *Actinopolyspora* or *Nocardiopsis*. Table 4 shows the differential characteristics of these four moderately halophilic species.

Besides these moderately halophilic species that have been characterized taxonomically and, according to the rules of the code of nomenclature of bacteria, are considered validly described species, there are several other moderately halophilic aerobic bacteria that have been used for other purposes, including physiological, biochemical, and biotechnological studies, but have not been studied taxonomically in detail. Typical examples are "*Pseudomonas halosaccharolytica*" (120, 121) and "*Micrococcus varians* subsp. *halophilus*" (241).

Recent studies based on 16S rRNA sequence analysis have permitted a determination of the phylogenetic position of most moderately halophilic bacteria. During last decade, the old technique of comparison of 16S rRNA oligonucleotide catalogs showed that *Spirochaeta halophila* belongs to the spirochete phylum and that some *Halomonas* and *Deleya* species are members of the gamma subclass of the *Proteobacteria* (341). They were placed in a new family, *Halomonadaceae* (76). More recently, complete 16S rRNA sequence analysis confirmed that *Spirochaeta halophila* is within the *Spirochaeta* cluster of the spirochete phylum, related to *Spirochaeta isovalerica*, *S. litoralis*, *S. bajacaliforniensis*, and *S. aurantia* (average similarity, 87.4%) (253). In addition, several studies have identified the phylogenetic position of most gram-negative moderately halophilic aerobic species currently described. Members of the genera *Halomonas*, *Deleya*, *Halovibrio*, and *Volcaniella*, as well as *Paracoccus halodenitrificans*, form a monophyletic group within the gamma subclass of the *Proteobacteria* (53, 56, 200, 204, 290). The levels of 16S rRNA sequence similarity among these species ranged from 91.5 to 100%; although several subgroups, which might represent separate genera, were resolved, they could not be differentiated on the basis of phenotypic or chemotaxonomic features. For these reasons, Dobson and Franzmann (53) proposed placing all members of the above four genera and *P. halodenitrificans* in a single genus, the genus *Halomonas*, and emended the description of the family *Halomonadaceae*. This family now comprises the species of *Halomonas* and *Zymobacter* and the moderate halophile, originally isolated from the Dead Sea, *Chromohalobacter marismortui* (200). All have 15 signature characteristics in their 16S rRNA

TABLE 2. Main characteristics of other aerobic or facultatively anaerobic gram-negative moderately halophilic bacteria[a]

Feature	*Salinivibrio costicola* (91, 201)	*Pseudomonas halophila* (65)	*Flavobacterium gondwanense* (52)	*Flavobacterium salegens* (52)	*Arhodomonas aquaeolei* (4)	*Spirochaeta halophila* (103)	*Dichotomicrobium thermohalophilum* (124)
Morphology	Curved rods	Rods	Rods	Rods	Short rods	Helicoidal	Prosthecate
Size (μm)	0.5 × 1.5–3.2	0.8–1.0 × 1.5–5.0	0.4–0.8 × 1.7–11.7	0.5–0.8 × 1.2–11.5	0.8–1.0 × 2.0–2.5	0.4 × 15–30	0.8–1.8 × 0.8–2.0
Pigmentation	Cream	Reddish-brown	Orange	Yellow	None	Red	Reddish-brown
Motility	+	+	−	−	+	+	−
Facultative anaerobe	+	−	−	−	−	+	−
Oxidase	+	+	+	+	+	ND	+
NaCl range (%)	0.5–12	0.1–20	0–5	0–20	6–20	0.5–8	0.8–22
NaCl optimum (%)	10	5	5	5	15	5	8–14
Temp range (°C)	5–45	4–37	0–30	ND	20–45	25–40	20–60
pH range	5–10	4.5–9.6	5–9	5–9	6–8		5.8–9.5
Acid production from:							
Arabinose	−	ND	−	+	−	ND	ND
Glucose	+	ND	−	+	−	+	−
Lactose	−	ND	−	+	−	ND	ND
Trehalose	+	ND	ND	ND	−	ND	ND
Mannitol	+	ND	ND	+	−	ND	ND
Hydrolysis of:							
Gelatin	+	+	d	+	−	ND	−
Casein	+	+	−	−	−	ND	−
Starch	−	−	+	+	−	ND	−
Esculin	+	+	+	+	−	ND	ND
Tween 80	d	ND	+	+	+	ND	ND
DNA	d	ND	+	+	−	ND	−
H_2S production	d	−	−	−	−	ND	−
Nitrate reduction	−	−	−	+	+	+	−
Nitrite reduction	−	−	−	−	−	ND	−
Phosphatase	−	ND	+	+	−	ND	ND
Type strain	NCIMB 701	DSM 3050	DSM 5423	DSM 5424	ATCC 49307	ATCC 29478	DSM 5002
Habitat	Salterns	Great Salt Lake	Hypersaline Antarctic lake	Hypersaline Antarctic lake	Petroleum reservoir producing fluid	Solar Lake	Solar Lake
G+C content (mol%)	49.4–50.5	57.0	35–39	39–41	67	62	62–64
Phylogenetic branch	Gamma *Proteobacteria*	ND	*Flavobacterium-Bacteroides*	*Flavobacterium-Bacteroides*	Gamma *Proteobacteria*	Spirochetes	ND

[a] +, positive; −, negative; d, differs among strains; ND, not determined.

sequences, including a distinctive cytosine residue at position 486 (53). *Arhodomonas aquaeolei* represents a deeply branching lineage in the gamma subclass of the *Proteobacteria*, most closely related to purple sulfur bacteria (particularly species of the genera *Ectothiorhodospira* and *Chromatium*). The 16S rRNA sequence analysis supports the placement of this single species in a separate genus (4).

Very recently, the phylogenetic position of six (*Salini*)*vibrio costicola* strains revealed that this moderate halophile constitutes a monophyletic branch that is distinct from other *Vibrio* species and from other species belonging to the gamma subclass of the *Proteobacteria* (202). Since other phenotypic and genotypic data supported these differences, placement of this species in a separate genus, *Salinivibrio*, has been proposed (201).

The 16S rRNA sequences of *Flavobacterium gondwanense* and *F. salegens*, two moderate halophiles isolated from a hypersaline Antarctic lake, contain the definitive flavobacterial signatures that unequivocally place them in the *Flavobacterium-Bacteroides* phylum (52). These species cluster with a group of organisms that contains the type species of the genus *Flavobacterium*, *F. aquatile* (with 89 and 90% sequence similarity between them and this species) (52).

Recent studies have determined the phylogenetic relationships of moderate halophiles within the gram-positive branch. Farrow et al. (64) showed that *Marinococcus halophilus* (formerly *Planococcus halophilus*) forms a distinct line of descent and is only distantly related to the genera *Planococcus*, *Sporosarcina*, and *Bacillus*. The 16S rRNA sequence data confirm the placement of *M. halophilus* in a separate genus. In addition, they indicated that *Sporosarcina halophila*, an endospore-forming motile gram-positive moderate halophile, was not closely related to *Sporosarcina ureae*, the type species of this genus. Later studies permitted the placement of *S. halophila* in a new genus, *Halobacillus*, as *H. halophilus*, closely related to other moderately halophilic species isolated from the Great Salt Lake, *H. litoralis* and *H. trueperi* (315). This study also confirmed the placement of *Salinicoccus roseus* in a separate genus, since it constitutes a deep branch not closely related to other gram-positive bacteria. *Bacillus salexigens* is closely related to *Bacillus pantothenticus*, a species that belongs to phylogenetic group I of the genus *Bacillus*, as well as to *Halobacillus halophilus*, *H. litoralis*, and the halotolerant species *Bacillus dipsosauri* (89). *Tetragenococcus muriaticus*, a recently described species isolated from a traditionally fermented Japanese fish sauce, is closely related to the halotolerant species *Tetragenococcus halophilus* (formerly *Pediococcus halophilus*), which showed a closer phylogenetic relationship to other lactic acid bacteria of the enterococci and lactobacilli than to pediococci (304).

While all these moderately halophilic species belong to the low-G+C group of the gram-positive phylum, only *Nesterenkonia halobia* (formerly *Micrococcus halobius*) is within the high-G+C group (218, 316), constituting a cluster that is clearly separate from other species of the genera *Micrococcus*, *Arthrobacter*, and *Kocuria.*

The recent phylogenetic studies of moderate halophiles have been based on the comparison of 16S rRNA sequence data. These studies have been very helpful for improving the classification of moderate halophiles according to a natural (phylogenetic) approach. In particular, results show that they are represented in many of the major bacterial phyla: spirochetes, *Proteobacteria*, *Flavobacterium-Bacteroides*, and low-G+C and high-G+C gram-positive organisms. A recent study of African soda lakes showed a wide phylogenetic diversity within the alkaliphilic (and in some cases halophilic) isolates (58). As with the moderate halophiles, these authors concluded that the alkaliphile phenotype is also polyphyletic and might have evolved many times (58).

ECOLOGY

Numerical Taxonomy Approaches to the Analysis of Natural Communities of Moderately Halophilic Bacteria

(i) Salt lakes and brines. The communities of moderately halophilic bacteria in thalassohaline (seawater-derived) hypersaline environments, such as saltern ponds for concentrating seawater, may to a large extent resemble the communities present in seawater. This is not too surprising, since many marine bacteria have a broad salt tolerance. It was even reported that the majority of 30 isolates of marine aerobic heterotrophic bacteria tested could grow at up to 20% NaCl and that some could even multiply in media containing 30% NaCl (72). Moderately halophilic bacteria could also be enriched from seawater by gradual salinity increases: when seawater was periodically amended with salt and nutrients, moderately halophilic bacteria outcompeted the slightly halophilic marine bacteria, which completely disappeared above 15% salt (347). A variety of halophilic bacteria were also isolated from sea sands and seaweeds (235). Thus, the sea contains many moderately halophilic or at least extremely halotolerant bacteria. In a study of Spanish saltern ponds of intermediate salinity (between 15 and 30% sea salts) (Alicante on the Mediterranean coast, Huelva on the Atlantic coast), the dominant types of colonies developing on agar plates were assigned by numerical taxonomy to the genera *Salinivibrio*, the *Pseudomonas-Alteromonas-Alcaligenes* group, *Acinetobacter*, and *Flavobacterium* (284–286). Most of these isolates should probably be reclassified in the family *Halomonadaceae*. Most isolates grew optimally at about 10% salts (the concentration that also yielded the largest number of CFU on agar plates) and could be found at salt concentrations up to about 25%. (*Salinivibrio* species dominated below 15% salt, while bacteria assigned to the *Pseudomonas-Alteromonas-Alcaligenes* group were especially abundant above 15%. *Flavobacterium* and *Acinetobacter* were found in smaller numbers and were evenly distributed up to 30%, while gram-positive cocci were found mostly above 25% salt (286). Most of the 140 isolates from the Huelva salterns clustered in eight phenons, two of which were identified as *Halomonas* (*Deleya*) (53), four resembled *S. costicola*, and the others were tentatively assigned to the genera *Flavobacterium* and *Acinetobacter* (187). Similar *Acinetobacter*-like bacteria (probably to be assigned to the *Halomonadaceae*) have been found in other hypersaline habitats (265).

Numerical taxonomic studies have been performed in inland, athalassohaline salterns near Granada, Spain (39, 41, 274), and Chile (262). The La Malá saltern near Granada is fed by brines from a subterranean well which is lower in Cl^- and higher in Mg^{2+}, Ca^{2+}, and K^+ than seawater. Of the 174 strains isolated, 74 were assigned to the genus (*Salini*)*vibrio*, 22 were assigned to *Alteromonas*, 43 were assigned to *Halomonas* (*Deleya*) (53), 7 were assigned to *Acinetobacter*, 13 were assigned to *Pseudomonas*, and 9 were assigned to *Flavobacterium* (37). An even more unusual inland saltern is the Salar de Atacama, Chile, located in the Atacama desert at 2,700 m above sea level. The most abundant isolates obtained from this environment were (*Salini*)*vibrio* strains, followed by *Acinetobacter*, *Marinomonas*, and *Alteromonas* (262).

Lake Assal in Djibouti (French Somaliland) contains 27.7% salts in its surface layers, increasing to 39.8% at a depth of 20 m. Of the 164 isolates obtained, 11 were moderate halo-

TABLE 3. Main characteristics of aerobic gram-positive moderately halophilic bacteria[a]

Feature	*Halobacillus*			*Bacillus*	
	H. halophilus (33, 315)	*H. litoralis* (315)	*H. trueperi* (315)	*B. halophilus* (342)	*B. salexigens* (89)
Morphology	Coccoid	Rods	Rods	Rods	Rods
Size (μm)	1.0–2.0 × 2.0–3.0	0.7–1.1 × 2.0–4.5	0.7–1.4 × 2.0–4.5	0.5–1.0 × 2.5–9.0	0.3–0.6 × 1.5–3.5
Pigmentation	Orange	Orange	Orange	None	None
Spore shape	S	E/S	E/S	E	E
Spore position	C/ST	C/ST	C/ST	C	ST/C
Sporangium swollen	−	ND	ND	−	+
Motility	+	+	+	+	
Facultative anaerobe	−	−	−	−	−
Oxidase	+	+	+	+	+
NaCl range (%)	2–20	0.5–25	0.5–30	3–30	7–20
NaCl optimum (%)	10	10	10	15	10
Temp range (°C)	15–40	10–43	10–44	15–50	15–45
pH range	7–9	6–9.5	6–9.5	6–8	6–11
Acid production from:					
Arabinose	−	ND	ND	−	ND
Glucose	−	+	+	+	+
Lactose	ND	ND	ND	−	−
Trehalose	−	+	+	+	−
Mannitol	−	+	−	−	+
Hydrolysis of:					
Gelatin	+	+	+	−	+
Casein	+	−	−	−	+
Starch	+	−	−	−	−
Esculin	−	−	−	+	+
Tween 80	−	−	−	ND	−
DNA	+	+	+	+	+
H_2S production	ND	ND	ND	−	+
Nitrate reduction	−	−	−	−	−
Nitrite reduction	−	−	−	−	−
Phosphatase	d	−	−	−	d
Voges-Proskauer	−	−	−	−	−
Menaquinone system	MK-7	ND	ND	MK-7	ND
Cell wall type	Orn–D-Asp	Orn–D-Asp	Orn–D-Asp	*m*-Dpm	*m*-Dpm
Type strain	DSM 2266	DSM 10405	DSM 10404	ATCC 49085	ATCC 700290
Habitat	Saline soils, salterns	Great Salt Lake	Great Salt Lake	Unknown (rotting wood from seawater)	Salterns, saline soils
G+C content (mol%)	40.1–40.9	42	43	51.5	36.3–39.5
Phylogenetic branch	Low-G+C gram-positive	Low-G+C gram-positive	ND	ND	Low-G+C gram-positive

[a] S, spherical; E, ellipsoidal; C, central; ST, subterminal. +, positive; −, negative; d, differs among strains; ND, not determined. MK-9, MK-8, MK-7, MK-6, menaquinone with nine, eight, seven, and six isoprene units, respectively. *m*-Dpm, *meso*-diaminopimelic acid.

philes requiring 3 to 15% salt, 7 were extremely halophilic archaea, 2 did not tolerate salt, and the great majority were slightly halophilic, requiring 1 to 5% NaCl (16). The authors concluded that "it is clear that the bacteria were, on the whole, common bacteria belonging to the same species which are currently isolated from large and small rivers and from seawater." This result is unexpected in view of the high salt concentrations prevailing in Lake Assal and the high temperature of the water (33 to 34°C), which seem more suitable to the development of extremely halophilic archaea than of marine-type slightly or moderately halophilic bacteria. Regretably, additional studies of this interesting but poorly accessible environment have not been reported.

A sadly neglected hunting ground for interesting new types of moderately halophilic bacteria is the Great Salt Lake, Utah, which has seen drastic changes in its salinity during the last decades. Most studies of its microbiology have centered around the red halophilic archaea and the *Dunaliella* communities (244, 258, 261). Attempts to enumerate viable bacteria in complex medium with 5, 13, and 20% NaCl yielded 3.6×10^5, 9.3×10^5, and 2.4×10^6 CFU/ml, respectively, with surface water from the north arm of the lake (22% salt), and 7.5×10^5, 1.1×10^5, and 1.5×10^3 cells/ml, respectively, when surface water from the south arm (8.5% salt) served as the inoculum (65). The data presented do not allow a differentiation between colorless bacteria and red archaea, but it was stated that moderately halophilic bacteria make up the predominant population in the south arm whereas extremely halophilic archaea predominate in the north arm. The Great Salt Lake yielded a number of new species: *Pseudomonas halophila*, *Halomonas variabilis* (53, 65), *Halobacillus litoralis*, and *Halobacillus trueperi* (315).

Although halophilic bacteria can easily be isolated from the Dead Sea by means of enrichment cultures (242, 349), no systematic studies have been performed on their abundance in this extremely hypersaline (presently around 34%), athalassohaline (around 1.8 M Mg^{2+}, 1.7 M Na^+, 0.4 M Ca^{2+}, and 0.14 M K^+) chloride lake. The first prokaryotes isolated from the

TABLE 3—*Continued*

Marinococcus		*Salinicoccus*		*Nesterenkonia halobia* (218, 316)	*Tetragenococcus muriaticus* (304)
M. halophilus (111, 189)	*M. albus* (111)	*S. roseus* (344)	*S. hispanicus* (188, 345)		
Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
1.0–1.2	1.0–1.2	1.0–2.5	1.0–2.0	ND	0.5–0.8
Yellow-orange	None	Pink red	Reddish-orange	None or yellow	White
−	−	−	−	−	−
−	−	−	−	−	−
−	−	−	−	−	−
+	+	−	−	−	−
−	−	−	−	−	+
−	+	+	+	+	−
2–25	2–25	0.9–25	0.5–25	3–25	1–25
10	10	10	10	10	7–10
15–37	15–37	15–40	15–37	20–40	15–40
ND	ND	6–9	5–9	5–10	5–9.6
−	−	−	−	−	−
+	−	−	+	+	+
−	−	−	−	−	−
+	−	−	−	−	+
+	−	−	+	−	+
+	−	+	+	+	ND
+	−	+	−	+	ND
−	−	+	−	+	ND
+	−	−	+	+	ND
−	−	+	−	−	ND
−	+	+	+	+	ND
−	−	−	−	−	ND
−	+	−	+	−	−
−	−	−	+	−	−
−	−	+	d	−	ND
−	−	−	−	−	ND
MK-7	MK-7	MK-6	MK-6	MK-9, MK-8, MK-7	ND
m-Dpm	*m*-Dpm	L-Lys–Gly_5	L-Lys–Gly_5	L-Lys–Gly–L-Glu	Lys–D-Asp
ATCC 27964	CCM 3517	ATCC 49258	ATCC 49259	ATCC 21727	JCM 10006
Salterns, saline soils	Salterns	Salterns	Salterns, saline soils	Salterns	Fermented squid liver sauce
46.4	44.9	51.2	45.6–49.3	ND	36.5
Low-G+C gram-positive	ND	Low-G+C gram-positive	ND	High-G+C gram-positive	ND

lake (which at the time contained 28 to 29% salt) were probably colorless bacteria (362). Some of the early isolates were characterized further and described as *Chromohalobacter* (originally *Chromobacterium*) *marismortui* (343), "*Pseudomonas halestorgus*" (subsequently lost), and *Halomonas* (originally *Flavobacterium*) *halmophila* (54, 60, 350). Another colorless isolate from the Dead Sea is *Halomonas israelensis* (also known as strain Ba_1) (128, 170).

Subterranean brines have also been subject to microbiological studies. Several euryhaline microorganisms belonging to the *Halomonadaceae*, possibly including some novel species, were isolated from Permian underground salt formations in the United States which were penetrated by meteoric waters which slowly solubilize the salt (356). A subterranean brine associated with an oil field in Oklahoma yielded *Arhodomonas aquaeolei*, an aerobic, organic acid-metabolizing, gram-negative, motile rod, growing in the range of 6 to 20% NaCl with an optimum of 15% (4).

(ii) Saline soils. The soil habitat is inherently inhomogeneous, and it can be expected that a wide range of salinities might be present in any one saline soil (101). Saline soils appear to yield mostly halotolerant rather than halophilic microorganisms, presumably reflecting adaptation to periodic episodes of relatively high dilution (267, 268). One early study stated that microorganisms may be unable to multiply in saline soil and that the microbiota of saline soil habitats are passive inhabitants brought by the wind. In this study, soils near the Red Sea, with a salt content varying from 25 to 30% on the surface to 1.5 to 2% at a depth of 50 cm, were examined. The highest bacterial counts were obtained in surface soil, using media without NaCl. The numbers obtained were much smaller than those commonly found in nonsaline soils. The authors stated that "the accepted assumption about the widespread distribution of salt-resistant and halophilic microorganisms in saline soils requires reconsideration" (116).

Later studies have unequivocally confirmed the abundance of halophilic bacteria in saline soils. The species composition in soils differs greatly from that of the aquatic environments discussed above: while *Salinivibrio* abounds in salt lakes and salterns, the dominant types encountered in saline soils belong to genera such as *Bacillus*, *Pseudomonas*, *Alcaligenes* (last two probably to be reclassified as members of the *Halomonada-*

TABLE 4. Main characteristics of moderately halophilic actinomycete species[a]

Feature	*Actinopolyspora*		*Nocardiopsis*	
	A. mortivallis (374)	*A. iraquiensis* (292)	*N. lucentensis* (371)	*N. halophila* (6)
Spore chain morphology	Short (no more than 10)	Short (no more than 15)	Short	Very long
Pigmentation	Yellowish white	Yellow-brownish	Yellowish brown	Yellow-coral red
Spore	Oval to cylindrical	Spheroidal	Cylindrical	Cylindrical
NaCl range (%)	5–30	5–20	ND	5–20
NaCl optium (%)	10–15	10–15	5–10	15
Temp range (°C)	10–50	16–40	ND	16–40
Acid production from:				
Glucose	+	ND	+	−
Mannitol	−	+	+	+
Xylose	+	−	−	+
Nitrate reduction	−	ND	+	ND
Hydrolysis of:				
Gelatin	+	ND	+	ND
Casein	+	ND	+	ND
Esculin	ND	−	+	−
Starch	ND	+	+	−
Tyrosine	ND	+	+	−
Tween 80	+	+	ND	+
Urea	−	−	−	+
Xanthine	+	+	+	+
Menaquinone system	MK-9 (H_4), MK-10 (H_4)	MK-9 (H_4)	MK-10 (H_8)	MK-10 (H_6, H_8)
Cell wall type	IV	IV	III	III
Phospholipid type	PIII	PIII	PIII	PIII
Mycolic acids	−	−	−	−
Type strain	JCM 7550	A.S.4.1193	DSM 44048	A.S.4.1195
Habitat	Saline soil	Saline soil	Saline soil	Saline soil

[a] +, positive; −, negative; ND, not determined. MK-10 (H_8, H_6, and H_4), octa-, hexa-, and tetrahydrogenated menaquinones with 10 isoprene units; MK-9 (H_4), tetrahydrogenated menaquinone with 9 isoprene units.

ceae), and *Micrococcus* (possibly *Nesterenkonia*) (268, 285). A saline soil near Alicante, Spain, with a Cl^- content between 2.4 and 12.7% yielded a high proportion of nonmotile rods on media containing 10 or 20% salt; this type of bacterium is only rarely isolated from aquatic hypersaline environments (265). The most abundant types were assigned to the genera *Alcaligenes*, *Acinetobacter*, *Flavobacterium*, and *Pseudomonas*. Many halotolerant gram-positive bacteria were also found. Facultative anaerobes were rare. Most isolates required moderately high salt (5 to 10%) for optimal growth but were also able to grow at low salinities (0.5 to 2%) (267). This probably reflects the ecological difference between water and soil environments, with water being relatively homogeneous and constant and soil being heterogeneous and affected by factors such as rainfall (267, 268). A study was made of the rhizosphere soil near xerophytic plants growing in hypersaline soils (5 to 10.7% NaCl) near Alicante, showing that the range of salt concentrations allowing growth of the organisms isolated did not correlate with the salinity of the soil from which they were isolated. Most isolates showed salt optima between 5 and 15% NaCl, but about half of the strains also grew at 0.9% NaCl. Plating on agar media containing 10% salt yielded mostly gram-positive rods, whereas gram-negative rods dominated between 10 and 20% salt and Gram-positive cocci developed above 20% salt. The gram-positive bacteria thus isolated were assigned to the genera (in order of abundance) *Bacillus*, *Micrococcus*, *Arthrobacter*, *Staphylococcus*, *Planococcus*, *Corynebacterium*, *Nocardia*, and *Actinomyces* (268).

Saline soils have been somewhat neglected compared to hypersaline aquatic environments. The recent isolation of novel halophilic *Actinopolyspora* and *Nocardiopsis* species from salty soils in Death Valley (Calif.), Alicante, and Iraq (6, 291, 370, 373) suggests that a wealth of interesting unknown halophilic microorganisms may be present in these soils.

(iii) Cold saline habitats. Extensive microbiological studies in the Antarctic, especially the cold saline lakes in the Vestfold Hills region and the saline soils of the Dry Valleys, have contributed some interesting insights into the extreme conditions under which halophilic bacteria may occur and thrive.

The Vestfold Hills region is a coastal, ice-free area in east Antarctica, which contains in excess of 300 lakes and ponds. These are relics of seawater catchments isolated some 6,000 years ago by uplift and trapped in valleys and depressions. Some of these lakes are hypersaline; the most saline lakes have a total salt concentration of up to 28%. Salinity may show seasonal variations due to meltstream influx and ice cover melt in the Austral summer. The best studied is Organic Lake, a meromictic lake with a maximum depth of 7.5 m. The lake is stratified, with salt concentrations increasing from 0.8 to 21%, and is anoxic below a depth of 4 to 5 m. The ice cover excludes wind-induced turbulence throughout winter. Thermal profile and the increasing salinity with depth prevent turnover in the ice-free summer period. Temperatures range from −14 to +15°C (73, 195, 196).

Many strains of moderate halophiles, belonging to genera including *Halomonas*, *Flavobacterium*, and *Cytophaga*, were isolated from the lake. Hexadecane- and phenanthrene-degrading bacteria were also found (196). Most isolates were able to grow from 0.5 to 20% NaCl and at temperatures as low as 0 to 5°C (55, 74). The species isolated include *Halomonas subglaciescola*, growing at salt concentrations between 0.5 and 20%, with a predicted minimum temperature of −3.3 to −9.2°C, an op-

timum at 20.0 to 23.4°C, and a maximum growth temperature of 29.7 to 32.3°C (74) (the observed minimum temperature was −5.4°C) (196); *Halomonas meridiana*, with two varieties: biovar I grows optimally at 1 to 3% NaCl and 28 to 40°C and tolerates NaCl up to 20 to 25% and temperatures up to 45°C, while biovar II grows best at 0.5 to 3% NaCl and 34 to 38°C and tolerates up to 25 to 30% NaCl and 47°C but is also able to grow at −5°C (134); and two yellow-orange isolates, named *Flavobacterium gondwanense* and *F. salegens*, both showing optimal growth at 5% NaCl and growing at up to 15 to 20% NaCl (52, 55).

The bacteria of Antarctic saline environments display an ability to grow at reduced temperatures compared with their taxonomic counterparts from tropical and temperate environments (195). It was postulated that since the saline lakes of the Vestfold Hills are geologically young, the resident bacteria have not yet evolved mechanisms that may enable them to grow in these environments in winter when temperatures fall below their minimum required for growth and that, given further evolution, organisms that can exploit these environments more fully may develop (73).

Also, the soils of the Antarctic Dry Valleys have yielded some interesting salt-tolerant bacteria, such as a halotolerant *Planococcus* that grows from 0 to 40°C in the presence of 0 to 2 M NaCl (207). Additional halotolerant gram-positive cocci were obtained from saline soils in geothermal regions in Antarctica. One such isolate, tentatively assigned to the genus *Micrococcus*, tolerates NaCl concentrations between 0 and 4.2 M (221). Since this strain originated from a thermal area with in situ temperatures of up to 40°C, it is not surprising that it does not share the psychrophilic or psychrotolerant properties of the other strains mentioned above: its optimum temperature is 37°C, and no growth was observed below 20°C.

(iv) Alkaline saline habitats. Stable alkaline hypersaline environments are not common and are the result of an unusual combination of geological, geographical, and climatic conditions (102). Most studies on the alkaline saline environments have concentrated on lakes such as Lake Magadi, Kenya, and the Wadi Natrun lakes, Egypt, which are dominated by extremely halophilic archaea.

A novel representative of the *Halomonas* group was recently isolated from sand of Venere Lake, Pantelleria Island, Italy. This aerobic pleomorphic rod, designated *Halomonas pantelleriense*, grows optimally at pH 9 (range, 7.5 to 11) and 10% NaCl (range, 1.25 to 15%) (290). Some interesting gram-negative halophilic bacteria were obtained from the alkaline (pH 9.8), saline (9% total salts) Mono Lake, Calif. One strain was isolated on a medium containing glycine betaine as the major carbon and energy source. Others were isolated on a medium containing dimethylsulfoniopropionate (DMSP). All isolates grew at NaCl concentrations from 1.5 to 3 M, and growth was much better at pH 9.7 than at pH 7. Glycine betaine is degraded by sequential demethylation via dimethylglycine to sarcosine, which is excreted into the medium. DMSP is either cleaved to dimethyl sulfide and acrylate or degraded by demethylation, with 3-methiolpropionate as the intermediate, yielding methanethiol (32, 51). Glycine betaine and DMSP may be found as substrates for bacterial degradation in Mono Lake, since they are produced by cyanobacteria and eukaryotic phytoplankton as osmotic solutes.

A haloalkaliphilic gram-positive bacterium was isolated from Lake Gabara in the Wadi Natrun, Egypt, and named *Bacillus haloalkaliphilus*; this isolate tolerates up to 4 M NaCl but grows best from 0.5 to 3 M (327, 361). Additional strains assigned to the species *B. haloalkaliphilus* have been isolated from brine, dried soil, mud and dung samples from the Wadi Natrun (78).

(v) Salted fish, meat, and other foods. Although moderately halophilic bacteria are often found on salted fish, meat, and other food products and regardless of their possible involvement in microbial spoilage at high salt concentrations, systematic studies on the occurrence of such bacteria on fish and meat products have rarely been performed.

Moderate halophiles can easily be isolated from materials such as salted and dried fish, fish intestines, soy sauce mashes, and other similar materials (235, 307). Recently, 128 strains of moderate halophiles were isolated from bachalao (dried salted codfish) and from fresh cod and curing salt used in its preservation. In fully cured wet and dry bachalao (which contains about 19% salt), between 10^3 and 10^7 moderate halophiles were found per g. Two primary colony types, smooth and rough, dominated in wet and dry bachalao, respectively. The bacteria forming the smooth colony type are similar to *Halomonas salina* and grew in 0.1 to 4.5 M NaCl at 15 to 37°C (348). In the curing of anchovies, *Pediococcus halophilus* became the dominant bacterium at the end of the curing process; this organism develops under both aerobic and anaerobic conditions. It showed optimum growth at 6.5 to 10% NaCl and tolerated over 15% NaCl (349).

(vi) Unusual habitats. Moderately halophilic bacteria may be found in some unusual environments, such as on desert plants and desert animals.

Atriplex halinus (family *Chenopodiaceae*) is a desert plant widespread in the Negev Desert, Israel, and in other desert environments. The leaves excrete salt through salt glands, the number and size of which depend on the amount of salt present during growth of the plant. Salt (predominantly NaCl) crystallizes on the leaves when the salt gland bladders burst. During the dry season, significant amounts of salts and organic material coat the leaf surface. The nightly occurrence of dew causes a diurnal wetting, so that the phylloplane microorganisms experience large fluctuations in salinity and water activity, including repeated desiccation. Between 1×10^4 and 5×10^5 bacteria were enumerated per cm^2 of leaf surface. The diversity of culturable bacteria was limited, with the dominant organism being an orange pigmented bacterium, identified as a *Pseudomonas* sp., growing from 0.05% to 20% NaCl with an optimum at 5% and 30°C (311).

Even more unusual is the isolation of a halotolerant *Bacillus* sp. from the nasal cavities of desert iguanas. These animals possess salt glands in their nasal cavities that allow them to excrete a concentrated KCl brine during osmotic stress. A *Bacillus* sp. was isolated, showing excellent growth in 2.2 M KCl and with an optimum growth temperature of 45°C (50).

Morphological and Physiological Diversity

The preponderance of nonspecialized heterotrophs among the known bacterial halophiles does not necessarily reflect their dominance in salt lakes, saline soils, and other saline habitats but, rather, may be due to the relative ease of culturing these bacteria (136). The halophilic property is probably widespread in the bacterial domain and may occur in a variety of morphological and physiological types. This is nicely illustrated by the studies of Hirsch, who differentiated 104 different morphotypes of bacteria in the hypersaline Solar Lake on the shore of the Sinai peninsula. This small and shallow (maximum depth, about 5 m) thalassohaline lake is stratified in winter, with an epilimnion containing 4.5 to 9% salt, increasing to about 19% at the bottom. In addition to salt stress, temperature may be an important selective factor, since in the upper hypolimnion heliothermal heating can increase the temperature to about 60°C in winter. During the summer season, the

lake is mixed and hypersaline (18 to 19%). Morphologically diverse bacteria were observed by direct examination of samples, in enrichments, and in pure cultures; they included cocci, rods, apple-shaped budding bacteria, long flexible filaments, spindle-shaped bacteria, short pointed filaments, and branched hyphae (123). A budding prosthecate bacterium with branching hyphae was obtained in culture and described as *Dichotomicrobium thermohalophilum* (124). Similar strains have been isolated from a coastal saline lake in Brazil. The nearly tetrahedral mother cells produce up to four hyphae at the tips, on which nonmotile buds are formed. The isolates grow from 0.8–4 to 18–22% salt, depending on the strain, with an optimum at 8 to 14%, and are moderately thermophilic (growing at up to 52 to 65°C). Their metabolism is strictly aerobic, and organic acids are used as carbon and energy sources. However, these bacteria were also found at greater depths in Solar Lake and could be isolated from the anaerobic hypolimnion at a depth of 3.5 m (123, 124). Solar Lake was also the source from which the facultative aerobe *Spirochaeta halophila* was isolated (103).

Many of the aerobic moderately halophilic bacteria can use nitrate as an alternative electron acceptor. Thus, *Halomonas elongata* can grow anaerobically by reducing nitrate to nitrite (357). Other well-known nitrate reducers are *H. halodenitrificans* and *Bacillus halodenitrificans*. The latter was isolated from a solar saltern in the south of France by enrichment in medium containing 1.06 M $NaNO_3$. It grows at NaCl concentrations between 0.35 to 4.25 M (optimum, 0.5 to 1.35 M) and is unusually tolerant to nitrite: growth is possible in 0.58 M $NaNO_2$. Since nitrous oxide reductase is absent, N_2O is the sole product of nitrate and nitrite reduction (49).

Considerable diversity also exists with respect to the carbon and energy sources used. Hydrocarbons can be used up to quite high salinities. A series of enrichment cultures successfully produced mineral oil degraders by using water from the Great Salt Lake, Utah, as an inoculum at salinities up to 17.2%. Experiments with radiolabeled hexadecane showed decreasing degradation rates with increasing salinity (360).

Aromatic compounds such as benzoate may be degraded by versatile halophilic bacteria such as *H. halodurans*, which cleaves aromatic rings by *ortho* cleavage (290). Even more exotic compounds may be utilized at high salinities, as illustrated by strain JD6.5, an *Alteromonas* type of organism growing at 2 to 24% salt and degrading several highly toxic organophosphorus compounds (38). Another *Halomonas* isolate degraded formaldehyde and proved highly tolerant to high formaldehyde concentrations (8, 250, 251).

Many additional metabolic functions may exist within the highly diverse group delineated by the common denominator "aerobic, halophilic bacteria". Thus, the moderately halophilic *Thiobacillus halophilus*, isolated from a Western Australian hypersaline lake (Lake O'Grady North), grows at salt concentrations of up to 4 M. It is a chemoautotroph that oxidizes reduced sulfur compounds (365). Aerobic halophilic methylotrophic bacteria were described as well (57). Many species of cyanobacteria are also moderate halophiles. However, they will not be discussed within the framework of this review, which deals primarily with heterotrophs. Whether moderately halophilic counterparts exist for all types of bacterial metabolism that occur in freshwater and marine environments is still unknown. One function that seems to be missing in the heterotrophic bacterial communities at high salt concentrations is the ability to fix molecular nitrogen. This fact was already recognized by Hof (127), who pointed out that N_2 fixers could be isolated on 0 and 3% salt but not on 6% and higher. Large numbers of bacteria able to grow in a nitrogen-free medium could be isolated from saline soil (Granada, Spain), but nitrogenase activity was not detected (275).

It is evident that very few attempts have been made to isolate specialized and unusual types of halophilic aerobic bacteria. Therefore, one may assume that many more physiological types remain to be discovered.

Measurement of In Situ Activities

Little is known about the in situ activity of halophilic bacteria in saline lakes, salterns, saline soils, and other habitats.

A few attempts have been made to assess microbial activities in the Great Salt Lake, Utah, by incubating water samples from the less saline southern part (8.5% salt) and the more saline northern half (22% salt) with [^{14}C]glucose, [^{14}C]glycerol, or [^{14}C]acetate and monitoring the appearance of the radioactive label as $^{14}CO_2$. Lowered rates of breakdown of the three substrates were found at the higher salinity (66), a finding that parallels the lowered dissimilation of [^{14}C]hexadecane by Great Salt Lake water samples with increasing salinity (360).

Measurements of [*methyl*-^{3}H]thymidine incorporation by heterotrophic communities in increasingly saline saltern ponds in Spain showed that the growth rate was highest between 5 and 10% salt. This was much higher than in the community of archaea present in ponds with salt concentrations exceeding 20% (104). A similar finding was reported from the Eilat saltern ponds at the coast of the Red Sea, where the estimated doubling times of the bacteria in ponds of low to intermediate salinity was 1.1 to 12 days, based on the thymidine incorporation rate (245).

Molecular Approaches to the Elucidation of the Community Structure of Halophilic Bacteria in Hypersaline Environments

In recent years, the characterization of 16S rRNA genes isolated directly from the environment has been used to obtain information on prokaryotic community structure. The technique has not yet been extensively applied to hypersaline environments. However, 16S rRNA genes were amplified by PCR from a saltern crystallizer pond in Spain. As expected, in view of the high salinity, archaeal sequences were recovered most frequently while bacteria represented only a minor component. One cluster of bacterial sequences showed about 82% identity to *Rhodopseudomonas marina* (alpha subclass of the *Proteobacteria*) (13, 14). To enable comparison of the prokaryotic communities in the salt concentration gradient presented by the salterns (preparation ponds of 6.4 and 9.2% salt, concentrator ponds of 13.3 and 21.6% salt, and an NaCl precipitation pond of 30.8% salt), fingerprinting of the community was performed with different restriction endonucleases. The highest similarities were found between the two concentrator ponds and between the two preparation ponds. The bacterial community decreased in complexity with increasing salinity. The bacterial genes isolated from the crystallizer pond showed little similarity to the genes isolated from the less saline ponds. Thus, it is improbable that the bacteria in the crystallizer pond represent only a carryover of inactive cells from the previous evaporation stage (13, 191).

Competition between Halophilic Bacteria and Archaea

The halophilic bacteria form a versatile group, adapted to life at the lower range of salinities and with the possibility of rapid adjustment to changes in the external salt concentration. In contrast, the halophilic archaea (family *Halobacteriaceae*) are generally found at higher salinities and their requirement

for high salt concentrations for the maintenance of structural cell components makes them strictly dependent on the constant presence of high salt concentrations (3 to 4 M for most species). Accordingly, the two groups occupy different niches, which seem to overlap very little (247). When heterotrophic bacteria from Spanish saltern ponds of increasing salinity were enumerated on agar plates, only a narrow salinity range (25 to 32% total salt) was found to contain the two groups (288). A similar conclusion was reached in studies in which amino acid incorporation by the heterotrophic communities in saltern ponds in Eilat, Israel, was measured. When inhibitors specifically directed against the halophilic archaea (bile salts such as taurocholate or deoxycholate and the protein synthesis inhibitor anisomycin) or bacteria (chloramphenicol or erythromycin) were tested, the contribution of each group could be distinguished. Up to a salinity of about 25%, all amino acid incorporation activity was inhibited by the bacterial inhibitors, while above 25%, inhibitors known to act on the archaea completely inhibited all activity (243, 244, 246). Similarly, aphidicolin, an inhibitor of DNA replication in halophilic archaea, completely inhibited thymidine incorporation in saltern brines with salinities exceeding 25% (245, 246).

Competition between halophilic bacteria and archaea has also been studied in laboratory model systems. When samples from the subterranean saline well that supplies the saltern ponds of La Malá near Granada, Spain, were subjected to gradual salinity changes with additional nutrient enrichment, marine-type slightly halophilic bacteria, halotolerant types, moderate halophiles, and extremely halophilic archaea were enriched, depending on the final salt concentration achieved. Marine and moderately halophilic types were most abundant between 3 and 30% salt. At 25°C, hardly any development of extremely halophilic archaea was observed at the higher salinities, but at 35°C, dense communities of archaea were obtained at 35 and 40% salt, suggesting that temperature is an important factor in determining the outcome of the competition (40). The importance of temperature was confirmed in chemostat studies in which competition was examined in continuous culture, with a mixed natural community from a Spanish saltern as the inoculum. Salt concentration, temperature, and nutrient concentration (dilution rate) were used as variables (287). At low salt concentrations, the moderately halophilic bacteria won the competition, while at the highest salinities, the pigmented archaea outcompeted the bacteria. Within the intermediate salt concentration range (20 to 30%), temperature was the decisive factor determining the outcome, with the bacteria being favored by low temperatures. Also differences in the affinity for low concentrations of organic nutrients determined the result of the competition experiments: bacterial strains that grew slowly in batch cultures often predominated in chemostat cultures at low dilution rates thanks to their higher affinity for nutrients. In nutrient-rich batch cultures, the halophilic bacteria generally grew faster than the archaea, even at the high NaCl concentrations preferred by the archaeal types (287). The average growth rate of pigmented archaea (39 strains tested) in complex medium was 0.02 h^{-1} at 25% salt, while nonpigmented bacterial strains (93 strains tested) grew much more rapidly (average optimal growth rate, 0.06 h^{-1} at 10% salt, with a broad optimum between 2 and 25%) (288).

Biogeochemical Importance

Several strains of moderately halophilic bacteria may be involved in the precipitation of $CaCO_3$ (calcite, aragonite) and other minerals (41). No direct evidence exists for the active involvement of these bacteria in the formation of mineral deposits in nature, either recent or ancient, but the phenomenon observed in cultures is of sufficient interest to be discussed here.

Most studies on the subject used *Halomonas halophila* as the model organism, and the minerals formed were identified by X-ray diffraction. In a test of 27 isolates, all caused the formation of $CaCO_3$ crystals as long as conditions allowed bacterial growth. Most crystals were spherical and consisted of calcite and magnesium calcite, in which $MgCO_3$ forms a significant part of the crystal (75 to 85% Ca and 25 to 15% Mg); the ratio between these minerals depended on the salinity and medium composition. High magnesium concentrations inhibited $CaCO_3$ precipitation by *H. halophila*. Growth at low salinity favored crystal formation, while at low temperature and/or at high salinity, crystal formation was repressed. The bacteria influenced the type of $CaCO_3$ crystal formed in vitro, and this effect may be species specific (42, 68, 280, 281). Calcification commences with a nucleus formed by aggregation of a few calcified bacterial cells and subsequent accumulation of more calcified cells and carbonate, which holds the bacteria together. This results in the formation of spherical bioliths of about 50 μm in diameter (282). To what extent the precipitation of $CaCO_3$ was triggered by a local increase in pH or whether the bacteria served only as crystallization nuclei was never ascertained.

Moderately halophilic isolates assigned to the genera *Flavobacterium* and *Acinetobacter* make calcite, and *Acinetobacter* also produces aragonite. High temperatures and low ionic strengths favor crystal formation. *Flavobacterium* makes magnesium calcite with 0.04 to 0.32 mol% magnesium; *Acinetobacter* produces magnesium calcite with up to 14% aragonite at the highest salinities (69). Similarly, 63 strains of *Salinivibrio* isolated from an inland saltern in Spain were found to be involved in crystal formation (279).

Bacteriophages

Like other prokaryotes, the aerobic moderately halophilic bacteria have bacteriophages. Few attempts have been made to quantitatively assess the occurrence of phages in hypersaline environments inhabited by halophilic bacteria and to estimate their importance in the regulation of the community density of their host organisms. Studies in which these bacteriophages are exploited to investigate the genetics of their hosts are lacking altogether.

Induction of lysogenic phages by mitomycin C led to the isolation of phage F9-11 from an *H. halophila* strain obtained from soil. This phage replicates over a wide range of salinities (2.5 to 15%) (20, 92).

A water sample from Lake Chaplin, Canada, produced a bacteriophage that lysed a bacterium designated *Pseudomonas* strain G3, which is able to grow from 0.25 to over 3 M NaCl. The phage also infects *S. costicola* and two unidentified halophilic bacteria and is stable in the absence of salt (150). Another phage (designated UTAK) active against an *S. costicola* strain was isolated from the salterns of Alicante, Spain. The burst size of this phage was maximal at 1 to 2 M NaCl (80 to 105 phages per cell) and decreased to an average of only 12.5 phages per cell at 0.5 M. It was thus suggested that intracellular phage replication may be controlled by the salinity of the medium (96). Additional bacteriophages were isolated from the saltern in the course of this study, attacking morphologically different hosts, but most of these phages were not studied further.

Two phages lysing *Tetragenococcus halophilus* involved in soy sauce fermentation were characterized: phages ϕ7116, hav-

ing an isometric head and a contractile tail, and ϕD-86, having an isometric head and a noncontractile tail. Both could propagate at all salinities in which their host could grow. Phage ϕD-86 was stable at all salinities between 0.03 and 2.6 M, while phage ϕ7116 was specifically unstable between 0.04 and 0.1 M salt while being stable in both the lower (0.01 to 0.03 M) and the higher (0.2 to 2.6 M) salt range. This effect, which is known for other (nonhalophilic) phages as well, is probably due to the salting-in effect of Na^+, causing destruction of phage protein and DNA-protein complexes (331).

Cultures of *Actinopolyspora halophila* grown on low-salt media (10 to 12%, near the lower limit required for growth) showed holes resembling viral plaques (95). This phenomenon was not investigated further.

All bacteriophages isolated thus far from the moderate halophiles are double-stranded DNA phages with distinct heads and tails. Most are almost equally stable in the presence and the absence of salt and can retain infectivity for weeks in dilute solutions (151), in contrast to the halophilic archaeal phages, which, similar to their hosts, are inactivated in the absence of salt. The bacterial phages are "halophilic" only to the extent that their hosts are: the phages multiply only when the halophilic host is growing. In view of the relatively low salt concentration within the cytoplasm of halophilic bacteria compared to the outside medium (see below), the process of attachment and infection of the host cells may occur at high salt concentrations while intracellular multiplication occurs in a low-salt medium (151).

Another approach to obtain information on the role that bacteriophages may play in regulating the community sizes of halophilic bacteria in nature is based on direct electron microscopic observation and enumeration of phages in environmental samples. This approach was used in a study of Spanish salterns of different salinities (104). While many infected cells (probably of halophilic archaea) were seen at salinities above 25%, infected cells were not observed in the lower salinity range. At the lower salinities, bacterivory by protozoa was estimated to be much more important than phage lysis. In the lower salinity range (up to about 15%) around 1×10^7 to 2×10^7 bacterial cells and 5×10^7 to 7×10^7 virus-like particles, most of them with icosahedral heads, were counted per ml of brine. Viral abundance, as well as prokaryote abundance, increased with salinity. Also in the Dead Sea, virus-like particles (head and tail or spindle-shaped) were abundantly found. In view of the dominance of halophilic archaea in the lake, these virus-like particles were most probably derived from lysis of archaea, but the possibility that bacterial viruses were involved cannot be ruled out (249).

Another factor which may cause a decrease in halophilic bacterial numbers, in addition to lysis by bacteriophages and predation by protozoa, is the existence of halophilic predatory bacteria of the genus *Bdellovibrio*. With *Vibrio parahaemolyticus* and *V. alginolyticus* as hosts, halophilic *Bdellovibrio* strains that grew from 1 to 15.5% salt were isolated (303). Thus, predatory bacteria should also be considered as potential regulators of the halophilic bacterial community size in nature.

PHYSIOLOGY

Requirement for Salt

The common denominator for all moderately halophilic bacteria is their requirement for salt and their ability to tolerate high salt concentrations. Salt requirement and tolerance are highly variable among the different species. Moreover, these parameters are by no means constant, since they may vary according to the growth temperature and the nature of the nutrients available (174). The salinity range of many isolates has been investigated in complex media. This fact led to the classification of some organisms as halotolerant rather than halophilic. Thus, *H. elongata* was originally described as extremely halotolerant (357). However, in minimal medium it requires at least 0.5 M NaCl, thus behaving like a true halophile (23). An examination of the specific requirement for Na^+ and Cl^- ions, as well as the tolerance toward other salts, is also necessary. In most cases, a minimum concentration of Na^+ is essential for growth. This may be due in part to the requirement for Na^+ gradients to drive transport processes in the cell membrane. Certain species may also possess a primary respiration-driven outward sodium pump (see below). *S. costicola* (optimal growth at 0.8 to 1.5 M NaCl, and growing up to 3.3 M in a peptone-based complex medium) had a minimum requirement for 0.5 M NaCl in media based on NaCl as the sole salt. Addition of high concentrations of compounds such as glucose or glycerol lowered the NaCl requirement to 0.3 M, but no further lowering of the sodium concentration required was achieved (1). Other cations may be tolerated in high concentrations. Thus, "*Micrococcus varians* subsp. *halophilus*" can grow in 1.5 to 2 M LiCl, RbCl, or CsCl in the presence of 60 mM Na^+ (146). There does not seem to be an absolute requirement for Cl^- ions: *H. elongata* grew as well on NaBr and $NaNO_3$ (but not on NaI or Na_2SO_4) as on NaCl (358). *H. halophila* grew well on NaCl, NaBr, Na_2SO_4 and $Na_2S_2O_3$ but not on other sodium salts (264). The moderately halophilic *Pseudomonas* sp. strain 40 can grow in 1 to 4 M NaCl, 1 to 2 M $NaNO_3$, or 1 M Na_2SO_4 but not in 1 to 4 M KCl (238).

Salt requirement and tolerance may be temperature dependent. In certain halophilic archaea such as *Haloferax volcanii*, the minimum and optimum salt concentrations shifted to higher values with increasing temperature (219), and a similar phenomenon was observed in halophilic bacteria as well. Thus, the optimum salt concentration for growth of *H. halophila* at 32 and 42°C was 7.5%, whereas the optimal concentration for growth at 22°C was 5% (264). *H. elongata* grew in complex medium at 20 and 30°C at salt concentrations between 0.05 and 3.4 M. At 40°C, no growth was obtained at 0.05 M, but growth was possible between 0.375 and 4.5 M. In defined medium with glucose and alanine as organic nutrients, salt tolerance was decreased, growth occurred within a narrower salt range than in complex medium, and a higher salt concentration was needed for optimum growth (358).

Marinococcus halophilus (NaCl range, 0 to 5.5 M; optimum, 1 M at 35°C) grew in the virtual absence of NaCl at 20°C. At 25°C, at least 0.5 M was required and could not be replaced by KCl or by nonionic solutes (168, 171, 174, 227, 228). Somewhat different behavior was observed in *S. costicola* (optimum, 1 M NaCl at 30°C): at higher or lower growth temperatures, both the optimum and the lower limit of NaCl concentrations were higher (3). While at 30°C cells grew from 0.5 to 5 M salt, at 20°C 0.2 M salt was sufficient for growth. This lower limit could not be reduced further (168, 171). Many halophiles may thus prove to grow at a wider range of NaCl concentrations when tested at a greater range of temperatures.

Minimal and Defined Media

Many of the moderately halophilic bacteria have simple growth requirements, and minimum growth requirements have been determined for several species. Thus, *H. halophila* grows well on a medium containing inorganic salts, including nitrate as the nitrogen source, and glucose as the only carbon and energy source (264). *H. halodenitrificans* could grow aerobi-

cally on any of a number of organic carbon sources in the presence of thiamine. Under anaerobic conditions (with nitrate as the electron acceptor), methionine had to be supplied as well. Methionine could be replaced by glycine betaine or by vitamin B_{12} but not by dimethylglycine. It was suggested that the bacterium may be deficient in the cobalamine-dependent path for methionine synthesis and is therefore unable to produce glycine betaine anaerobically (126). However, *H. halodenitrificans* also produces ectoine as compatible solute anaerobically and cannot synthesize glycine betaine de novo (83); hence, this explanation for the effect of methionine may not be valid. *S. costicola* has more complex growth requirements. The earliest designed synthetic medium contained glucose, L-cysteine or cystine, glutamate, arginine, valine, isoleucine, and salts (70). Glucose, cyst(e)ine, and NaCl were essential, and omission of any of the other components led to decreased growth. A simpler formulation was based on glucose, glutamate, two vitamins (biotin and thiamine), choline (as a precursor of glycine betaine), and salts (148).

Most moderate halophiles have more demanding nutritional requirements at high salt concentrations. Complex media stimulate growth at high salt concentrations. The effect may be due to the presence of compatible solutes or their precursors that can be accumulated or to the fact that other growth factors may be synthesized more slowly under the high-salt conditions (136). Thus, the salt tolerance of *S. costicola* in defined medium could be extended by including 2% sodium glutamate (148), and its growth in 4 M (but not in 3 M) salt required the presence of nutrients such as glycine betaine (296). The widest salt range for growth was found in proteose peptone and tryptone medium, Casamino Acids alone gave a narrower range (0.4–2.5 M), while in a defined medium no growth was obtained above 2.2 to 2.3 M (71).

Tolerance to Heavy Metal Ions

Surveys of heavy metal sensitivity and tolerance to 10 heavy metal ions in moderate halophiles (224), both from culture collection strains and from fresh isolates, showed a very heterogeneous response among the taxonomic groups (the *Halomonas* group, *Acinetobacter*, *Flavobacterium*, moderately halophilic cocci), as well as among the strains included in each group. All were sensitive to mercury, silver, and zinc and tolerant to lead. The response to arsenic, cadmium, chromium, and copper was very heterogeneous. *Acinetobacter* strains proved the most metal tolerant, and *Flavobacterium* strains were the most sensitive. The influence of salinity and yeast extract concentrations in the test medium on the toxicity of the heavy metals tested was also examined. In general, lowering the salinity led to enhanced sensitivity to cadmium and, in some cases, to cobalt and copper. However, increasing the salinity resulted in a decrease only in the cadmium, copper, and nickel toxicities. Reduction in the yeast extract concentration resulted in an increased sensitivity to all metals, but only a slight decrease in the toxicities of nickel and zinc was found when the yeast extract concentration was increased (224).

Different *S. costicola* strains were compared for heavy metal tolerance. All proved sensitive to cadmium, copper, silver, zinc, and mercury. All tolerated lead, and most were also tolerant to nickel and chromium, so that multiple tolerance to the three metal ions chromium, nickel, and lead emerged as the major pattern (90). On the basis of these studies, several metal concentrations were proposed to discriminate between heavy metal-tolerant strains and those that were sensitive (222), thereby facilitating the isolation of metal-tolerant strains from polluted hypersaline habitats. In a recent study, the isolation and taxonomic characterization of a large number of heavy metal-tolerant halophilic strains from different geographical sites in Spain has been attempted. A total of 222 metal-tolerant (to mercury, cadmium, copper, chromium, or zinc) moderately halophilic strains were selected for a detailed taxonomic analysis. Most isolates were assigned to the genus *Halomonas*, and approximately 30% of the strains displayed multiple resistances (278).

Salinity-dependent cadmium tolerance was documented in *Pseudomonas* sp. strain 40. In 1 M NaCl, poor growth was obtained in the presence of 2 mg of $CdCl_2$ per ml and no growth was possible at 2.5 mg/ml. However, in 2 to 4 M NaCl and 2.5 mg of $CdCl_2$ per ml, moderate growth was observed. $NaNO_3$ and Na_2SO_4 enhanced cadmium toxicity. Cadmium ions react with chloride ions to form complexes whose nature depends on the chloride concentration: at 1 M NaCl, most of the cadmium appears as a mixture of $CdCl_2$ (35%) and $CdCl_3^-$ (45%); at 2 M NaCl, the anionic complexes $CdCl_3^-$ (47%) and $CdCl_4^{2-}$ (33%) predominate and are probably less toxic (238).

Internal Ion Concentrations

To cope with the high and often changing salinity of their environment, the aerobic halophilic bacteria, similar to all other microorganisms, need to balance their cytoplasm with the osmotic pressure exerted by the external medium.

Osmotic balance can be achieved by the accumulation of salts, organic molecules, or a combination thereof. A fourth possibility, that the cell is able to control water movement in and out and maintain a hypoosmotic state of their intracellular space, has been proposed for *S. costicola* and *H. elongata* (308, 351, 359).

Much of the controversy in the literature about the nature of the real intracellular environment of the halophilic bacteria originates from the difficulties in the estimation of the cell volume. Intracellular solute concentrations are generally determined by analysis of cell pellets and thus depend on the precise assessment of what fraction of the pellet volume is occupied by the intracellular space. Volume determinations are based mostly on the distribution of radioactive marker molecules labeling the total water space, the water space excluding the cytoplasm, and the water space excluding the whole cell volume, including the outer layers and periplasmic space (133). Cell-impermeable markers that have been successfully used so far include inulin (105, 193, 308, 359), dextran (46, 203, 298), and blue dextran (146). Permeable solutes such as tritiated water or ethylene glycol are also used to calculate the total water space in the cell pellets (170). An exact determination of the intracellular and extracellular water spaces is essential. Errors are especially large for sodium and chloride, which are generally abundant in the medium, and thus a small error in the determination of the spaces results in a large error in their apparent concentrations. Accurate estimation of the cytoplasmic volume also suffers from the lack of reliable methods to differentiate between the periplasmic space and the osmotically active cytoplasmic space (133). Small molecules such as raffinose (105), sorbitol, and sucrose (308), which have been used in this type of experiment, can be expected to penetrate the outer membrane and become distributed in the periplasmic space as well. This periplasmic space can have a considerable size: in *S. costicola*, it was estimated to occupy 38% of the total cellular space (308). All calculations are based on the (not necessarily true) assumption that the molecules used as markers are not taken up and metabolized by the cells or bound to the envelopes and other cellular structures. Indeed, it was suggested that dextran and inulin may bind to cell envelopes

(130) and that smaller fragments of unpurified dextran may be taken up by the cells, causing large errors in the calculation of the cytoplasmic solute concentrations. That a proper knowledge of the intracellular water space under different growth conditions is essential and that a comparative approach based, e.g., on the ion content per unit of cell protein is insufficient is clearly shown by the finding that in *H. elongata* the cell volume per unit of protein is inversely related to salinity, decreasing from 2.62 to 2.06 μl/mg of protein in cells grown from 0.175 to 1.37 M NaCl (203). A similar phenomenon was observed in *H. canadensis*: cells grown at 0.6 M NaCl had a volume of 4.94 μl/mg of protein, decreasing to 2.69 μl/mg of protein at 4.35 M (193).

During the analysis of cell pellets obtained by centrifugation, anaerobic conditions may develop in the densely packed cells during handling and washing, potentially leading to loss of substantial amounts of potassium and gain in the amount of sodium (308). The effect is reversible: when dense cell suspensions of *S. costicola* were aerated in the presence of an energy source, potassium ions were taken up while sodium was released (168). Perhaps variations in harvesting and handling may explain the differences in the estimated intracellular ion concentrations in *H. halodenitrificans* as published by different authors (31, 298) (Table 5). Centrifugation of cells through a layer of silicone oil may avoid some of the problems involved in conventional centrifugation techniques. Oils of different densities must be used to adjust for the density of the cells, which depends on the salt concentration of the medium (171). The method has not been widely applied in the study of moderate halophiles. A different approach toward the estimation of intracellular ion concentrations, yet to be applied to the aerobic halophilic bacteria, is the use of X-ray microanalysis in the electron microscope. This method, in which individual cells are analyzed, was recently used to measure ion contents in the halophilic anaerobe *Haloanaerobium praevalens* (252).

The phase of growth can also have a major influence on the results of the internal ion concentration measurements; stationary-phase cells may have a much higher intracellular sodium concentration than exponentially growing cells. This may also explain some of the unusually high apparent intracellular sodium concentrations reported in the literature.

Table 5 summarizes some of the reported estimates of intracellular ion concentrations of halophilic bacteria. Most analyses are limited to Na^+ and K^+, intracellular Cl^- concentrations have seldom been determined, and data on divalent cations are scarce. Great variations in the intracellular ion concentrations are obvious, both among different species of moderate halophiles and within the same species, depending on the growth conditions and on the method used. A few general trends are clear, however (176). (i) The intracellular K^+ concentration is generally higher than that in the medium. (ii) The Na^+ concentration inside the cells is generally lower (to different extents) than that outside. (iii) The apparent intracellular Na^+ and K^+ concentrations increase with increasing external NaCl concentration in a nonlinear fashion. (iv) Generally the sum of the concentrations measured is insufficient to balance the osmotic pressure of the medium. However, taking into account the presence of organic osmotic solutes as well (see below), such a balance may be achieved.

In certain gram-positive bacteria, the apparent intracellular cation concentrations are similar to those of the growth medium. This was reported for the haloalkaliphilic *Bacillus haloalkaliphilus* (361). However, the reported value of 0.37 g of intracellular water per g (dry weight) is rather high and may overestimate the cytoplasmic ion concentrations. Moreover, this strain produces ectoine and other yet unknown organic osmotic solutes (83). In "*Micrococcus varians* subsp. *halophilus*," the apparent intracellular Na^+ concentration was approximately equal to that in the medium over the range from 1 to 2 M, while in cells grown at 4 M NaCl, 2.1 M Na^+ was measured intracellularly (28). The presence of high intracellular Na^+ concentrations in this organism was confirmed by Kamekura and coworkers, who also showed that other monovalent cations added to the growth medium (K^+, Li^+, Rb^+, and Cs^+) were not excluded from the cytoplasm (146, 176).

In many *Halomonas* species (*H. elongata*, *H. canadensis*, and *H. halodenitrificans*), the sum of the apparent intracellular Na^+ and K^+ concentrations is much lower than the medium concentration. In *H. halodenitrificans*, the sum of intracellular Na^+ and K^+ concentrations remained low and constant (about 0.1 M Na^+ and 0.3 M K^+ in exponentially growing cells) over a wide range of medium NaCl concentrations. In stationary-phase cells, a drastic increase in the intracellular Na^+ concentration was observed (up to 0.5 and 1.1 M in cells grown in 1 and 3 M NaCl, respectively) and the intracellular K^+ concentration decreased to about 0.1 M (298). In *H. canadensis*, the intracellular Na^+ concentration increased when the cells reached the stationary phase (193). In "*Pseudomonas halosac-*

TABLE 5. Intracellular ionic concentrations of moderately halophilic bacteria

Species	Medium concn of:			Intracellular concn of:			Reference
	Na	K	Cl	Na	K	Cl	
Halomonas elongata	0.06	0.02		0.04	0.002		359
	1.38	0.02		0.31	0.02		
	3.4	0.01		0.63	0.02		
Halomonas canadensis	0.6	0.04		0.05	0.34		193
	4.4	0.04		0.62	0.58		
Stationary	4.4	0.04		1.01	0.66		
Halomonas israelensis	2.0			1.14			97
Halomonas halodenitrificans							
Exponential	1.0	0.04	1.0	0.31	0.47	0.055	31
Stationary	1.0		1.0	0.51	1.01		
Exponential	3.0		3.0	1.07	0.12		
	1.0			0.11	0.30		298
	3.0			0.13	0.33		
"*Pseudomonas halosaccharolytica*"	1.0	0.006	1.0	0.90	0.71	0.71	192
	2.0	0.006	2.0	1.15	0.89	0.98	
	3.0	0.006	3.0	1.04	0.67	0.70	
Salinivibrio costicola	1.0	0.004		0.68	0.22		31
	0.6	0.008		0.51	0.52		308
	1.0	0.008		0.58	0.66		
	1.6	0.008		1.09	0.59		
	2.0	0.008		0.90	0.57		
	0.6			0.65	0.72		351
	2.0			1.29	0.55		308, 359
(molal)	1.0	0.009		0.89	0.82		308
Defined medium	2.0	0.009		1.29	0.55		
Complex medium	3.0	0.009		1.78	0.37		
Stationary	1.0	0.04	1.0	0.68	0.22	0.14	
"*Micrococcus varians* subsp. *halophilus*"	1.0			1.17	0.03		28
	4.0			2.11	0.03		
Exponential	3.0	0.012		2.88	0.21		146
Bacillus haloalkaliphilus (molal)	3.4			3.52	0.31	2.7	361

charolytica," the apparent internal salt concentration was relatively independent of the salt concentration in which the cells were grown, but in this organism the measured sum of the intracellular concentrations was quite high (1.4 to 1.9 M $Na^+ + K^+$ in cells grown between 1 and 3 M NaCl) (192). In *S. costicola*, the intracellular $Na^+ + K^+$ concentration was significantly lower than outside only at the highest medium salinities. However, upon treatment with cetyltrimethylammonium bromide, the cellular Na^+ and K^+ concentrations did not equilibrate with the external medium, possibly indicating that the ions may partially occur in a bound state (308).

Below we summarize a few data on the intracellular abundance of individual ions.

(i) Sodium. The apparent intracellular Na^+ concentrations are often far too high to enable the generally salt-sensitive cytoplasmic enzymes to be active (see below). However, the assessment of the true intracellular Na^+ concentration is problematic, as discussed above. In addition, Na^+ and other ions may be bound to the outer cell layers, in amounts increasing with external salinity (133). It was thus suggested that much of the cell-associated Na^+ in "*P. halosaccharolytica*" is not cytoplasmic (192).

(ii) Potassium. In most halophilic bacteria, K^+ is accumulated to a few tenths of 1 M (133). *H. elongata* seems to be an exception, with K^+ concentrations as low as 20 mM (359). In any case, in contrast to some archaeal halophiles, cytoplasmic potassium contributes relatively little to the achievement of an osmotic balance.

(iii) Magnesium. Intracellular Mg^{2+} concentrations have been determined only seldom in halophilic bacteria. In *H. elongata* grown in medium containing 24 mM Mg^{2+}, the intracellular Mg^{2+} concentrations varied from 9 to 23 mM depending on the growth conditions (359), which is not particularly high compared to 30 mM in *Escherichia coli* and 102 mM in *Halobacterium salinarum* (*cutirubrum*) (45, 46).

(iv) Calcium. *H. elongata*, growing in media with 0.7 mM Ca^{2+} and in the presence of 0.05 to 3.4 M NaCl, was reported to contain between 3.1 and 12 mM Ca^{2+} intracellularly, most of it probably bound (353).

(v) Manganese. A single measurement of intracellular Mn^{2+} concentrations in *S. costicola* (by using ^{54}Mn added to the medium as label) gave a value of 0.6 mM Mn^{2+}/kg of cell water (46).

(vi) Chloride. Estimations of intracellular chloride concentrations within cells of moderately halophilic bacteria are greatly variable, from relatively low values (55 and 139 mM in *H. halodenitrificans* and *S. costicola*, respectively, grown in 1 M NaCl) (31) to values as high as 0.7 to 1 M in "*P. halosaccharolytica*" grown at NaCl concentrations between 1 and 3 M (192). The measured intracellular Cl^- concentrations are in most cases much lower than the combined Na^+ and K^+ concentrations (133). Thus, *S. costicola* grown in 1 M NaCl and 6 mM KCl will have a combined intracellular Na^+ and K^+ concentration of about 0.6 M but only about 0.1 to 0.2 M Cl^- (171). When the cells are grown at higher salt concentrations, the apparent intracellular Cl^- concentration may be increased (1.5 M in cells grown in 3 M) (141). The old assumption that Cl^- is the main counterion for the intracellular cations in moderate halophiles is not necessarily true, and since no other anions have been detected at high concentrations within the cells, it has been speculated that most of the cellular cations may be associated with negative charges present on proteins, cell envelopes, and other macromolecules (170–172).

To verify the theory that part of the Na^+ and other cations may be tightly bound to different cellular structures, bulk analysis of ions is insufficient (160). Indications that a significant fraction of the intracellular Na^+ does not occur freely dissolved in the cytoplasm but is rather strongly bound, came from a number of studies in which ^{23}Na nuclear magnetic resonance spectroscopy (NMR) was used to obtain information on the state of the Na^+ ions. Sodium NMR relaxation times can indicate changes in the intracellular environment in which the Na^+ is embedded. The need for thick cell suspensions, easily leading to the development of anoxic conditions, is a problem inherent in this method (94). By using NMR to probe the ionic environment inside *H. israelensis* cells, three types of cell-associated Na^+ were detected. One fraction (about 40% of the total) is free, dissolved in the cytosol, and can exchange with the extracellular Na^+. The other 60% is bound; part of it could exchange with the "free" intracellular Na^+, while another part could not exchange and was "invisible" to the NMR. Since the shift reagent [dysprosium bis(tripolyphosphate)] used to discriminate between extracellular and cell-associated Na^+ was not effective at the highest NaCl concentrations, the approach could be applied up to about 2 M salt only. In the concentration range tested, the "NMR-visible" intracellular Na^+ concentration never exceeded 15 to 20% of the medium concentration (97, 299). It should be noted that the use of shift reagents may lead to faulty results as they form complexes and therefore disturb the equilibrium across the membrane.

The state of intracellular lithium was also investigated by NMR. Li^+ can replace part of the intracellular Na^+. The interaction of Li^+ ions with the bacteria is different in cells grown in high salt and in low salt: in low salt (0.5 M), almost all (92%) of the Li^+ was "seen" by the NMR, while in 3 M salt-grown cells, only 55% of the Li^+ was detected. Goldberg et al. suggested that three types of Li^+ ions existed: free, weakly bound, and strongly bound (98).

NMR methods were also applied to study *S. costicola* grown at NaCl concentrations between 0.6 and 2 M. Apparent intracellular Na^+ concentrations (excluding strongly bound Na^+ which is not detected by the NMR) were always below 25% of the medium concentration (94). It was calculated that the fraction of the free intracellular Na^+ was about 40% of the total, which yielded an estimate of the total intracellular Na^+ concentrations as being between 0.14 and 0.435 M (94), much lower than those obtained by bulk analyses in cell pellets (308).

Additional evidence for a substantial binding of Na^+ ions to cellular structures came from a study in which cell envelopes of "*P. halosaccharolytica*" were incubated in 2 M NaCl and 5.5 mM KCl. It was found that 149 mg of Na^+ and 14 mg of K^+ were bound per g of protein, about twice as much Na^+ and eight times less K^+ as found per gram of protein in whole cells (192).

It has often been speculated whether the exclusion of salt and the maintenance of a steep ionic gradient by the moderately halophilic bacteria is achieved through the constant pumping of ions by active, energy-dependent mechanisms (see below) or by tightening of the permeability barrier of the membrane. This important question does not seem to have been pursued in depth. Continuous-culture experiments with *H. elongata* strongly indicate that the maintenance energy of these organisms is relatively independent of the salinity of the medium, providing evidence in favor of an ion-tight membrane (82).

Ion Pumps in the Cell Membranes

A low intracellular ionic environment can be achieved only by energy-dependent mechanisms. This was recognized as early as 1954 by Baxter and Gibbons: "...adaptation at the

cellular level, in a mechanism that is probably energy-dependent that maintains the intracellular salt concentration at a level considerably below that of the environment" (9).

To achieve low intracellular Na^+ ion concentrations against a constant influx of Na^+ ions leaking inside through a not completely impermeable membrane, in addition to Na^+ entering the cells during cotransport with amino acids and other substrates (see below), mechanisms of Na^+ extrusion have to be present in the cell membrane. Two possible mechanisms have been suggested: activity of Na^+/H^+ antiport and presence of a primary respiration-driven Na^+ pump. It is generally accepted that Na^+/H^+ antiport activity is an important mechanism for maintaining low intracellular sodium concentrations. The Na^+/H^+ antiporter activity of *S. costicola* has been relatively well studied (105, 106, 332). However, there is still considerably controversy about the occurrence and relative importance of primary Na^+ pumps in different representatives of the very heterogeneous group of moderate halophiles. *S. costicola* cells maintain a proton motive force (the combined action of the pH gradient over the membrane and the membrane potential) varying from 170 to 100 mV (inside negative) in cells grown at pH values varying from 5.7 to 9.0. Cells maintain their intracellular pH close to 7.5, and thus the reversed pH gradient is compensated at least in part by an increased membrane potential at high medium pH. The primary respiration-driven outward proton pump supplies the driving force for the Na^+/H^+ antiport activity for Na^+ efflux. Protonophores, such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 3,3′,4′,4-tetrachlorosalicylanilide (TCS), respiration inhibitors, and monensin, which facilitates Na^+/H^+ exchange, dissipate the proton motive force, thereby abolishing Na^+/H^+ antiport activity (105). When *S. costicola* cells, kept anaerobically at pH 6.5, were given a pulse of oxygen, protons were extruded, both in the presence and in the absence of Na^+ ions. At pH 8.5, an acidic response was observed in absence of Na^+ but alkalinization was noted in its presence, suggesting that an Na^+/H^+ antiport mechanism is functional at pH 8.5 and that the establishment of an Na^+ gradient participates in pH homeostasis. All these effects are abolished by protonophores. At pH 8.5, growth is prevented by CCCP (at the rather high concentration of 50 μM [see below]) and by TCS (106). During growth on lactate as the energy source, Na^+ translocation in *S. costicola* was inhibited by CCCP but little affected by valinomycin. Amiloride, an inhibitor of the eukaryotic Na^+/H^+ antiporter system, inhibited lactate-dependent Na^+ translocation. It was concluded that lactate-dependent H^+ extrusion enables the generation of a Na^+ gradient mediated by the secondary Na^+/H^+ antiporter (332). All of the evidence reviewed above supports the idea that H^+ extrusion is the primary process, enabling the secondary conversion of the proton motive force into a sodium motive force via the antiporter.

However, primary Na^+ pumps have been postulated to be involved in the extrusion of Na^+ from certain moderate halophiles ever since the first respiration-driven Na^+ pumps were identified in *Vibrio alginolyticus* and the moderate halophile *S. costicola* (328). The evidence for the existence of such a pump is based in part on the fact that growth of this organism in alkaline media is not inhibited by the protonophore CCCP. Thus, growth was postulated to occur in the absence of proton circulation across the cell membrane. The Na^+-specific pump, with an optimum in the alkaline pH range, then maintains the intracellular cation environment, in addition to the Na^+/H^+ antiporter, which is operative at low pH (328). The primary outward Na^+ pump in *S. costicola* was identified to be the NADH oxidase (NADH:quinone reductase) segment of the respiratory chain. At pH 8.5, the formation of a membrane potential was inhibited by 2-heptyl-4-hydroxyquinoline (NQNO), which inhibits the Na-motive NADH oxidase. When NADH served as electron donor, Na^+ translocation was resistant to CCCP and stimulated by valinomycin (as noted above, when lactate was used as the electron donor, Na^+ translocation was inhibited by CCCP and little affected by valinomycin). Amiloride had little effect on NADH-dependent Na^+ translocation (332).

In a comparative study of eight moderate halophiles, all six gram-negative species tested (*H. variabilis*, *H. halophila*, *H. canadensis*, *S. costicola*, "*P. beijerinckii*," and "*P. halosaccharolytica*") showed Na^+-dependent NADH:quinone reductase activity. Buildup of a membrane potential linked to NADH oxidation in inverted membrane vesicles was not completely inhibited by CCCP alone but was abolished by a combination of CCCP and the Na^+-conducting ionophore monensin. Succinate oxidation and the terminal oxidase step of the respiratory chain did not show any specific requirement for Na^+ ions. No Na^+-dependent NADH oxidase could be demonstrated in the gram-positive *Marinococcus halophilus* and "*Micrococcus varians* subsp. *halophilus*," since in these organisms the membrane potential generated during NADH oxidation was completely dissipated by CCCP (334). However, Nikolayev and Matveyeva (225) also postulated the presence of a primary Na^+ pump in "*Micrococcus varians* subsp. *halophilus*," based on the low level of inhibition of alanine transport by CCCP at alkaline pH.

Extensive studies on ion metabolism in *H. israelensis* also suggested the importance of a primary Na^+ pump in the regulation of intracellular Na^+ concentrations (152–154, 156). The presence of an uncoupler-stimulated Na^+ pump was postulated to explain the drop in respiration rate of up to 80% observed when the pH was increased from 6.5 to 8.5 in the absence of Na^+. Catalytic amounts of Na^+ prevented this drop. The rate-limiting step in the electron transport at high pH was located in the NADH-oxidoreductase section of the respiratory chain, and Na^+ was suggested to release the kinetic control (152). In the presence of Na^+, a pH gradient of reversed polarity was formed, in both the absence and presence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), driven by the Na^+/H^+ antiport activity. Inverted membrane vesicles responded as expected from the behavior of intact cells. It was concluded that extrusion of Na^+ ions is the primary event and that proton counterflow and Na^+/anion symport phenomena are secondary events which minimize the buildup of a membrane potential generated by the primary Na^+ pump (156). Respiration initiates efflux of Na^+ from preloaded cells, driven by an Na^+ pump insensitive to uncouplers such as FCCP and the ATPase inhibitor *N*,*N*′-dicyclohexylcarbodiimide (DCCD) (153, 154). Movement of Na^+ is thus directly coupled to electron transport. The uncoupler-insensitive primary Na^+ pump may play an important role in the regulation of intracellular salt concentrations.

When K^+ ions were omitted from the medium, a shift in pH from 6.5 to 8.5 caused an increase in respiration rate in *H. israelensis*, in contrast to the above-described drop in respiration in the absence of Na^+. When both Na^+ and K^+ were present, the pH did not affect the respiration rate (152). Stimulation of respiration by K^+ was most pronounced at acidic pH and was accompanied by an alkalinization of the cytoplasm. The effect of K^+ was observed only in intact cells. Possibly to compensate for the electrogenic entry of K^+, more protons are extruded by the primary proton pump, leading to internal alkalinization. The step in the electron flux affected by K^+ has not yet been identified (152).

MacLeod (184) suggested an alternative explanation not involving a primary respiration-driven Na^+ pump for at least

part of the observations presented above. Many of the conclusions relating to the nature of the ion pumps are based on the effect of ionophores such as CCCP on cells or membrane preparations. What is often not taken into account is that CCCP functions poorly at alkaline pH and may be altogether ineffective in complex media containing such ingredients as proteose peptone and tryptone. Cells in complex media may not be inhibited by high CCCP concentrations, while 5 μM CCCP causes complete growth inhibition in defined medium (106). The fact that CCCP causes only a partial collapse of the membrane potential in *S. costicola* at high pH (328) may thus be due to the ineffectiveness of this protonophore under the conditions used. In *S. costicola*, CCCP proved effective at higher concentrations and TCS was almost as effective at pH 8.5 as at pH 7 (106). MacLeod therefore suggested that NADH oxidation at pH 8.5 in *S. costicola* leads to proton extrusion, which drives the formation of a membrane potential through an Na^+/H^+ antiporter, and he argued that no compelling evidence exists that a protonophore-resistant primary electrogenic Na^+ pump operates in *S. costicola* at alkaline pH (184). To our knowledge, the above studies have not been pursued recently, and therefore the question of the relative importance of Na^+/H^+ antiporters and primary respiration-driven Na^+ pumps in the extrusion of sodium and the maintenance of low intracellular Na^+ concentrations in moderately halophilic bacteria remains unanswered.

Little information is available on transport systems for other inorganic ions in the moderate halophiles. A K^+ transport system was characterized in a *Halomonas* strain isolated as a contaminant from a *Dunaliella* culture, enabling cells to accumulate the necessary K^+ concentration following a hyperosmotic shock. The K_s of the transporter was 21.5 μM; thus, its affinity is lower than that of the well-characterized Kdp system of *E. coli*. However, high-affinity K^+ transport systems are probably not essential for *Halomonas* and other moderately halophilic bacteria, which generally live in seawater-derived brines, in which K^+ can hardly be considered limiting (36). A potassium extrusion system was described in *H. israelensis*, probably based on a K^+/H^+ antiporter. K^+ and Rb^+ ions were found to rapidly penetrate the cell membrane. Under energized conditions, a powerful pump extrudes this K^+ and Rb^+. At low pH values, the presence of Na^+ is not required for K^+ extrusion. However, millimolar concentrations of Na^+ are needed for effective K^+ and Rb^+ extrusion at high pH values, again possibly pointing to the involvement of primary Na^+ pumps. The K^+ pump was postulated to have a function in the regulation of intracellular salt concentrations and/or internal pH (310).

Little is known about the mechanisms of extrusion of anions from the cells of moderately halophilic bacteria. No information was found in the literature beyond the assumption that the membrane potential generated by Na^+ efflux may be the driving force causing efflux of permeant anions (153, 154).

Transport of Organic Compounds

The most widely studied transport system in moderate halophiles is the transport of α-aminoisobutyrate (AIB), a nonmetabolizable amino acid analog. In *S. costicola*, AIB transport is competitively inhibited by Gly, Ala, and to a lesser extent Met (107, 108). In *H. elongata*, competition was observed with Ala, Gly, Ser, D-Ala, L-homoserine, and D-Ser and to a lesser extent with Met, Leu, Phe, and His (190). The transport activity is Na^+ dependent. Sodium at concentrations below 0.2 M increased the apparent affinity of the *S. costicola* transport system. NaCl concentrations between 0.2 and 1 M increased the V_{max}, while at NaCl concentrations above 1 M, the V_{max} decreased without affecting the K_m (107, 108). In cells grown in the presence of 1 or 2 M NaCl, AIB transport was active at higher salinities than in cells grown in 0.5 M NaCl. Low-salt-grown cells adapted to AIB transport at high salinities after 6 h of incubation at high salt in a process independent of protein synthesis (175). A similar phenomenon was reported for *H. elongata*, which showed optimum AIB uptake at an NaCl concentration of 0.38 M in low-salt-grown cells and 1.37 M in high-salt-grown cells (190, 296). Adaptation of AIB transport in *S. costicola* to the highest salinities (4 M NaCl) required the presence of nutrients, probably related to the accumulation of compatible solutes, and glycine betaine was especially stimulatory (172, 175). Transport showed an optimum at pH 8.5 to 9 and required the presence of Na^+ and a membrane potential (107, 108, 183). Na^+ was required for AIB transport in all the gram-negative bacteria in which a respiration-driven primary Na^+ pump was demonstrated (*H. halophila*, *H. canadensis*, *H. variabilis*, *S. costicola*, "*P. beijerinckii*," and "*P. halosaccharolytica*"), while two gram-positive bacteria lacking the respiration-driven Na^+ pump (*Micrococcus halophilus* and "*M. varians* subsp. *halophilus*") did not require Na^+ for AIB uptake (333).

NaCl caused a two- to threefold stimulation of the transport activity of proline in *H. israelensis*. As in the case of AIB transport in *S. costicola* and *H. elongata*, the maximum uptake rate of proline was observed at salt concentrations similar to those at which the cells were grown (257).

Transport of different amino acids was compared in a variety of moderate halophiles by Nikolayev et al. (225, 226). In *S. costicola*, *Nesterenkonia halobia*, and *H. halodenitrificans*, all amino acid transport was found to be Na^+ dependent, but in some cases Li^+, K^+, NH_4^+, or Cs^+ could replace Na^+. In *S. costicola* transport of Asp in the presence of Li^+ was 10% of that with Na^+, while Ala transport was strictly Na^+ dependent. For Arg transport, K^+, NH_4^+, and Cs^+ could replace Na^+ to different degrees. In *H. halodenitrificans*, Arg transport in the presence of Li^+, NH_4^+, or K^+ was only 1.5 to 2 times lower than when Na^+ was present. Asp, Arg, and Ala could be transported by "*M. varians* subsp. *halophilus*" at salt concentrations of up to 2 M. The transport of Asp and Arg was inhibited by NaCl, while that of Ala was stimulated (226).

Organic Osmotic Solutes

Since the intracellular ion concentrations measured in the moderate halophiles are generally insufficient to provide osmotic balance with the external medium, much effort has been dedicated to the search for organic compounds accumulated by the cells. Only relatively recently were the principal organic osmotic solutes of the moderately halophilic bacteria identified. Techniques such as natural-abundance ^{13}C-NMR and high-pressure liquid chromatography are now routinely used to probe the intracellular environment of the moderate halophiles. These techniques led to the identification of the tetrahydropyrimidines ectoine and hydroxyectoine (Fig. 1), and to the recognition of the role of glycine betaine, which is not synthesized by most representatives of the group but can be readily taken up if available in the outside medium. Before the importance of these compounds was realized, unsuccessful attempts to identify intracellular organic compounds at high concentrations led to the view that these bacteria may be able to manage without the traditional osmotic balance and may maintain a hypoosmotic cytoplasm (351). The discovery that ectoine and hydroxyectoine, two compounds that have long resisted detection, may be present inside the cells in molar

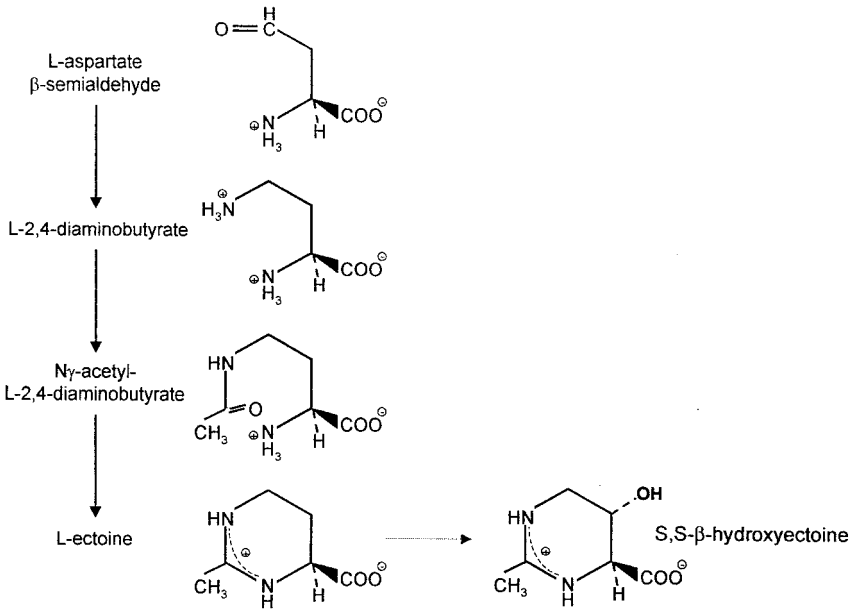

FIG. 1. Biosynthetic pathway for ectoine and hydroxyectoine based on enzymological studies. The proposed formation of hydroxyectoine (dotted line) from ectoine is still hypothetical. Modified from reference 85 with permission of the publisher.

concentrations abolished the need to hypothesize a hypoosmotic intracellular space (363). The observation that cells of *S. costicola* swelled rapidly upon treatment with penicillin (254) is also incompatible with the concept of a hypoosmotic cytoplasm, since it will not support the turgor pressure necessary for cell growth.

The organic intracellular solutes are called compatible solutes, since they provide osmotic balance without interfering with the metabolic functions of the cells (17, 132). We do not intend present here an in-depth discussion of the action of organic solutes, including the question why some are more "compatible" than others in enabling activity of salt-sensitive enzymes at low water activities. For a survey of the theories proposed, we refer the reader to the reviews by Galinski (80–82). Compatible solutes are polar, highly soluble molecules and uncharged or zwitterionic as physiological pH. They are strong water structure formers and as such are probably excluded from the hydration shell of proteins. This "preferential exclusion" probably explains their function as effective stabilizers of the hydration shell of proteins. Water near the interfaces is structurally different (more dense), allowing the osmotic solutes to migrate toward the less dense water fraction. This phenomenon of nonspecific exclusion is often described in terms of increased surface tension of water, with the presence of solutes affecting the forces of cohesion between water, minimization of entropy, and reinforcement of the hydrophobic effect. Compatible solutes display a general stabilizing effect by preventing the unfolding and denaturation of proteins caused by heating, freezing, and drying (79–82, 85).

(i) Amino acids. Most early attempts to detect organic osmotic solutes in moderately halophilic bacteria were aimed at the quantitation of intracellular amino acids. The search for amino acids was to some extent inspired by the finding that in vitro protein synthesis by ribosomes of *S. costicola* is already inhibited by relatively low chloride concentrations but proceeds uninhibited or even somewhat stimulated in the presence of 0.6 M potassium glutamate (141, 173). Also, a number of tricarboxylic acid cycle enzymes are strongly inhibited by Cl^- but function well in 1 M sodium or potassium glutamate or higher.

With the exception of a few specialized groups of gram-positive bacteria that accumulate proline (see below), amino acids do not seem to be of great quantitative importance as osmotic solutes in moderate halophiles. In *H. elongata*, the total intracellular amino acid pool increased with medium salinity (16, 105, and 358 mM in cells grown in 0.05, 1.37, and 3.4 M NaCl, respectively). In the 358 mM amino acid pool, Glu was found at the highest concentration, followed by Ala and Gln (359). In *H. halophila*, intracellular Gly and Asp concentrations increased with salinity (44). However, the data presented (in micrograms of amino acid per milligram of protein) do not allow an exact calculation of cytoplasmic concentrations. Quite high glutamate concentrations were detected in *S. costicola* (428, 1,203 and 389 mM in cells grown in 0.51, 1.7, and 3.4 M NaCl, respectively) (170).

Proline is the main organic osmotic solute in certain gram-positive species (*Salinicoccus roseus* and *Salinicoccus hispanicus*) (87, 306). In other gram-positive halophiles, amino acids do not contribute greatly to the osmotic balance. Thus, *Bacillus haloalcaliphilus* maintains an intracellular amino acid concentration of about 70 mmol/kg of water (361). The halotolerant *Planococcus* strain A4a, isolated from the dry valleys of Antarctica, showed an increase in the concentrations of intracellular amino acids with salinity: cells grown in 0 and 1.5 M NaCl contained intracellular Glu, Asp, and Gln concentrations of 102, 6, and 8 mM and 71, 49, and 39 mM, respectively. In 1.5 M salt-grown cells, the total intracellular amino acid concentration was about 340 mM, in addition to 605 to 640 mM K^+ (207).

(ii) Glycine betaine. Glycine betaine was recognized in the early 1980s as the most important organic osmotic solute in photosynthetic purple bacteria and halophilic cyanobacteria. Its possible role in the osmotic stabilization of halophilic heterotrophic bacteria had been suggested as early as 1968 (273). When grown in complex growth media, most halophilic bacteria accumulate glycine betaine or its precursor, choline, from

the medium, and under these conditions glycine betaine is often found as the sole or main osmotic solute (131). However, with the possible exception of the actinomycete *Actinopolyspora halophila*, none of the halophilic or halotolerant heterotrophic bacteria seem to be able to synthesize the compound de novo (80).

Certain halophilic bacteria can use glycine betaine not only as an osmotic stabilizer but also as a carbon and energy source. A number of haloalkaliphilic isolates from Mono Lake, Calif., grew on glycine betaine by sequential demethylation via dimethylglycine to sarcosine, which was then excreted into the medium. At high salinities, glycine betaine catabolism was suppressed and the compound was taken up solely to serve as an osmotic solute, thereby allowing extension of the salt range, enabling growth in 2.5 to 3–3.5 M NaCl (51). An unidentified *Halomonas* sp. grew on glycine betaine as the sole carbon and energy source by oxidation via dimethylglycine and sarcosine to glycine. Growth on glycine betaine was possible only up to 2 M NaCl. For growth at higher salt concentrations, the presence of another carbon and energy source such as glucose was required, and catabolism of glycine betaine was inhibited, so that it served as an osmotic solute only (36).

(iii) Ectoine and hydroxyectoine. Ectoine, first detected as an osmotic solute in photosynthetic purple bacteria of the genus *Ectothiorhodospira*, is derived from N-acetylated diaminobutyrate by intramolecular dehydration to form a pyrimidine derivative. As a result of the delocalization of the π electrons, the amino functions are not reactive, and therefore the compound has escaped detection for a long time (82). Ectoine and its β-hydroxy derivative are now recognized as the most widespread compatible solutes found in the domain *Bacteria* (see Table 6).

The biosynthesis of ectoine proceeds in three enzymatic steps, starting with aspartate semialdehyde, with diaminobutyrate and *N*-γ-acetyldiaminobutyrate as intermediates (259) (Fig. 1). All three enzymes, diaminobutyrate transaminase, diaminobutyrate acetyltransferase, and *N*-acetyldiaminobutyrate dehydratase ("ectoine synthetase"), have been identified in *H. elongata* (81, 259). Hydroxyectoine was hypothesized to be synthesized in a similar pathway, but the intermediates and enzymes involved are unknown (180). Some of the enzymes involved in ectoine synthesis in *H. elongata* have been isolated and partially characterized. The 2,4-diaminobutyrate transaminase has an apparent molecular mass of 260 kDa and probably consists of six subunits. The presence of K^+ ions (1 to 50 mM) increased the activity threefold and also stabilized the enzyme (323). An alternative biosynthesis pathway for ectoine, starting with glutamate, was suggested to be operative in *Brevibacterium linens* (15) and in *Streptomyces parvulus* (134).

Ectoine was found in high concentrations in different *Halomonas* species and is the dominant solute in cells grown in defined medium lacking glycine betaine or its precursor, choline (24, 363). Intracellular ectoine concentrations can be sufficiently high to balance the osmotic pressure of the medium in combination with the intracellular ions and other dissolved organic compounds present. *H. elongata* grown in 10% NaCl had an estimated ectoine concentration of 2.25 M, which, in addition to about 0.4 M cations and negative counterions, would give about 3.05 M, a value close to osmotic equilibrium (363). The ectoine concentration in *H. israelensis* is probably much lower: 0.1 to 0.2 M ectoine was measured in cells grown in 1 to 2 M salt (277). Also, *S. costicola* produces ectoine, but its intracellular concentrations never approach those of the NaCl in the medium. It was estimated that the "true" intracellular environment of *H. israelensis* and *S. costicola* may osmotically balance 60 to 80% of the extracellular solute concentration (160).

(iv) Other organic osmotic solutes. Amino acids, glycine betaine, and the tetrahydropyrimidines are by no means the only organic compounds found in moderately halophilic bacteria. The following are also found. (i) *N*ε-acetyllysine and *N*δ-acetylornithine were identified by ^{13}C-NMR in a number of gram-positive bacteria such as *Halobacillus halophilus* and *Planococcus* spp. The second compound is also known as an intermediate in arginine biosynthesis. Intracellular concentrations may be in the molar range (up to 0.5 μmol/mg [dry weight]). In a strain designated M96/12b, proline is the dominant osmotic solute in the exponential growth phase and is replaced by *N*δ-acetylornithine in stationary-phase cells (364). (ii) Polyamines cannot be expected to play a significant role in osmoregulation, since they were never found in high concentrations. Certain moderately halophilic bacteria may lack polyamines altogether: no polyamines were detected in *S. costicola* (109, 139). *Halomonas* and *Chromohalobacter* contain spermidine (0.3 to 2.1 μmol/g of wet cells, which is in the concentration range generally found in nonhalophilic bacteria) (109). When grown in a complex medium, *S. costicola* can incorporate polyamines (putrescine, cadaverine, spermidine, and spermine) from the medium. The presence of these polyamines did not increase the osmotic stability of the cells upon salt downshock (139).

(v) Distribution of organic osmotic solutes within moderately halophilic bacteria. An overview of the occurrence of organic osmotic solutes in different moderately halophilic bacteria is given in Table 6. A few general principles emerge from this table and from additional studies (80, 82, 87, 306, 330). (i) Cells grown in 10% salts generally have an organic solute content between 0.6 and 1.3 μmol/mg dry weight, equivalent to about 10 to 20% of the cellular dry weight. (ii) Apart from glycine betaine, all N-containing osmotic solutes are derived from the glutamate or the aspartate branch of amino acid biosynthesis. (iii) Uptake of osmotic solutes from the medium is preferred over de novo synthesis. (iv) All bacteria tested take up glycine betaine, but only *Actinopolyspora halophila* synthesizes the compound de novo. (v) Ectoine and hydroxyectoine are the predominant osmotic solutes in representatives of the gamma subclass of the *Proteobacteria* and in many gram-positive species. (vi) Charged amino acids such as glutamate never accumulate at concentrations exceeding 0.4 M. (vii) Proline, *N*δ-acetylornithine, and *N*ε-acetyllysine are found in low-G+C gram-positive bacteria such as some *Bacillus* species and the related genera *Salinicoccus* and *Halobacillus*. The interchangeability between proline and *N*δ-acetylornithine probably points to a common biosynthetic pathway. (viii) In most cases, cells do not accumulate a single osmotic solute but maintain intracellular cocktails of different compounds. Glutamate is often found as a minor compound in organisms that contain primarily ectoines (36, 165). The types of osmotic solutes present in the cells may depend greatly on the growth conditions. Thus, *H. israelensis* contained mainly trehalose when grown below 0.6 M salt, but at higher salt concentrations ectoine became the major solute (277).

(vi) Uptake and action of exogeneously supplied glycine betaine. When grown in complex media, all heterotrophic, aerobic, moderately halophilic bacteria tested were found to contain glycine betaine at concentrations increasing from around 0.2 M to 0.65–0.85 M to over 1 M in cells grown in 3, 10, and 20% salts, respectively (131, 133). Glycine betaine is either taken up from the medium (171) or derived from choline by means of an O_2-dependent choline oxidase (producing betaine aldehyde), and an NAD-dependent betaine aldehyde dehydro-

TABLE 6. Organic osmotic solutes within moderately halophilic bacteria[a]

Species[b]	Glycine betaine	Ectoine	Hydroxyectoine	Trehalose	Others
Micrococcus halobius		++	+	+	Glucose, glutamate
"Micrococcus varians subsp. *halophilus"*		++	+		Glucose, glutamate
Marinococcus halophilus		++	++		Glucose
Marinococcus albus		++	+		Alanine
Planococcus citreus					Proline, *N*-acetylornithine, *N*-acetyllysine
Halobacillus halophilus		++			Proline, *N*-acetyllysine
*Salinicoccus roseus**	+				Proline
*Salinicoccus hispanicus**	+				Proline
Halomonas halophila		++	+		Glutamate
Halomonas elongata		++	+		Glutamate, glucose
Halomonas halmophila		++	+		Glutamate, alanine
Halomonas halodenitrificans		++			Glutamate, glucose
Halomonas salina		++	+		Glutamate
*Halomonas variabilis**	++	++	++	+	
Halomonas eurihalina	++	+			Glutamate
Salinivibrio costicola	++				Glutamate
*Chromohalobacter marismortui**		+	++		
"Pseudomonas halosaccharolytica"		++	+		
Bacillus haloalkaliphilus	+	++			*N*-Acetylornithine
Bacillus halophilus		++			Proline, *N*-acetylornithine

[a] Data were derived from references 43, 87, and 306.
[b] ++, present in high concentrations; +, present in minor amounts; *, yeast extract was present in the medium, enabling the accumulation of glycine betaine.

genase. *H. elongata* and *S. costicola* possess both enzymes, and hybridization experiments with specific gene probes have detected genes similar to the *betA* and *betB* genes of *E. coli* (22). The choline oxidase of *S. costicola* is membrane bound, and its activity was strongly induced by the presence of choline and inhibited by Cl^- ions. The betaine aldehyde dehydrogenase is located in the cytoplasm. Its activity was stimulated by 0.5 M NaCl, and the enzyme could function at up to 2 M salt. The intracellular glycine betaine concentration increased with external salt and with the availability of choline (29, 171). A high-affinity transport system for glycine betaine and the enzymes of the choline-betaine pathway were characterized in *H. elongata* (23). Thin-layer chromatography and ^{13}C-NMR analysis showed that exogeneous choline is taken up and transformed in *H. elongata*, demonstrating the existence of the choline-glycine betaine pathway (23).

The first indication of the importance of glycine betaine in the physiology of moderately halophilic bacteria came from a study of respiration in *H. israelensis*. Glycine betaine was found to improve the salt resistance of the respiration system and to accelerate succinate oxidation. Stimulation was more pronounced the higher the NaCl concentration of the medium (273, 309). Labeled glycine betaine was accumulated by the cells. The authors stated that, "Apparently the prerequisite for the betaine effect is that the cells be free of, or contain little of, a genuine factor associated with salt resistance, and betaine acts by replacing it. Therefore the high-salt-grown cells, not influenced by betaine, presumably contained the genuine factor, which they may have acquired either through accumulation from their environment or by a process of synthesis and retention," and, "Since it was shown that betaine accumulation was also salt-dependent, it seems that salt resistance was directly related to intracellular betaine concentration." (273). The real nature of the stimulatory effect of glycine betaine is not yet completely clear, and it was suggested to be due neither to stimulation of substrate uptake nor to counteracting of plasmolysis; instead, it seemed to involve an effect on the cytoplasmic membrane, distinct from its function as a compatible solute (296). Ken-Dror et al. (155) suggested that the effect of glycine betaine on respiration may be explained by isoosmotic adaptation due to its accumulation in the cytosol (deplasmolysis) or by facilitation of penetration of Na^+ into plasmolyzed cells. Na^+ was found to be required for the proper functioning of the respiratory chain, and no glycine betaine accumulation was observed in the absence of Na^+.

(vii) Accumulation of other osmotic solutes by transport from the medium. Glycine betaine is not the only compound that can be taken up from the medium and serve as an osmotic solute. Other more unusual compounds can be accumulated for the same purpose. Two haloalkaliphilic isolates from Mono Lake designated strains ML-D and ML-G, produce ectoine in minimal medium. Strain ML-D also synthesizes hydroxyectoine. Under nitrogen limitation, exogenously supplied dimethylsulfoniopropionate (DMSP) (normally produced by marine algae as an osmotic solute) substituted for ectoine in strain ML-G and became the major intracellular solute. Strain ML-D could accumulate arsenobetaine (produced by the brine shrimp in Mono Lake) for the same purpose (32). Not only can DMSP serve as an osmotic solute in heterotrophic bacteria, but also it may be catabolized. Two moderately halophilic alkaliphiles isolated from Mono Lake could grow on DMSP, one strain by cleaving it to DMS and acrylate, the other by demethylation with 3-methiolpropionate as an intermediate, yielding methanethiol. At high salinities, DMSP catabolism was suppressed and the compound was preferentially taken up and used for osmotic purposes (51).

ENZYMES

When discussing enzymatic activities of moderately halophilic bacteria, and especially their relation to salt, we must discriminate three distinct categories of activities: (i) intracellular enzymes, which are not exposed to the salt concentration of the medium but sense the "true" intracellular environment—in most cases characterized by low ion concentrations and the presence of organic osmotic solutes; (ii) membrane-bound activities, including transport proteins, which sense both the intracellular environment and the outer medium; and (iii) truly extracellular enzymes, exposed to the external hypersaline con-

ditions. For earlier reviews on the subject, we refer the reader to the papers by Cazzulo (27), Kamekura (138), and Kushner and Kamekura (176).

Cytoplasmic Enzymes

The pyruvate kinase of *S. costicola* has a high percentage of the acidic amino acids Asp and Glu and a low proportion of hydrophobic amino acids and is enriched in Gly and Ser, all typical characteristics of halophilic proteins (47, 48). Stability was improved at high salinities. However, while a low concentration (0.25 M) of NaCl or KCl was somewhat stimulatory, virtually no activity was detected above 1 M salt. This would imply that if the intracellular Na^+ and K^+ concentrations are as high as reported (Table 5), the enzyme can be expected to be strongly inhibited under physiological conditions (48), assuming no sparing effect of compatible solutes.

The phosphoenolpyruvate carboxykinase of *S. costicola* did not show a great excess of acidic over basic amino acid residues, and in this respect it resembled the enzyme from animals and yeast. However, an unusually high content of Gly and Ser was found (16.7 and 10.2%), which supposedly maintains the balance between hydrophilic and hydrophobic forces. Oxaloacetate decarboxylation and $^{14}CO_2$-oxaloacetate exchange were activated by NaCl and KCl at concentrations up to 1 M (with the activities being about 2.5 times as high as in the absence of salt), while the optimum for carboxylation of phosphoenolpyruvate was found at 0.025 to 0.05 M salt. In view of these properties, it was suggested that under physiological conditions the enzyme probably acts in the direction of phosphoenolpyruvate synthesis (300, 302).

Other enzymes from *Salinivibrio* that have been characterized are the NADP-specific malic enzyme, glycerol dehydrogenase, threonine deaminase, and aspartate transcarbamylase. The malic enzyme was found to be unstable in dilute solutions but stable in 1 M NaCl or KCl or in 25% glycerol. Maximum activation occurred by 0.1 M NH_4Cl or KCl (not by NaCl), and higher concentrations were inhibitory, with about 23% of the optimal activity remaining at 0.85 M NaCl or KCl (301). The glycerol dehydrogenase was most active at 0.25 to 0.5 M salt, and KCl was preferred over NaCl. The enzyme was very salt tolerant, with about 60% of its maximal activity remaining in 3 M KCl. In this respect, it is not greatly different from a similar enzyme from *E. coli* (9). The threonine deaminase was most active in the absence of salt, with as little as 30% of the optimal activity remaining in 3 M NaCl or KCl. However, salts did stabilize the enzyme. Regulatory properties were not greatly influenced: feedback inhibition by isoleucine was observed both in the absence of salt and in 3 M NaCl or KCl, so this enzyme is expected to function well over a wide range of salinities (168, 169). The aspartate transcarbamylase showed much more pronounced halophilic properties: it proved stable at 1.5 M NaCl, but activity was rapidly lost at lower salt concentrations. Optimal activity was measured at 1.5 M NaCl or 1.0 M KCl, but high activity was still present at 0.15 M NaCl. The enzyme differs from its nonhalophilic (*Saccharomyces*) and halophilic archaeal (*Halobacterium salinarum* [formerly *H. cutirubrum*]) counterparts in the salt response patterns of activity and regulation: ATP and CTP were inhibitory, with the strongest inhibition in the low-salt range. Above 0.5 M salt, hardly any regulatory effect of CTP was observed. This observation is important since enzymes must not only be active but also be subject to allosteric regulation (5).

The properties of the alanine dehydrogenase of *H. elongata* depend greatly on the salt concentration at which the cells were grown. The enzyme isolated from cells grown at 50 mM salt was optimally active at salt concentrations between 0 and 20 mM, while the enzyme isolated from 1.37 M and 3.4 M salt-grown cells had its optimum at 340 and 500 to 600 mM, respectively (19). The estimated intracellular salt concentrations of the cells grown at the respective salinities were 42, 312, and 630 mM; hence, the enzyme seems to be well adapted to the actual conditions inside the cells. No simple explanation was put forward for the phenomenon, but for cells grown at 0.05 and 1.37 M salt, two bands with alanine dehydrogenase activity were detected on native gels, while for cells grown in 3.4 M, only one band was seen (19). Experiments involving dissociation during different enzyme purification methods and sequence analyses would clarify the effect. Low salt concentrations were optimal for the functioning of isocitrate, malate, succinate and α-ketoglutarate dehydrogenases from *H. halodenitrificans*, while lactate dehydrogenase and cytochrome oxidase (being membrane-bound enzymes) were more active in the presence of salt (10). Also in the alkaliphilic *Bacillus haloalkaliphilus*, the intracellular malate dehydrogenase and isocitrate dehydrogenase were inhibited by salt, with about 20% of the optimal activity remaining above 1 to 2 M NaCl (361). Organophosphorus acid anhydrase activity was detected in a halophilic isolate (designated JD6.5, probably belonging to the genus *Alteromonas*) isolated from a warm, hypersaline (24% salt) spring near the Great Salt Lake, Utah (38). No information was given about the salt requirement or the sensitivity of the enzyme.

The complex enzymatic machinery required for protein synthesis by the ribosomes presents an excellent object for the study of the influence of salt on intracellular activities, and therefore extensive research efforts have been dedicated to in vitro protein synthesis by *S. costicola* ribosomes (30, 141, 171, 173, 368). These studies included the measurement of poly(U)-directed phenylalanine incorporation and [^{14}C]valine incorporation, with *E. coli* phage R_{17} RNA as the template, leading to the formation of a protein with the same electrophoretic mobility as the valine-rich R_{17} coat protein. Optimum activity was obtained at low salt concentrations (0.1 to 0.3 M, with ammonium glutamate being the most stimulatory). Choride salts proved especially inhibitory (with NH_4Cl, KCl, and NaCl being increasingly toxic), and hardly any activity was observed in the presence of 0.6 M Cl^-. Choride was found to prevent the attachment of the 50S ribosomal subunit to the 30S subunit-mRNA complex and also displaced already bound ribosomes. The accuracy of the translation process was not affected. However, the inhibitory effect of Cl^- could be partially reversed by glycine betaine or glutamate. When Cl^- was replaced by other anions, such as glutamate, sulfate, or acetate, excellent in vitro protein synthesis activity was found at cation concentrations as high as 0.6 M, both in *S. costicola* and in *H. canadensis* (30, 141, 171, 173, 368). Not all components of the protein-synthesizing machinery are equally chloride sensitive: the phenylalanyl-tRNA synthetase of *S. costicola* functions well at 0.5 M NaCl. The ribosomal proteins of *S. costicola* are slightly more acidic than those of *E. coli* but much less so than those of the halobacteria. The *S. costicola* ribosomes showed an unusual sedimentation behavior in sucrose gradients: association of subunits to monosomes took place only at high salt concentrations (367). A study of the binding of dihydrostreptomycin to the 30S ribosomal subunits of *S. costicola* showed more binding in cells grown at high salt concentrations, suggesting that some ribosomal properties may vary with the salt concentration in which the cells were grown (161).

Stress Proteins

Upon sudden exposure to high NaCl concentrations (2 to 2.5 M), low-salt-grown *H. halophila* cells showed a transient cessation of cell division. During the initial phase of the adaptation to the new conditions, protein synthesis and amino acid uptake were inhibited. During the adaptation period, changes in the patterns of pulse-labelled proteins were observed. Distinct changes were also seen in the protein profiles upon downshock from 2.5 to 1 M salt, but only minor changes were found upon downshock from 1 to 0.5 M (59). High-salt-related proteins of 39, 24, 20, and 15.5 kDa have been identified in *H. elongata*, and the synthesis of these proteins increased with increasing salinity. A different set of proteins (60, 42, 15, and 6 kDa) was induced under low-salt conditions (209).

A study of protein turnover in *S. costicola* by monitoring the breakdown of pulse-labeled proteins showed that in cells growing exponentially in 1 or 1.5 M NaCl the turnover is about 5% per h and in cells growing in 0.5 M NaCl the turnover is as high as 9% per h, compared with 1 to 2% per h for *E. coli*. A shift from high-salt to low-salt conditions gave rise to an increase in the turnover rate of pulse-labeled proteins. It was suggested that the low salt concentration alters the "native" protein conformation and increases the susceptibility of proteins to proteolysis. Hipkiss et al. postulated that the increased protein turnover at low salinity may set a limit to growth at such concentrations (119).

The appearance of heat shock proteins was examined in *Chromobacterium marismortui*. When cells were grown in 1 M salt, transfer to 42°C resulted in complete inhibition of the synthesis of the normal proteins and induced the formation of heat shock proteins. In cells grown in 2.5 M salt, a heat shock at 42°C still allowed the synthesis of some of the normal proteins. The higher the salt concentration at which the cells were grown, the higher the upper temperature limit at which heat shock proteins could be synthesized (150). A similar phenomenon was observed in *H. halophila* (149).

Also, the sensitivity of *H. halophila* to oxidative stress (sensitivity to H_2O_2) depends on the NaCl concentration of the medium. Cells grown in 1 M salt were much more resistant to peroxide than were 2.5 M salt-grown cells. The effect could not be explained by differences in catalase activity. Exposure to 50 μM H_2O_2 resulted in the induction of several new proteins. The number of these proteins and the kinetics and extent of their induction were influenced by salinity, with the adaptive response to nonlethal concentrations of H_2O_2 being faster in 1 M than in 2.5 M NaCl (220).

A manganese superoxide dismutase of *H. israelensis* was also characterized. This enzyme did not require salt for activity (373).

Membrane-Bound Enzymes

Membrane-bound enzymes are in contact with the high salt concentrations in the medium, and therefore they can be expected to be active at high salt concentrations or even to require high salt concentrations for full activity.

A membrane-bound 5′-nucleotidase of *S. costicola* was optimally active at 2 M NaCl or KCl or higher. This enzyme splits ATP, ADP, and AMP, which are dephosphorylated to adenosine, which is subsequently transported into the cells. Its activity is distinct from that of the F_0F_1 ATPase also present in the membrane and can be differentiated from that of the proton channel ATPase by its higher magnesium requirement and its insensitivity to DCCD (11, 12). Also the DCCD-inhibited F_0F_1 ATPase of *S. costicola* requires high salt concentrations for both activity and stability, with optimal activity reported at 0.5 M KCl. KCl concentrations of up to 3 M caused relatively little inhibition, but 3 M NaCl produced 75% inhibition (26, 117).

The NADH:quinone oxidoreductase of *H. israelensis* depends on Na^+ for activity. Na^+ ions increased the rate of quinone reduction severalfold, but oxidation of the quinol with oxygen was not affected by Na^+ (153). Succinate respiration in this organism was Na^+ dependent and showed a maximum rate between 0.2 and 0.8 M Na^+ (272).

The membrane-bound nitrate reductase and nitrite reductase activities of *H. halodenitrificans* were separated by treatment with detergent and ammonium sulfate fractionation. The nitrite reductase was identified as a cd-type cytochrome (99, 100). Both a membrane-bound and a cytoplasmic nitrite reductase activity were detected. When phenazine methosulfate and ascorbate served as electron donors, the membrane-bound enzyme produced nitrous oxide and the cytoplasmic enzyme produced nitric oxide. In the presence of methyl viologen and dithionite, the cytoplasmic enzyme produced ammonia. After solubilization, the membrane-bound enzyme behaved like the cytoplasmic one and produced nitric oxide or ammonia in the presence of the above-mentioned electron donors. All activities are probably due to one *cd*-type cytochrome enzyme, whose products depend on its location and on the nature of the electron donors (186). Manometric activity measurements in resting-cell suspensions showed nitrite reduction to be optimally active at 0.38 to 0.75 M NaCl, with about 20% of the maximum activity remaining in the presence of 3 M NaCl (283). A membrane-bound assimilatory nitrate reductase was characterized in *H. israelensis*. This enzyme accepts electrons from the electron transport chain in a pathway that includes a *b*-type cytochrome and was found to be very similar to dissimilatory membrane-bound nitrate reductases known from other organisms. The assimilatory nitrite reductase was soluble, repressed by ammonium ions, and induced by nitrate, and it used reduced ferredoxin as its preferred electron donor (125).

Other membrane-associated enzymes activated by salt are the lactate dehydrogenase of *H. halodenitrificans* (optimum concentration, 0.8 M NaCl) (10, 283) and the alkaline phosphatase of "*P. halosaccharolytica*" (370). Alkaline and acid phosphatases were detected in *H. elongata*, with both enzymes being located on the cell envelope. In low-salt (50 mM)-grown cells, little activity was detected, and at higher salinities (1.4 and 3.4 M), the activities of both enzymes were optimal at the same salt concentration at which the cells were grown. These enzymes thus seem to "adapt" to the NaCl concentration in the medium in a mechanism that was not further elucidated. The effect was not due to the presence of isozymes, since only one band of each enzyme was detected on gels (18).

Extracellular Enzymes

A considerable amount of effort has been dedicated to the study of extracellular salt-tolerant enzymes of the moderately halophilic bacteria, especially toward the use of such enzymes in biotechnological processes.

Halophilic amylases were characterized from a moderately halophilic *Acinetobacter* (236), *N. halobia* (240), "*M. varians* subsp. *halophilus*" (158), and other *Micrococcus* isolates (157, 233, 234). A moderately halophilic *Acinetobacter* strain isolated from sea sand had two amylases. Both were most active at pH 7 and 50 to 55°C in 0.2 to 0.6 M NaCl or KCl. Activity was lost upon dialysis against water but could be stabilized by the addition of 10 mM $CaCl_2$. Cells grown in 1 to 2 M NaCl made the largest amount of enzyme, and only a small amount of amylase was produced during growth in low-salt medium or at 4 M NaCl (236). An amylase was purified from *N. halobia*. It was

optimally active at pH 6 to 7 in 0.25 M NaCl or 0.75 M KCl and at 50 to 55°C. The products of starch degradation were maltose, maltotriose, maltotetraose, and small amounts of glucose (240). "*M. varians* subsp. *halophilus*" produced the highest amylase activity when grown in 2 M NaCl. When the organism was grown at lower salt concentrations, the excreted enzyme was inactivated by the low salt concentration but could be reactivated by dialysis against 2.5 M NaCl. The enzyme was stabilized by 10 mM $CaCl_2$. The purified amylase has two components with apparent molecular masses of 86 and 60 kDa. Both were optimally active at 0.75 to 1 M NaCl or KCl and pH 6 to 7 at 55°C (in the absence of $CaCl_2$) or 60°C (in the presence of 50 mM $CaCl_2$) (158). The amylases from two other micrococci showed maximum activity in 1 M NaCl at pH 7.5 and 50°C (157) and in 1.4 to 2 M NaCl at pH 6 to 7 and 50°C (233, 234).

Little information has been published on extracellular proteases from moderately halophilic bacteria. An unidentified pseudomonad was reported to secrete a protease with a maximum activity at 18% salt (337).

"*M. varians* subsp. *halophilus*" produces a nuclease (nuclease H) when grown in 1 to 4 M NaCl or KCl. The purified enzyme has both DNase and RNase activities. Enzyme production is maximal in media containing 2.5 to 3.5 M salt. The enzyme was also synthesized in media containing RbCl, CsCl, or Na_2SO_4 but not in media containing LiCl, $NaNO_3$, NaBr, or NaI (147). Activity was optimal when the organism was grown in 2.9 M NaCl or 2.1 M KCl at 40°C. Activity was lost by dialysis against water but was restored upon dialysis against buffer containing 3.4 M NaCl (142, 143). The protein had a large (21 mol%) excess of acidic over basic amino acids (145). Inclusion of high magnesium (40 mM or higher) or phosphate (over 14 mM) concentrations in the medium abolished extracellular nuclease activity and caused flocculation of the cells. Under these conditions, the enzyme was adsorbed to the surface of the flocs (144, 372). The nuclease shows considerable biotechnological potential (see below).

Another halophilic nuclease (an exonuclease, releasing 5′-mononucleotides from both DNA and RNA) was produced by *Bacillus halophilus*. The enzyme was optimally active in 1.4 to 3.2 M NaCl or 2.3 to 3.2 M KCl and was stimulated by Mg^{2+} and Ca^{2+} ions. Activity was lost by dialysis against water, but 10 mM Ca^{2+} stabilized the protein and dialysis against 3.5 M NaCl restored most of the activity. The optimum pH was 8.5, and the optimum temperature was 50 or 60°C when DNA and RNA served as the substrate, respectively (239).

Amino Acid Composition of Proteins from Moderately Halophilic Bacteria

Halophilic archaea of the family *Halobacteriaceae* contain high intracellular salt concentrations, and their proteins are adapted to the presence of molar concentrations of K^+, Na^+, and Cl^-. This adaptation is reflected in the bulk amino acid composition of these microorganisms, showing a high content of acidic amino acids (Glu and Asp), and a low content of basic amino acids. A low proportion of hydrophobic amino acids is offset by a high frequency of amino acids such as Gly and Ser (177).

The aerobic halophilic bacteria tolerate high salt concentrations, but organic osmotic solutes provide most of the osmotic balance. However, the apparent intracellular salt concentrations can be fairly high (Table 5). Therefore, one should expect that extracellular and membrane-bound proteins of the halophilic bacteria may display halophilic characteristics with amino acid compositions similar to those found in the halophilic archaea, while there is little a priori reason to assume that the cytoplasmic proteins should show such adaptations. However, in view of reports of high apparent intracellular salt concentrations in certain species (Table 5), some degree of halophilic character may be expected in the intracellular enzymes as well.

Comparative studies showed that ribosomal proteins from moderate halophiles often had a slightly higher content of acidic amino acids than did the comparable proteins from *E. coli* and other nonhalophiles. Thus, the N-terminal sequences of the ribosomal A proteins of *S. costicola* and *H. canadensis* were slightly more acidic than that of the *E. coli* protein but much less so than that of the similar protein from *H. salinarum* (*cutirubrum*), with the ratios of basic to acidic amino acids being 0.36 in *S. costicola* and 0.41 in *H. canadensis*, compared to 0.54 in *E. coli* and 0.05 in *H. salinarum* (62, 63). The values for whole ribosomes of *H. canadensis* were 1.23 and 1.16, respectively, compared to 1.58 in *E. coli* and 0.69 in *H. salinarum* (61). The average hydrophobicity of the ribosomal proteins of *S. costicola* and *H. canadensis* did not differ greatly from that for *E. coli* and was much higher than that for *H. halobium* (194).

Determinations of the abundance of different amino acids in the bulk protein of *H. elongata* showed an excess of acidic amino acids and a low frequency of basic amino acids, with the values being intermediate between those for *E. coli* and the archaeon *Haloferax mediterranei*. Convergent evolution of amino acid usage was suggested to have led to this "halophilic" character of the *H. elongata* proteins (93). However, this convergent evolution did not lead to changes in the frequencies of the hydrophobic amino acids. The ratio of the occurrence of hydrophobic amino acids (Ala, Val, Leu, Ile, Phe, and Met) to that of the borderline hydrophobic amino acids (Ser and Thr) was 3.56 in *H. elongata*, compared with 3.58 in *E. coli*, 2.73 in *H. mediterranei*, and 2.46 in membranes of *H. salinarum* (248).

CELL ENVELOPES

The cytoplasmic membrane forms the barrier between the cytoplasm (generally low in salt) and the environment with its high and possibly fluctuating salinity. It can thus be expected that the properties of this membrane are regulated by the outside salt concentration to adjust such functions as ion permeability and the activity of integral membrane proteins (294). The envelope of the moderate halophiles is involved in regulating the cytoplasmic ionic environment by both binding and pumping ions, and haloadaptation is at least in part a response of the cell envelope to osmotic stress (296).

Salt-dependent changes in the properties of the cell membrane have been identified on the levels both of the types of phospholipids that dominate in the membrane structure and of the types of fatty acid chains present in the lipids.

Polar Lipids

Studies of the effect of salinity on polar lipid composition of the membranes have consistently shown that the higher the salinity, the higher the content of negatively charged phospholipids at the expense of neutral phospholipids. With increased salt concentrations, the amount of phosphatidylethanolamine (PE) (uncharged) is decreased and there is a concomitant increase in the amounts of the negatively charged phosphatidylglycerol (PG) and/or diphosphatidylglycerol (cardiolipin, CL) (294, 351, 354).

To explain the shift toward a higher negative-charge density on the membrane at increasing salt concentration, it was first postulated that the increase in the amount of anionic lipids

served to allow charge balance at the membrane surface exposed to high Na^+ concentrations (121, 122). However, a simple calculation showed that millimolar concentrations of salt would suffice to provide the negative-charge shielding (296). Moreover, a similar increase in the amount of negatively charged polar lipids was observed when nonionic solutes were added to the medium to increase its osmotic value. Another idea proposed is that the high content of negative phospholipids may contribute to the regulation of the selective permeability of the membrane to cations (231). It is now assumed that the change in polar lipid composition provides a mechanism for preserving the membrane bilayer structure. PE containing unsaturated fatty acids tends to form nonbilayer phases, while PG forms bilayers. Addition of PG to PE in the appropriate amounts could suppress the formation of a hexagonal-II phase and thus counteract the effect of increased salinity. A functional membrane requires a suitable proportion of bilayer-forming and non-bilayer-forming lipids. Modification of the membrane phospholipid ratio is necessary to preserve the integrity of the membrane bilayer in the face of an increased tendency of PE to form nonbilayer phases as a consequence of raised external salinity. The tendency to form nonbilayer phases could activate specific phospholipid-synthesizing enzymes located in the membrane, resulting in a rise in the proportion of lipids such as PG that prefer the lamellar phase, to counteract the disruptive forces of the nonbilayer-phase-forming lipids (293, 294, 322).

(i) *Salinivibrio costicola*. The major lipids of *S. costicola* are PG and PE, with lesser amounts of lyso-PE, CL, a glycolipid, and two other phospholipids, tentatively identified as lyso-CL and lyso-PG. When the medium salinity was increased over the range 0.5 to 3 M, the level of PG increased and that of PE decreased. Thus, the PG/PE ratio increased from 0.5:1 to 1:1 when the medium salinity was increased from 1 to 3 M. The growth phase of the culture had little effect on the lipid composition (110, 322). The altered salt dependence of *S. costicola* at temperatures below the optimum may be due to a modification in the membrane lipid phase behavior and stability brought about by changes in lipid composition (3).

The effect of salinity on the phase behavior of total lipid extracts from *S. costicola* was compared with the phase behavior of binary mixtures of PG and PE. Although many similarities were detected, certain differences were observed, suggesting that the minor lipid components do play a part in determining the phase behavior of the membrane (322).

Salt-sensitive mutants of *S. costicola* have been isolated with a phospholipid composition similar to the wild type when grown in 1 M NaCl but showing only a partial change in phospholipid composition upon growth at elevated salt concentrations. These data provide evidence that a correct phospholipid composition may be needed for haloadaptation (162).

High concentrations of the chaotropic anion SCN^- (1 M as KSCN but not as NaSCN) caused lysis of *S. costicola*. The protective effect of Na^+ is probably due to specific interactions of Na^+ with components of the cell membrane, thereby rendering their structure resistant to the action of chaotropic anions, which act by breaking hydrophobic bonds (335).

(ii) *Halomonas elongata*. In cells grown at 3.4 M NaCl, the CL content was four times as high as in cells grown at 0.05 M NaCl; the change was mainly at the expense of PE, while the PG content did not change greatly. Thus, the anionic lipids PG and CL contributed 57% of the polar lipids at high salt concentrations and 45% at low salt concentrations (354).

The hydrophobic-hydrophilic cell surface character of *H. elongata* changed with medium salinity, as shown in phase partitioning studies. Mid-exponential-phase cells growing at 3.4 M NaCl were more hydrophilic than cells at 0.05 M NaCl, and high-salt-grown cells were more hydrophilic than low-salt-grown cells in all stages of growth. Surface hydrophobicity tended to increase as the cells approached the stationary phase. The hydrophobicity of the cell was not influenced by trypsin treatment, in contrast to many other microorganisms in which trypsin treatment converts hydrophobic to hydrophilic cells. The increase in the proportion of charged phospholipids at higher NaCl concentrations may in part explain the hydrophilicity. At the high salinity, the hydrophilic cell surface makes the cell more attractive to water molecules in a water-poor environment and the hydrated cell surface may help the cell obtain cytoplasmic water and thereby prevent desiccation (114). Electron microscopic studies showed that an increasing medium salinity had a profound effect on the cell envelope of *H. elongata*, with the cell wall becoming more compact internally and coherent at higher salinity (354). The cell wall peptidoglycan is unique in that it contains leucine, which contributes to the overall hydrophobicity of the cell. To study the differences in cell wall composition, rabbits were immunized with live *H. elongata* cells grown at different NaCl concentrations. Antiserum prepared against low-salt-grown cells reacted with cells grown at all salt concentrations, but antiserum against high-salt-grown cells was specific for this type of cells. Two possible explanations for the finding were put forward: either the low-salt-grown cells possessed a unique antigen, or some type of conformational change in the surface antigens caused the rabbits to respond to a determinant that was not exposed in low-salt-grown cells (355).

(iii) *Halomonas israelensis*. The major lipid components of *H. israelensis* are PG and PE, with α-glucosyl-PG (256) and an acidic glucuronic acid-containing glycolipid (glucuronosyldiglyceride). In stationary-phase cells, the CL level was increased at the expense of PG (317). The biosynthesis of glucosyl-PG was studied in a cell-free system and was found to proceed from UDP-glucose and PG. Mg^{2+} and Ca^{2+} were required for activation, and KCl and NaCl were inhibitory, even at the low concentration of 0.1 M (319). Labeled glucuronosyldiglyceride was formed from UDP-[^{14}C]glucuronic acid and added diglycerides in a cell-free preparation. KCl and NaCl were inhibitory, with 0.5 M causing more than 80% inhibition (318).

(iv) *"Pseudomonas halosaccharolytica."* The major phospholipids of "*P. halosaccharolytica*" are PE, PG, CL, and an unidentified phosphoglycolipid—a glucosyl derivative of PG containing phosphate, glycerol, fatty acids, and glucose in the ratio 1:2:2:1. When grown in the lower-salinity range (0.5 to 1 M NaCl), PE was twice as abundant as PG, while at high salinity (3 M or more), the amounts of PE and PG were approximately equal (121, 122, 230, 231). At a combination of high temperature and high salt concentration, acidic phospholipids were especially abundant (73.6 mol%), due to an increase in CL and glucosyl-PG levels with a decrease in the PE level (112). Upon entering the stationary growth phase, the content of CL increased at the expense of PG. The cytoplasmic membrane contained a higher proportion of acidic polar lipids than did the outer membrane (50 and 40% respectively, in cells grown in 2 M NaCl). When 1% glucose was added to the medium, the phosphoglycolipid content increased to 25% of the total lipid. Thus, the phospholipid pattern resembled that of other moderate halophiles discussed above, with the phosphoglycolipid being possibly identical to the α-glucosyl-PG identified in *H. israelensis* (230). As in *H. israelensis*, UDP-glucose served as the glucosyl donor for its biosynthesis (229). The glucuronosyldiglyceride of *H. israelensis* was not detected in "*P. halosaccharolytica*" (230).

The outer membrane proteins of "*P. halosaccharolytica*"

show a 20 to 26 mol% excess of acidic amino acids over positively charged ones, a level similar to that found in the extremely halophilic archaea. When the medium salinity was increased from 0.85 to 2 M, there was a decrease in the level of a major 43-kDa outer membrane protein and an increase in the level of a 50-kDa protein. A shift from 2 to 3 M salt resulted in a decrease in the level of the 50-kDa protein and the appearance of a 41-kDa protein (120–122).

(v) Other gram-negative halophilic bacteria. The membranes of the gram-negative facultatively anaerobic halophilic rod-shaped bacterium designated *Vibrio* strain HX, now renamed *Halomonas canadensis* (128) contained PE and PG (together constituting more than 90% of all lipids) with small amounts of CL and traces of a glycolipid and other lipids. The PE/PG ratio decreased from 1.6 to 1.0 when the medium NaCl concentration was raised from 1 to 4 M (2).

(vi) Gram-positive moderate halophiles. In the gram-positive species, the increase in the anionic lipid fraction with salinity is generally due to an increase in the amount of CL rather than of PG. The effect may be nonspecific, since an increase in the amount of CL at the expense of PG is often associated with slowly growing cells, and growth rates are often reduced at high salt concentrations (293, 294, 296). Glyco (phospho)lipids are comparatively rare in the gram-negative bacteria but are often found in gram-positive bacteria (295). PE was reported to be absent in *Bacillus haloalkaliphilus* (327). Studies of lipid metabolism in *Planococcus* sp. strain A4a showed an increase in the amounts of anionic phospholipids and a decrease in the amount of PE in the presence of increasing salt concentrations (205). When the organisms were grown at high concentrations of monovalent cations, the CL/PG ratio was increased, while this ratio remained unchanged when $MgCl_2$, $CaCl_2$, or $MgSO_4$ was used to increase the medium salinity (206). Elevated temperatures and salinity had an antagonistic effect on phospholipid composition: a rise in temperature caused an increase in the PE content and a decrease in the CL content (205, 293).

(vii) Adaptation of the polar lipid composition to changing salinities. The effect of sudden shifts in salt concentration on lipid metabolism have been investigated in depth in *S. costicola* (1, 163, 295–297) and in "*P. halosaccharolytica*" (113, 121, 122, 231). Adaptation to the new increased salinity occurred in three phases. Within a few minutes, shrinkage of the cells with plasmolysis was observed and growth and phospholipid synthesis were severely inhibited. Within a few hours, growth resumed, as did the biosynthesis of PG, while PE synthesis remained at a low level, resulting in a decrease in the PE/PG ratio. The duration of this lag period depends on the extent of the salinity shift. Synthesis of new proteins is not required during the first two phases of the adaptation. Only when the correct membrane composition has been achieved can the organism resume growth. During the third phase, growth and lipid biosynthesis were fully adapted to the new salt concentration. The alteration in membrane phospholipid composition may be a necessary physiological response for adaptation to salinity changes (163). No phospholipid turnover is involved in the changes in lipid composition, with all changes being due to changes in the relative biosynthesis rates.

After a salt upshock from 1 to 3 M, the first increase in the concentration of PG relative to PE is observed within minutes (296). The changes are due to an up-to-30-fold decrease in PE synthesis, which recovered only slowly afterward, while PG synthesis showed only a 5-fold initial decrease and recovered rapidly (297). These changes in biosynthesis rates were found to be fully reversible when the external NaCl concentration was restored to the previous level (1).

Pulse-labeling and pulse-chase experiments with labeled acetate or phosphate showed that when a culture was shifted from 1 to 3 M salt, PG was synthesized more rapidly than PE during the lag phase, with both being lower than before the salt shift. Following a shift from 3 to 1 M, the incorporation of label into PE rose sharply within 5 min and then slowly declined while the incorporation of acetate into PG decreased to about 50% of the value for PE (297).

It was argued that part of the effects of salt upshock may be due to inhibition of fatty acid synthetase by salt entering the cell, which would starve the phospholipid biosynthesis system of a supply of fatty acyl chains. Since the phospholipid biosynthetic enzymes are located in the cytoplasmic membrane, the biosynthetic enzymes may respond directly to alterations in membrane fluidity and/or phase when the external solute concentration is suddenly changed (297). The salt upshock is probably sensed via osmotic pressure effects, since nonionic solutes such as sucrose can cause similar effects on growth and phospholipid synthesis. Glycerol, which is much more permeable than sucrose, was less osmotically stressful and did not inhibit PG biosynthesis (1, 295, 297). Thus, osmotic pressure changes, rather than alterations in ion concentrations, are responsible for triggering the haloadaptive changes in this organism.

In "*P. halosaccharolytica*," pulse-label experiments with precursors such as serine, acetate, and glycerol showed similar effects of salt on phospholipid biosynthesis. The radioactivity incorporated in PE decreased with increasing NaCl concentrations, while incorporation in PG remained constant between 0.5 and 3.5 M salt (113, 121, 122, 231; for a critical assessment of these experiments, see reference 294).

Effect of Salt Concentration on Fatty Acid Composition

The adaptation to growth in different salt concentrations is often reflected in changes in the fatty acids of the membrane lipids. These changes are markedly different in gram-negative and gram-positive moderate halophiles. Since the fatty acid composition of membranes is also influenced by temperature, complex interrelations between salinity and temperature can be expected.

(i) Gram-negative halophilic bacteria. In gram-negative halophilic bacteria, the content of cyclopropane fatty acids and unsaturated fatty acids generally increases with salt concentration, with a decrease in the abundance of branched-chain fatty acids. The effect has been reported for such organisms as "*P. halosaccharolytica*" (231), *H. halmophila* (211), *H. halophila* (210), and *H. canadensis* (2). *S. costicola* was never shown to produce cyclopropane fatty acids. Cyclopropane fatty acids are made by the addition of a methyl group (donated by *S*-adenosylmethionine) across the double bond of a monounsaturated fatty acid, which makes the central part of the acyl chain more rigid.

An increase in unsaturated and cyclopropane fatty acid content is expected to cause an increase in membrane fluidity. However, this interpretation should be taken with some caution, because in some species the major change is from unsaturated to cyclopropane fatty acids. Our understanding of the functions and properties of cyclopropane fatty acids in membranes is limited. The physical properties of cyclopropane fatty acids (e.g., 17:0 cyclo) are intermediate between the corresponding saturated fatty acid (17:0) and the unsaturated fatty acid from which it was derived (16:1). Thus, an increase in the abundance of cyclopropane fatty acids at the expense of unsaturated fatty acids would tend to decrease membrane fluidity (293, 294).

A survey of the occurrence of fatty acids among members of

the *Halomonadaceae* and the genus *Flavobacterium* showed the major fatty acids to be 18:1 ω7c, 16:1 ω7c, and 16:0. Cyclopropane fatty acids were found in minor amounts (313). Other studies (75, 210, 255) showed a high abundance of cyclopropane fatty acids in *Halomonas* spp. The differences may be due to variations in oxygen tension, medium composition, and age of the culture. *Flavobacterium salegens* and *F. gondwanense* from Organic Lake, Antarctica, had a high abundance of 15:0 iso, 15:0, 15:1 ω10c iso, 15:1 ω10c anteiso, and 16:0. The structure of the branched-chain C_{15} monounsaturated fatty acids is unusual and may be used as a taxonomic marker for these bacteria. This biomarker is to be exploited in ecological studies of Antarctic lakes (313). Another study, including *Salinivibrio*, *Halomonas*, *Alteromonas*, *Chromobacterium*, *Flavobacterium*, and *Pseudomonas* isolates, showed 16:0, 16:1, and 18:1 (the predominant fatty acid in most species examined) to be present in all the strains tested while cyclopropane fatty acids were detected in 26 of 40 strains tested (212).

H. halophila showed an increasing content of cyclopropane fatty acids with increasing salt concentrations (from 7 to 25% of the fatty acids when NaCl was increased from 0.5 to 3.4 M at the optimum growth temperature of 32°C), with a concomitant decrease in the content of monounsaturated fatty acids. It was suggested that the cyclopropane fatty acid synthetase is activated or induced by high levels of salt (210). When the salt concentration was raised from 5 to 15%, the content of 17:0 cyclo increased from 0 to 8.5%, that of 19:0 cyclo increased from 4.2 to 14.9%, and that of the saturated branched-chain 15:0 decreased from 42.9 to 31.9% (211). *H. canadensis* contained twice as high a concentration of cyclopropane fatty acids when grown at 4 M as at 0.7 M salt (2). When the medium NaCl concentration was raised from 1 to 3 M, the endogenous activity of cyclopropane fatty acid synthetase measured in lysates was doubled. NaCl was inhibitory to both fatty acid synthetase (82% inhibition by 3 M NaCl) and cyclopropane fatty acid synthetase (97% inhibition by 1 M NaCl), but both activities were stimulated up to 100-fold by 2 to 3 M glycine betaine (165).

The metabolism of cyclopropane fatty acids was most thoroughly studied in "*P. halosaccharolytica*" (112, 121, 122, 129, 130, 167, 214, 230, 231). This species contains 16:0, 16:1, 18:0, 18:1, and 17:0 cyclo and 19:0 cyclo as the major fatty acids (230). The content of cyclopropane fatty acids increased with salinity. A corresponding decrease occurred in the content of monounsaturated species. It was suggested that cyclopropane fatty acid synthetase was induced by high levels of NaCl (120–122). The proportion of cyclopropane fatty acids also increased with temperature (231) and upon reaching the stationary phase (214).

The biosynthesis of cyclopropane fatty acids in "*P. halosaccharolytica*" was studied in depth by using labeled *S*-adenosylmethionine as the precursor in cell extracts (214). The cyclopropane fatty acid synthetase either is an integral membrane protein or is associated with the membrane. The activity of cyclopropane fatty acid synthetase was inhibited by NaCl or KCl but stimulated up to 12-fold by glycine betaine. Maximum activity was reached at 3 M NaCl, a phenomenon similar to that reported for *H. canadensis* (165). Shift-up experiments (from 0.7 to 2.1 M NaCl) showed that the enzyme activity responded immediately to increased glycine betaine concentrations and that enzyme induction is not required to achieve the salt-dependent alterations in cyclopropane fatty acid content observed in vivo. Other compatible solutes tested (trehalose and sucrose) were less effective than glycine betaine (ectoine was not tested because it was not available). It was suggested that the compatible solute accumulating upon salt upshock may directly stimulate or protect enzyme activity.

Electron paramagnetic resonance studies of rotational movements of lipids in the membranes in intact "*P. halosaccharolytica*" cells, using stearic acid with *N*-oxyl spin labels at different positions in the carbon chain, showed the presence of an unusually thick (up to a depth of 12 carbons from the hydrophobic surface) viscous surface region in the membrane bilayer. At the 12th carbon from the polar surface, the viscoelastic properties changed drastically. The membrane thus has two viscous outer regions and one fluid central region, with the border of the viscous region being located near the double bonds or the cyclopropane rings located at carbon 11 to 12 of the acyl chains. Increasing the salinity caused an increase of the rigidity, i.e., of the microviscosity of the polar surface in the lipid bilayer. It was suggested that the presence of thick viscous regions may be related to the need to withstand high osmotic pressure or to the requirement for a low permeability to Na^+ (121, 122, 129, 130, 167).

S. costicola does not produce cyclopropane fatty acids. Its main fatty acids are 16:1 *cis* 9, 16:0, and 18:1 *cis* 11, with minor amounts of 14:0, 17:1, and 18:0. Unsaturated fatty acids are synthesized using the anaerobic pathway, common to most prokaryotes. The content of 16:0 was lowest at the optimal salinity and increased at the low- and high-salt extremes of growth (0.5 and 3 M); 18:1 showed an opposite trend, with the highest content of monounsaturated fatty acids being found at the optimum salinity. For this organism, the growth phase had little effect on the fatty acid composition (110, 320, 322). Double-bond positional isomeric differences were found in the fatty acid compositions of PG and PE (the presence of isomers 16:1 *cis* 11 and 18:1 *cis* 13 in phospholipids of cells grown in 3 M but not in 1 M NaCl), indicating that salinity may affect the specificity of the fatty acid synthetase. Salinity had a greater influence on the fatty acid composition of PG than of PE, suggesting that PG and PE biosynthesis are controlled separately by a selective effect of NaCl which occurs after the CDP-diacylglycerol branch point (321).

(ii) Gram-positive halophilic bacteria. In the genera *Marinococcus*, *Nesterenkonia*, and *Halobacillus*, branched-chain fatty acids such as 15:0 and 17:0 are dominant (215). The concentration of these branched-chain fatty acids changes with salinity (294). In *Marinococcus halophilus*, an increase in salinity caused a response similar to that found with increased temperature: an increase in the content of saturated fatty acids (mainly 18:0) and a decrease in the content of the branched-chain 15:0 anteiso. The content of the unsaturated 16:1 increased from 40.9 to 48.8% when the salt concentration was raised from 5 to 15%. Cyclopropane fatty acids were not detected (213). In *Planococcus* strain A4a (ATCC 35671), 15:0 anteiso is the major fatty acid, and the branched-chain fatty acids make up more than 85% of the fatty acid content at all salinities (205).

GENETICS

The lack of suitable genetic tools has classically hampered the genetic manipulation of moderately halophilic bacteria. This fact was stated clearly in 1993 by Kushner (174): ". . .practically nothing is known about their genetics. There seems to be a silent agreement among molecular biologists, who think of halophilic bacteria at all, that these will be very close to the non-halophilic eubacteria in gene organization and expression. This implies that such attributes will not be affected by the high salt concentration around, and sometimes in, the cells. If true, this would be so strange to be worth establishing. I suspect,

however, that the halophilic eubacteria will eventually prove to have their own fascinating genetic, as well as physiological properties." Some 5 years have passed since those lines were written, and only recently were the first genetic systems developed and optimized for moderately halophilic bacteria, providing the tools to examine their genetic properties.

Genome Analysis and Physical Maps

So far, few studies on the analysis of the genome organization of moderately halophilic bacteria have been reported. In a recent study of *S. costicola* E-367, large restriction fragments of genomic DNA were separated by pulsed-field gel electrophoresis after digestion with *Sfi*I and *Mbo*I. The genome size was estimated to be 2,505 kb (*Sfi*I) or 2,259 kb (*Mbo*I), i.e., about half the size reported for *E. coli* but similar to that determined for *Vibrio cholerae* (199). The genome sizes of five other *S. costicola* strains, as determined by *Sfi*I digestion, ranged from 2,100 to 2,600 kb. Analysis of the restriction profiles led to the conclusion that strain E-367 harbored three plasmids (see below), as well as a megaplasmid. The DNA of *S. costicola* is likely to be highly methylated because digestion with *Mbo*I (which recognizes the DNA sequence GATC, and hence the dam methylase, but is inhibited by methylation) yielded very few cleavage sites. Different *Mbo*I restriction patterns were observed when the strain was grown at different salinities, suggesting that the methylation system may be affected by the salinity of the medium. The genome of 14 *Halomonas* and *Chromohalobacter* strains was analyzed by the same technique (197). The infrequently cutting restriction endonucleases *Spe*I and *Swa*I yielded characteristic fingerprints for each strain. The genome size for 11 *Halomonas* strains tested ranged from 1,170 to 2,490 kb, and that for the *Chromohalobacter* strains ranged from 1,170 to 2,210 kb. The macrorestriction fingerprint could be a useful tool to elucidate the taxonomic position of bacteria belonging to the *Halomonas-Deleya* complex.

Native Plasmids and Derived Cloning Vectors

The first plasmid from a moderately halophilic bacterium (pMH1, isolated from a strain of *H. elongata* [Fig. 2]) was reported in 1992 (67). This plasmid was also found to be harbored by *H. halmophila*, *H. halophila*, and *S. costicola*. Plasmid DNA was used to transform *E. coli* on media with different antibiotics. Digestion of the plasmid DNAs from transformants resistant to the antibiotics kanamycin, neomycin, and tetracycline showed a single plasmid profile identical to that pMH1. Therefore, it was concluded that this plasmid encodes resistance to these three antibiotics. The common occurrence of pMH1 in the four strains may reflect their close relationship within the *Halomonadaceae* and their presence in the same habitats. The occurrence of pMH1 in the parent strains was confirmed by Southern hybridization analysis.

To construct cloning vectors, screening for such plasmids was carried out with members of the genus *Halomonas* (339). Eight *Halomonas* strains were examined for the presence of plasmids and megaplasmids. Three cryptic plasmids, designated pHE1 (isolated from *H. elongata* ATCC 33174), pHI1 (from *H. israelensis*), and pHS1 (from *H. subglaciescola*), were isolated (Table 7). No megaplasmids were detected.

Because of its small size, plasmid pHE1 was selected for further characterization and construction of a shuttle vector for *Halomonas* strains. Its restriction map showed 10 unique restriction sites (Fig. 2). Hybridization experiments excluded the existence of sequences homologous to pHE1 in DNA from other *Halomonas* strains, and no homology to pMH1 was found. pHE1 does not confer resistance to 10 common antimicrobial agents, and it is unable to replicate in *E. coli*. Therefore, a number of mobilizable pHE1-derived hybrid plasmids that could be maintained both in *H. elongata* and *E. coli* were constructed. To generate chimeric pHS plasmids, an antimicrobial resistance gene suitable for selection of moderate halophiles was included by cloning the omega cassette, carrying streptomycin/spectinomycin resistance, into the *E. coli* vector pBluescript KS. To enable the mobilization from *E. coli* to *Halomonas* spp., the *oriT* of the broad-host-range IncP plasmid RK2 was incorporated, and to ensure replication in *Halomonas*, plasmid pHE1 was included. Thus, shuttle vector pHS15 was generated as the result of a *Bam*HI-*Bgl*II fusion of plasmid pHE1 in a recombinant product of pBluescript and pKSΩoriT. The vector was readily mobilized by the RK2 derivative pRK2013 to all *Halomonas* strains tested (339).

A cryptic narrow-host-range plasmid from *C. marismortui*, designated pCM1, that contains four restriction sites, was isolated (198) (Fig. 2). Lack of a selectable marker in pCM1 precluded an attempt at direct transformation into *E. coli*. Therefore, plasmid regions competent for self-replication were identified by cloning into suicide plasmids. Three suicide vectors were constructed from R6K plasmid-based pGP704, which can replicate only in strains that produce the Pir protein, an R6K-specific essential replication protein, and the trimethoprim resistance (Tp^r) gene from plasmid pAS396. Fragments of pCM1 were cloned into Tp^r derivatives of the suicide vector pGP704, and the resulting recombinant plasmids were used to transform *E. coli* CC118(pir). Recombinant plasmids were subsequently transferred by conjugation into different moderate halophiles and tested for their ability to propagate in the new hosts, which are not permissive for the R6K replicon of the vector. One construct, consisting of a 6.1-kb fragment of pCM1 cloned into one of the suicide vectors, was able to replicate as an autonomous Tp^r replicon in the different hosts tested.

The minimal replicon of pCM1 was localized on a 1,600-bp region which includes two functionally discrete regions, the *oriV* region and the *repA* gene. The *oriV* region, located on a 700-bp fragment, contains four 20-bp direct repeats, or iterons, adjacent to a DnaA box that is indispensable for efficient replication. In addition, it requires *trans*-acting functions. The *repA* gene, encoding a replication protein of 289 residues, is similar to the genes encoding the replication proteins of other gram-negative bacteria. Figure 3 shows the deletion analysis of the pCM1 replicon. No expression products of *repA* were obtained with either a prokaryotic in vitro DNA-directed transcriptional system or an in vivo system, possibly due to product instability (198).

Novel cloning vectors have been constructed, based on the combination of the minimal replicon of pCM1 with the useful properties of plasmid pUC18 (small size, high copy number, multiple cloning sites, etc.), as well as with the Tp^r gene (an antimicrobial selection marker different from that of pSH15). Conjugation mediated by the transfer machinery of plasmid RP4 is an efficient and reliable method for the introduction of foreign DNA into moderate halophiles. Two vectors, pEE3 and pEE5, were found to be suitable for use as narrow-host-range cloning vectors. Plasmid pEE5, a relatively small (7.0-kb) shuttle vector, contains unique recognition sites for five different restrictases and permits the selection of white or blue pigmented colonies, after introduction of the alpha-peptide of LacZ from plasmid pNot18 (Fig. 2) (202).

S. costicola E-367 carries three plasmids designated pVC1, pVC2, and pVC3 (199) (Fig. 2). On the basis of its small size and the presence of four unique restriction sites, pVC1 is a potential candidate for the construction of cloning vectors useful for *S. costicola*. Hybridization experiments showed that

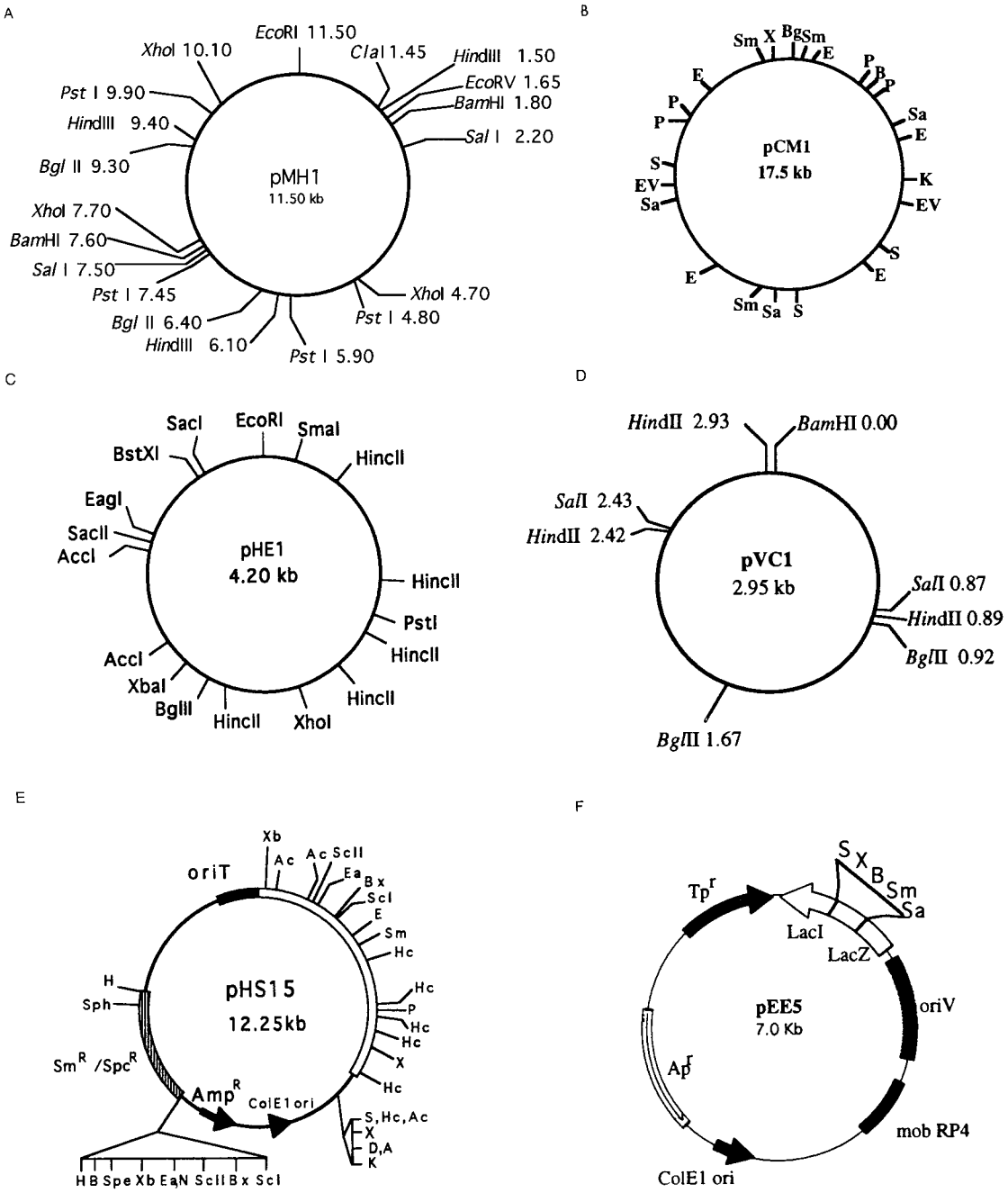

FIG. 2. Restriction maps of four plasmids and two derivative cloning vectors from the moderately halophilic bacteria *H. elongata* ATCC 33173 (A), *C. marismortui* ATCC 17056 (B), *H. elongata* ATCC 33174 (C), *S. costicola* E-367 (D), pHE1 derivative (E), and pCM1 derivative (F). Reprinted from references 67, 198, 199, 202, and 339 with permission of the publishers).

pVC1 is harbored only by *S. costicola* E-367 and is absent from 15 other *S. costicola* strains tested. No DNA homology among plasmid pVC1 and plasmids pMH1, pHE1, pCM1, and pVC1 could be detected.

Genetic Transfer

So far, conjugation is the only genetic transfer mechanism described for the moderate halophiles. Natural transformation has not been reported, and approaches such as electroporation (197) or $CaCl_2$ treatment (338) have either been unsuccessful or given nonreproducible results. Although some bacteriophages have been described for moderate halophiles, transduction methods have not yet been developed.

A major difficulty in the use of moderate halophiles for genetic transfer experiments is that at their optimal salinity they generally tolerate high concentrations of most antimicrobial agents (223, 270, 271). However, a decrease of the salinity resulted in an enhanced sensitivity of *Halomonas*, *Chromohalobacter*, and *Salinivibrio* strains to many antimicrobials (34, 166, 352). For nalidixic acid, spectinomycin, and tetracycline, the effect of salinity was less pronounced and strain dependent.

TABLE 7. Native plasmids and derived cloning vectors from moderately halophilic bacteria

Plasmid	Source	Size (kb)	Characteristics	Reference
pMH1	*H. elongata* ATCC 33173 *H. halmophila* ATCC 19717 *H. halophila* CCM 3662 *S. costicola* NCIMB 701	11.5	Km^r, N^r, Tc^r	67
pHE1	*H. elongata* ATCC 33174	4.2	Sequenced, RepA protein, *mob* genes	339
pHI1	"*H. israelensis*" ATCC 43985	48	Cryptic	339
pHS1	*H. subglaciescola* UQM 2927	ca. 70	Cryptic	339
pCM1	*C. marismortui* ATCC 17056	17.5	Replication origin sequenced, RepA protein	198
pVC1	*S. costicola* E-367	2.95	Cryptic	199
pVC2	*S. costicola* E-367	ca. 19	Cryptic	199
pVC3	*S. costicola* E-367	ca. 21	Cryptic	199
pEE3	pCM1 derivative	6.6	pCM1 replication functions, mobilizable, Ap^r, Tp^r	202
pEE5	pCM1 derivative	7.0	pCM1 replication functions, mobilizable, Ap^r, Tp^r, *lacZ*	202
pHS15	pHE1 derivative	12.25	pHE1 replication functions, mobilizable, Ap^r, Sm^r, Sp^r	339

All moderate halophiles tested showed a high sensitivity to rifampin and trimethoprim, regardless of the salt concentration (34). These findings greatly simplify the design of selection media and facilitate the use of genes encoding resistance to some antimicrobial agents, especially aminoglucosides, as genetic markers for plasmids or transposons.

Conjugation has been successfully used to transfer both native plasmid-derived vectors (198, 202, 338, 339) and broad-host-range plasmids for gram-negative bacteria (166, 338) from *E. coli* to *Chromohalobacter*, *Halomonas*, and *Salinivibrio*. Factors affecting the transfer frequency, such as cell growth phase, mating time, donor-to-recipient ratio, and composition and salinity of the mating medium, were evaluated to optimize the conditions for conjugation between *E. coli* and moderate halophiles (338). Table 8 summarizes the host range of the main cloning vectors reported. Whereas pHS15 (pHE1-derivative), pKT230 (IncQ), and pVK102 (IncP) were able to replicate in all hosts tested (338), pEE5 and pEE3 did not replicate in *S. costicola* (202), pGV1124 (IncW) was maintained only in *H. elongata*, and pCU1 (IncN) could not be established in any of the hosts tested. Since instability problems can be overcome by growing the plasmid-carrying cells under selective conditions (that is, in the presence of the antibiotic whose resistance is encoded by the plasmid), the vectors belonging to the incompatibility groups IncQ (pKT230) and IncP (pVK102), as well as the three shuttle vectors, seem to be suitable tools for use in moderate halophiles.

Conjugation between moderate halophiles has been demonstrated by using the self-transmissible IncP plasmid RK2 (338). Transfer of this plasmid between *Halomonas* species was found at frequencies up to 1.2×10^{-3} to 2.8×10^{-4}. Intergeneric conjugation between *H. elongata* and related species such as *H. eurihalina* (transfer frequency of 2.0×10^{-5}) and *H. halophila* (5.0×10^{-5}) on the same medium was also demonstrated.

Reporter Genes

The first gene reporter system in moderately halophilic bacteria was described in 1995 (7). The expression of the ice nucleation gene *inaZ* of the plant-pathogenic bacterium *Pseudomonas syringae*, a system that has proved useful in gram-negative bacteria, was used as a reporter for promoter activity and gene expression in several moderate halophiles. A promoterless version of *inaZ* was subcloned in vector pSH15 in both orientations and at two different restriction sites. Con-

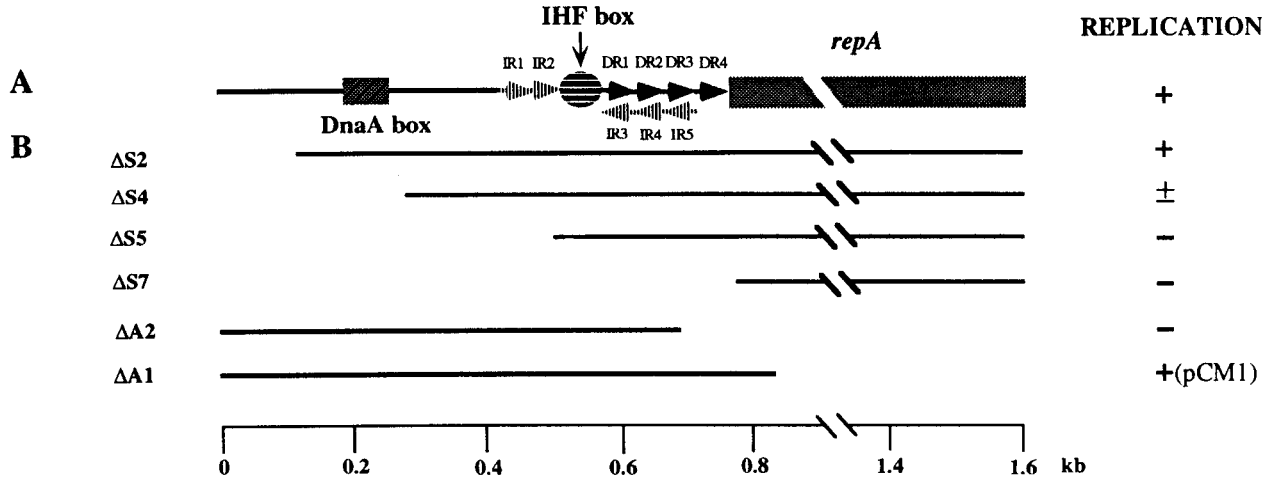

FIG. 3. Deletion analysis of the replicon of plasmid pCM1. (A) Schematic illustration of the organization of the 1.6-kb basic replicon. (B) Consequence of deletions on replication. + able to replicate; +(pCM1), able to replicate only in the presence of pCM1; −, not able to replicate; ±, defective replication. Reprinted from reference 198 with permission from the American Society for Microbiology.

TABLE 8. Host range, among moderate halophiles, of native plasmid-derived vectors and cloning vectors of the incompatibility groups IncP, IncQ, IncW, and IncN

Host	Conjugation frequency (no. of transconjugants/recipient cell) of:					
	pHS15	pEE5/pEE3	pVK102 (IncP)	pKT230 (IncQ)	pGV1124 (IncW)	pCU1 (IncN)
C. marismortui ATCC 17056	3.2×10^{-3}	10^{-2}–10^{-3}	3.3×10^{-4}	7.2×10^{-3}	0	0
H. halophila CCM 3362	6.3×10^{-4}	10^{-4}–10^{-5}	8.3×10^{-2}	2.5×10^{-1}	0	0
H. canadensis ATCC 43984	2.9×10^{-3}	ND[a]	2.0×10^{-3}	2.4×10^{-2}	0	0
H. halodurans ATCC 29629	2.5×10^{-3}	ND	6.2×10^{-2}	2.1×10^{-1}	0	0
H. israelensis ATCC 19717	8.3×10^{-2}	10^{-5}–10^{-6}	5.0×10^{-1}	3.3×10^{-2}	0	0
H. elongata ATCC 33173	1.6×10^{-1}	10^{-4}–10^{-5}	7.6×10^{-2}	9.5×10^{-2}	4.2×10^{-6}	0
H. elongata ATCC 33174	4.0×10^{-2}	ND	3.2×10^{-3}	5.0×10^{-2}	3.9×10^{-6}	0
H. meridiana DSM 5425	1.4×10^{-1}	ND	6.8×10^{-3}	7.4×10^{-2}	0	0
H. subglaciescola UQM 2927	1.1×10^{-2}	10^{-4}–10^{-5}	5.0×10^{-2}	3.9×10^{-2}	0	0
H. eurihalina ATCC 49336	4.1×10^{-2}	10^{-3}–10^{-4}	6.0×10^{-3}	5.0×10^{-2}	0	0
S. costicola NCIMB 701	1.7×10^{-4}	0	7.0×10^{-4}	1.3×10^{-3}	0	0

[a] ND, not determined.

structed plasmids were mobilized from *E. coli* to several moderately halophilic strains by triparental matings in which the RK2 *tra* genes were provided in *trans* by the helper plasmid pRK600. All moderately halophilic transconjugants harboring a pHS15 derivative expressed ice nucleation activity. One orientation of the *inaZ* constructs gave much higher activity in *H. elongata* and *H. eurihalina*, indicating that *inaZ* was possibly introduced in the correct orientation downstream of putative native promoters. Also, a significantly higher ice nucleation activity was found in a recombinant construct carrying a tandem duplication of *inaZ* in the same orientation, indicating that *inaZ* is appropriate for gene dosage studies. The gene was also transferred and expressed in *H. elongata* and *H. eurihalina* under the control of two heterologous promoters, P_{bla} (the promoter of the β-lactamase of *E. coli*) and P_{pdc} (the promoter of the pyruvate decarboxylase gene of *Zymomonas mobilis*), yielding an ice nucleation activity comparable to that obtained from the expression of the native putative promoters. It was thus demonstrated that foreign genes can be introduced and expressed in moderate halophiles, facilitating genetic improvement and strain construction. It was also shown that *inaZ* can be expressed in a broad spectrum of moderate halophiles (7).

The presence of two promoters in the native plasmid pHE1 of *H. elongata*, controlling the transcription of opposite orientations, was postulated (7). By using the promoter analysis vector pKK232-8, one of these promoters was further characterized by direct subcloning, deletion analysis, and assaying for ice nucleation and chloramphenicol acetyltransferase activities (324). The sequence of the region showing promoter activity revealed the existence of two possible consensus sequences (−10 and −35) for σ^{70}-dependent promoters of *E. coli*. Accordingly, this putative promoter was functional in this host. The identified promoter, the first characterized from a moderate halophile, showed comparable activity to that of the known promoters P_{bla} and P_{pdc} when expressed in *H. elongata* and *H. eurihalina*. Availability of a small pHE1 DNA fragment containing a promoter sequence may be useful for the construction of expression vectors for moderate halophiles.

Isolation of Mutants

Salt-sensitive mutants of *S. costicola* unable to grow at 2.5 to 3 M NaCl were isolated following UV irradiation and penicillin selection. Some of these mutants also showed an altered temperature range (162).

A transposon mutagenesis procedure was developed for *H. elongata* (166). The suicide plasmids pSUP101 and pSUP102-Gm were used to introduce the transposons Tn*5* and Tn*1732* into *H. elongata* via *E. coli* SM10-mediated conjugation. Tn*5* proved unsuitable for transposon mutagenesis in this moderate halophile. However, Tn*1732*, a transposon of the Tn*3* family, was useful for the isolation of mutants. Insertions occurred at different sites in the chromosome. Several auxotrophic mutants with a single requirement were generated, as well as different salt-sensitive mutants. Some of the salt-sensitive mutants were able to grow in 16% NaCl medium when supplemented with glycine betaine. It was suggested that these mutants may be defective in the genes for ectoine synthesis or its regulation (see below). One of the mutants was found to have a reduced ectoine and hydroxyectoine content (166).

The killing action and induced mutagenicity of hydroxylamine have been investigated in *H. elongata* and *H. meridiana* (25). The induced mutagenicity was assessed by measuring the frequency of appearance of mutants resistant to streptomycin (223). A number of single and double auxotrophic mutants of *H. elongata* were obtained, as well as different salt-sensitive mutants of both *H. elongata* and *H. meridiana*. Some of the mutants appeared to be affected in the synthesis of ectoine or in the genes responsible for the regulation of compatible solute synthesis, and others were probably deregulated mutants (25).

Genetic Basis of Osmoregulation

Accumulation of organic compatible solutes, either by de novo synthesis of compounds such as ectoine and hydroxyectoine or by transport of osmoprotectants from the medium, such as glycine betaine or its precursor, choline, has been studied at the genetic level (23, 35, 82).

Ectoine is produced from aspartate semialdehyde by the action of L-diaminobutyric acid transaminase, L-diaminobutyric acid acetyltransferase, and L-ectoine synthase (259) (Fig. 1). The genes encoding these enzymes have been isolated from *Halomonas* sp. strain KS-3 (208), from *H. elongata* DSM 3043 (24), and from *Marinococcus halophilus* (180). To clone the ectoine synthase gene from *Halomonas* sp. strain KS-3, Min-Yu et al. (208) amplified a 90-bp product by using primers based on the N-terminal amino acid sequence of the protein. This region was used as a probe against genomic DNA, and a 4.2-kb fragment that gave a positive hybridization signal was cloned in pBluescript II SK and the nucleotide sequence of the structural gene encoding the ectoine synthase (*ectC*) was determined. The ectoine synthesis genes of *M. halophilus* were cloned by functional expression in *E. coli*: colonies of *E. coli* XL1-Blue carrying a genomic DNA library of *M. halophilus* in

the vector pHSG575 were screened on minimal medium with elevated salinity; one clone, carrying the recombinant plasmid pOSM1, was able to grow at 5% NaCl thanks to ectoine production. Sequencing of a 4.4-kb fragment of pOSM1 revealed three open reading frames involved in ectoine synthesis: *ectA*, encoding L-diaminobutyric acid acetyltransferase; *ectB*, encoding L-diaminobutyric acid transaminase; and *ectC*, encoding ectoine synthase. The *M. halophilus ectC* gene had 47% identity to the corresponding gene of *Halomonas* sp. strain KS-3 (208). The genes were osmoregulated in *E. coli*, as demonstrated by an increasing cytoplasmic ectoine concentration in response to salinity (180).

To identify the ectoine biosynthetic genes of *H. elongata* DSM 3043, Cánovas et al. (24) isolated mutants blocked in its synthesis. Two of these Tn*1732*-induced mutants accumulated ectoine precursors, as shown by ^{13}C-NMR and mass spectroscopy. Mutant CHR62 accumulated low levels of diaminobutyric acid, while mutant CHR63 contained high concentrations of *N*-γ-acetyldiaminobutyric acid, suggesting that strain CHR62 could be defective in the diaminobutyric acid acetyltransferase gene and strain CHR63 could be defective in the ectoine synthase gene. The genes were isolated by colony hybridization, using a 370-bp chromosomal fragment flanking the Tn*1732* insertion in mutant CHR63 as a probe against a genomic library of *H. elongata*. One cosmid clone thus identified was able to complement the mutations in both CHR62 and CHR63, confirming that it carried the wild-type genes encoding the enzymes for the synthesis of *N*-γ-acetyldiaminobutyric acid and ectoine. Preliminary data on the sequence of the isolated region revealed that the predicted protein encoded by the ectoine synthase gene of *H. elongata* is highly homologous to the corresponding protein sequences of *Halomonas* sp. strain KS-3 and *M. halophilus* (22). The organization of the ectoine synthesis genes in *H. elongata*, most probably contained in one operon, seems to differ from that in *M. halophilus* (22, 24).

The genes encoding the transport system for glycine betaine in *H. elongata* DSM 3043 have recently been isolated (22). For this purpose, a gene bank of this organism was transferred by conjugation to an *E. coli* strain unable to take up glycine betaine and transconjugants were selected for growth in minimal medium containing 0.75 M NaCl and 1 mM betaine. A number of cosmid clones carrying the genes for glycine betaine transport were obtained. To isolate the genes of the choline-betaine pathway of *H. elongata*, the same gene bank was used to complement *E. coli* MKH13 harboring plasmid pJB004 (carrying the *E. coli* choline transport *betT* gene). Three cosmid clones were isolated that conferred to MKH13(pJB004) the ability to oxidize choline to glycine betaine, as shown by thin-layer chromatography of cytoplasmic extracts of the transconjugants. The isolated region has been sequenced, showing that it contains three genes highly homologous to the *betI*, *betB*, and *betA* genes of *E. coli* (22).

At least in some bacteria, halotolerance may be linked to the presence of certain plasmids. Preliminary studies have indicated that when *H. elongata* was cured of its single plasmid, it lost its ability to grow in 3.4 M NaCl (351). Similarly, a euryhaline strain designated "*Spirillum luteum*" was able to grow at up to about 1.2 M salt. However, after plasmid curing by acriflavine treatment, growth could be obtained at up to about 0.7 M only. It was concluded that salt resistance in this organism is probably controlled by a plasmid (216, 217). It is to be regretted that these studies were not pursued in further depth.

BIOTECHNOLOGICAL APPLICATIONS

Moderately halophilic bacteria have the potential for exciting and promising applications. Not only do many of them produce compounds of industrial interest (enzymes, polymers, and osmoprotectans), but also they possess useful physiological properties which can facilitate their exploitation for commercial purposes. First, most of them can grow at high salt concentrations (352), minimizing the risk of contamination. Second, they are easy to grow, and their nutritional requirements are simple: the majority can use a large range of compounds as their sole carbon and energy source (176). Moreover, as discussed above, many of the genetic tools developed for the nonhalophilic bacteria can be applied to the moderate halophiles, and hence their genetic manipulation seems relatively simple. In spite of all this, the moderately halophilic bacteria have not yet been used extensively for biotechnological purposes. In this section, we discuss some of the current industrial applications of these halophiles and emphasize some expected future developments.

Fermented Foods

Tetragenococcus strains are involved in the fermentation of soy sauce. In soy sauce manufacture, ground wheat and soy grains are suspended in water with about 19% NaCl and incubated for up to 9 months in the dark (172). Halophilic lactococci (*Tetragenococcus halophilus*) are used as starters for the fermentation and typically develop densities of up to 10^8 CFU/ml in soy sauce mash with about 3 M NaCl (289, 331). *Tetragenococcus muriatianus* is involved in the preparation of fermented liver sauce (304).

In the preparation of Thai fish sauce (nam pla, a food condiment widely used in Southeast Asia), moderate halophiles and halotolerant bacteria are used (*Bacillus* spp., coryneform bacteria, and, more rarely, pseudomonads, most of these tolerating up to 20 to 30% salt). Extremely halophilic red archaea are also found during the process (326).

Enzymes

As described above, a number of extra- and intracellular enzymes from moderately halophilic bacteria have been isolated and characterized. These include hydrolases (amylases, nucleases, phosphatases, and proteases), which are currently of commercial interest. Nuclease H produced by "*M. varians* subsp. *halophilus*" may be useful for the industrial production of the flavoring agent 5′-inosinic acid and 5′-guanylic acid (140) by using a bioreactor system with a column of flocculated cells (241). Esterases with high affinity for short-chain esters are made by a strain of *H. elongata* growing in Danish bacon curing brines (118), and the isolation of novel *Bacillus* species such as *B. salexigens*, which produces an extracellular nuclease in saline medium (89), may also lead to the development of industrially promising processes.

Compatible Solutes

Moderate halophiles accumulate high cytoplasmatic concentrations of low-molecular-weight organic compounds to cope with the osmotic stress and to maintain positive turgor pressure. The ability to produce and accumulate high concentrations of these compounds makes moderate halophiles useful for the biotechnological production of these osmolytes. Some compatible solutes, especially glycine betaine and ectoines, have gained considerable attention in recent years, because they may be used as stress protectants (against high salinity,

thermal denaturation, desiccation, and freezing) and stabilizers of enzymes, nucleic acids, membranes and whole cells (80, 82, 181). The industrial use of these compounds in enzyme technology (biosensor technologies, PCR, etc.) and for pharmaceuticals and cosmetics is a most promising field (84–86, 346).

Ectoine and hydroxyectoine exert a remarkable protective effect on a number of labile enzymes such as lactate dehydrogenase and phosphofructokinase. In a study with several compatible solutes (glycine betaine, trehalose, glycerol, proline, ectoines, and sugars), hydroxyectoine showed the highest efficiency of protection of lactate dehydrogenase against freeze-thaw treatment and heat stress whereas ectoine was the most effective freeze-stabilizing agent for phosphofructokinase (80, 181). Ectoine and hydroxyectoine can as yet be produced only biologically. The production of ectoine for industrial purposes by using *H. halodenitrificans* has recently been established (325). Cells were grown in an anaerobic fed-batch fermentation process, in a synthetic medium with glycerol at high concentration as the carbon source. Once a high cell density was reached, ectoine was extracted by "bacterial milking", a process previously developed with *H. elongata*, in which an osmotic down-shock from 10 to 2% NaCl is employed, resulting in excretion of about 80% of the intracellular ectoine to the surrounding medium (88, 305). Subsequent exposure of the cells to a hyperosmotic shock (from 2 to 10% NaCl) restored the original level of ectoine in a period of 10 h. Thus, a yield of 2 g ectoine per liter of medium per day was obtained. Ectoine and hydroxyectoine can also be produced by the gram-positive moderate halophile *Marinococcus* strain M52. A maximum yield of 35.3 g per liter cell dry weight was reached (77). The use of a dialysis reactor, in which cells were grown in an inner chamber fed with fish peptone and glucose, resulted in a dramatic increase in yield: up to 132 g of cell dry weight with about 20% hydroxyectoine was obtained (164). Another organism used for the production of ectoine is the actinomycete *Nocardiopsis lucentensis* A5-1, isolated from a saline soil near Alicante, Spain (179, 371).

Advances in fermentation technology and genetic engineering of moderate halophiles will allow large amounts of these compatible solutes to become available. As stated above, the genes involved in the synthesis of ectoine and its regulation in halophilic bacteria have recently been cloned (24, 179, 207), opening the way to its overproduction. It may also enable the transfer of genes for salt and drought tolerance from moderate halophiles to agricultural crops, such as wheat, rice, and barley, enabling them to grow in more saline soils.

Polymers

Bacterial polysaccharides are of great value as enhancers of oil recovery because of their surfactant activity and bioemulsifying properties. Since the conditions in oil deposits are often saline, the use of salt-resistant surfactants may be advantageous. Pfiffner et al. (260) isolated more than 200 bacterial strains capable of producing extracellular polysaccharides from oilwells and oil well-associated environments. The predominant type was a facultative anaerobic, as yet unidentified *Bacillus* species, growing at up to 12% NaCl. It produced a polysaccharide with pseudoplastic behavior, resistant to shear and thermal degradation, and showing higher viscosities at dilute concentrations and elevated temperatures than commercial polymers such as xanthan gum. The polymer is a charged heteropolysaccharide containing glucose, arabinose, mannose, ribose, and small amounts of allose and glucosamine. Production was optimal under anaerobic conditions in media with 4 to 10% NaCl.

H. eurihalina F2-7 produces large amounts of an extracellular polyanionic polysaccharide (263). The polymer, consisting of 42% carbohydrates (mostly hexoses) and 15% protein, is a potent emulsifying agent, exhibiting pseudoplastic behavior. It forms gels of high viscosity at acid pH. In view of these properties, the polysaccharide (EPS V2-7) may find broad applications in pharmaceuticals, in the food industry, and in biodegradation processes (21). Having a high content in sulfate groups, this polysaccharide was shown to have remarkable in vitro immunomodulating activity, enhancing the proliferative effect of human lymphocytes as a response to the presence of the anti-CD3 monoclonal antibody in blood (258).

Degradation of Toxic Compounds

Hypersaline waters and soils are often contaminated with heavy metals or other toxic compounds from anthropogenic sources. Hypersaline wastewaters are generated during the manufacture of chemicals such as pesticides, pharmaceuticals, and herbicides and during oil and gas recovery processes. Conventional microbiological treatment processes do not function at high salt concentrations, and therefore the use of moderately halophilic bacteria should be considered (250, 251).

Hydrocarbon-degrading moderate halophiles have been isolated from a variety of environments, including the Great Salt Lake (360) and Antarctic saline lakes (196). Woolard and Irvine (366) reported the utilization of a biofilm of a moderately halophilic bacterium isolated from a saltern at the Great Salt Lake, Utah, for the treatment of hypersaline wastewaters containing phenol. By using a batch biofilm reactor, more than 99% of the phenol was removed from a waste containing 15% salt. Benzoate and other aromatic compounds can be degraded by *H. halodurans* by cleavage of aromatic rings (291). A moderately halophilic bacterium isolated from a hypersaline spring in Utah degrades highly toxic organophosphorus compounds. The microorganism (strain JD6.5) grew at 2 to 24% salt and was tentatively identified as an *Alteromonas* strain (38). The enzyme responsible, organophosphorus acid anhydrase, was purified and characterized. Five additional halophilic bacteria, showing hydrolytic activity against several organophosphorus compounds and related chemicals, were isolated (37). Such enzymes may have considerable potential for the decontamination and mineralization of chemical warfare agents.

Moderate halophiles belonging to the family *Halomonadaceae* have been recently isolated from highly saline sites contaminated with the herbicide 2,4-dichlorophenoxyacetic acid; they were able to utilize chloroaromatic compounds as sources of carbon and energy. One of the isolates, strain I-18, showed high activities of catechol 1,2-dioxygenase, muconate cycloisomerase, and dienelactone hydrolase at about 1.0 M NaCl and pH 8.4 to 9.4 (185). This strain was also able to utilize other aromatic compounds including benzoic acid, 3-chlorobenzoic acid, and 4-chlorophenol.

Other Potential Applications

Moderately halophilic bacteria may have numerous additional potential uses in biotechnology, yet to be exploited. (i) It has been claimed that moderate halophiles may be used to remove phosphate from saline environments, as a cheaper alternative to chemical approaches (275). (ii) Moderate halophiles could be used in the recovery of hypersaline waste brines derived from the olive oil industry and leather- or fur-curing processes. (iii) They may be screened for the production of bioactive compounds such as antibiotics. (iv) Biological surfac-

tants derived from moderate halophiles may have a variety of applications in industry. Thus, Yakimov et al. (369) recently isolated a moderate halophile which synthesizes a novel glycolipid belonging to a powerful novel class of biosurfactants. The bacterium (strain MM1, a member of the *Halomonadaceae*) was isolated from saline water and sediment samples by enrichment on *n*-hexadecane. The purified surfactant is a powerful surface-active agent, showing a strong positive effect on chlorinated biphenyl degradation, probably due to the solubilization of these compounds in water. (v) New restriction endonucleases and other enzymes for molecular biological studies are expected to be present in microorganisms from hypersaline habitats and will be discovered and exploited. (vi) Many moderate halophiles produce orange or pink colonies, probably due to the production of carotenoids as a protective mechanisms against photooxidation processes. Carotenoids have major applications in the food industry as food-coloring agents and as additives in health food products. Therefore, investigations of the utilization of moderate halophiles as producers of carotenoids could be of great interest.

Finally, we may note that in the last few years, considerable economic effort has been made to facilitate the exploitation of the potentialities of extremophilic microorganisms such as moderate halophiles. Thus, in Europe, a consortium of 39 teams funded by the BIOTECH-Programme of the European Commission has recently finished a 3-year project aimed at isolating and/or selecting the extremophiles with the best potential for industry. In early 1997, a new project was initiated to utilize these microbes as cell factories for the production of commercially interesting compounds. It may be expected that the moderately halophilic bacteria will shortly be used routinely in biotechnology as their astonishing properties are increasingly exploited.

CONCLUSION AND FUTURE DIRECTIONS

Studies carried out, specially during the last decade, have increased our current knowledge about different aspects of moderately halophilic bacteria, such as their physiology, ecology, taxonomy or phylogenetic relationships with other microorganisms, and, to a lesser extent, their genetics. Besides, there are several fields in which their industrial applications are more promising, and, as in the case of other extremophilic microorganisms, they have an important biotechnological potential as a source of compatible solutes, enzymes, and other compounds of industrial interest. Moreover, moderately halophilic bacteria constitute an excellent model for the molecular study of the osmoregulatory mechanisms that permit them to grow over a wide range of salt concentrations. This aspect has very exciting potentialities, such as, for instance, their possible application in agriculture to construct salt-resistant plants carrying procaryotic genes encoding enzymes for the synthesis of osmoprotective compounds. For all these studies, the availability of genetic tools and an in-depth knowledge of their molecular genetics will be necessary, and thus, this field could be identified currently as one of the most attractive for researchers.

Systematic and phylogenetic studies have defined a large number of species to be included within the moderately halophilic bacteria, distributed over at least half of the major phylogenetic branches of the bacteria. Molecular ecology techniques available nowadays should be used to determine in more detail the ecological distribution of these halophilic microorganisms and the roles they play in hypersaline environments, as well as their contribution to microbial transformation processes. The use of such techniques would enable the elucidation of the biodiversity of moderately halophilic bacteria and the identification of species that constitute the predominant populations in these extreme habitats.

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