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Characterization of the Deoxyribonucleic Acid of Various Strains of Halophilic Bacteria

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Bacteria classified as extreme halophiles, in the genera *Halobacterium* and *Halococcus*, contain deoxyribonucleic acid (DNA) which displays two components in a CsCl equilibrium density gradient. The base composition of the major DNA component ranges from 66 to 68% guanine plus cytosine (GC), whereas that of the satellite DNA comprising some 11 to 36% of the total, is between 57 and 60% GC. Purification of the bacterial cells in a CsCl density gradient and other more conventional strain purification procedures both indicated that the presence of the satellite DNA component is not a result of mixed cultures.

In the classification proposed by Larsen (23), extreme halophiles are defined as those microorganisms for which optimal growth occurs at an NaCl concentration between 20 and 30%. The group of extremely halophilic bacteria is quite small, comprised mainly of members of two genera of morphologically different obligate aerobes. The bacteria of both genera are reddish in color, owing to the presence of carotenoid pigments. Members of the genus *Halobacterium* are gram-negative slender rods which are sometimes polarly flagellated. They have a specific requirement for the presence of at least 15% NaCl in the growth medium (23), but if the NaCl concentration is below 5 or 10%, they become spherical and lyse (1, 2, 38). The genus *Halococcus* proposed by Larsen (23) is otherwise known as the *Sarcina-Micrococcus* group. These are nonmotile, nonsporeforming cocci which require a concentration of 5 to 10% NaCl for growth. Unlike the halobacteria, however, these organisms do not lyse in solutions of low sodium ion concentration.

The intracellular salt concentration of the extreme halophiles is very high, ranging from 10 to 20% (11, 12, 17). Potassium, the major cation component of the internal salt (9), is specifically required in high concentration to maintain the structural integrity of the ribosomes (7) and to obtain the maximal rate of synthesis in an in vitro protein synthesizing system (6). The extensive work of Baxter and Gibbons (3-5), dealing with the effect of salt concentration on the activities of several enzymes from extremely halophilic bacteria, clearly demonstrates that these enzymes are adapted to function at high salt concentrations.

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In addition to these unique biochemical attributes of the extremely halophilic bacteria, Joshi et al. (22) observed a satellite component in the deoxyribonucleic acid (DNA) of two species of extreme halophiles in CsCl equilibrium density gradients. The purpose of this investigation was to determine whether the presence of a satellite DNA represents another general property of extremely halophilic bacteria. The accompanying communication deals with the nature of this satellite DNA component and the genetic relationships among the various halophilic bacteria (32).

MATERIALS AND METHODS

Bacteria and media. Strains of bacteria used and their sources are listed in Table 1. Additional strains were isolated by the procedure described by Eimhjellen (16), which consists of incubating pieces of salted fish partially submerged on fragments of filter paper in high salt medium. After 10 to 14 days at 37 C, the colonies which appear are streaked onto high-salt-agar medium. The resultant colonies are then purified by further plate streaking. Isolates I, II, III, and V were selected for use in this study. Based upon their rod shape, the presence of carotenoid pigments, their requirement for at least 15% salt for growth, and optimal growth rate in over 20% salt, isolates II and III can be considered members of the genus *Halobacterium*. The rod-shaped isolates I and V both lacking carotenoids grow in less than 5% salt. The optimal rate of growth occurs in salt concentrations of less than 20%. These isolates, therefore, belong to the group of moderate halophiles (23).

The extreme and moderate halophiles were grown in high-salt medium containing 0.5% tryptone (Difco), 0.5% yeast extract (Difco), 2.0% $MgSO_4 \cdot 7H_2O$, 25% NaCl, 0.2% $CaCl_2$, and 0.001% $FeCl_2$. The *p*H was 7.5 to 7.8. Because of the high phosphate content of

this medium, it was not suitable for the labeling of nucleic acids with ^{32}P. For this purpose a medium similar to that described by Dundas et al. (14) was used; it contained: 0.5% salt-free, vitamin-free casein acid hydrolysate (Nutritional Biochemicals Corp., Cleveland, Ohio), 0.5% yeast extract (Difco), 24% NaCl, 0.5% KCl, 0.5% $MgSO_4 \cdot 7H_2O$, 0.5% $MgCl_2 \cdot 6H_2O$, 0.1% $CaCl_2$, 0.5% NH_4Cl, and 0.2 ml of the vitamin mixture described by Raymond and Sixtrom (33). This medium was also found to be the best for the growth of *Halococcus morrhuae*. The cultures were incubated at 37 C.

The photosynthetic extreme halophile, SL-1, was grown on medium A plus 22% NaCl (33) in 1-liter glass-stoppered bottles submerged in a water bath at 44 C. The light source was a 150-w General Electric flood lamp placed approximately 15.24 cm from the culture. *Rhodopseudomonas spheroides* was grown as described for *Rhodomicrobium* by Duchow and Douglas (13). *Escherichia coli* and *Pseudomonas aeruginosa* were grown as described previously (31).

Determination of bacterial cell densities. The density of selected bacterial cells was determined by centrifugation in 25 ml of preformed CsCl density gradients. These linear gradients were made by mixing 12.5 ml of the high-salt medium for halophilic bacteria minus the tryptone and yeast extract (1.174 g/ml) with 12.5 ml of this solution containing 32 g of CsCl per 100 ml (1.379 g/ml). The gradient was formed with a sucrose gradient maker similar to that described by Britten and Roberts (8). The densities of individual fractions were determined by weighing 10-ml portions.

Cultures of bacteria in the exponential phase of growth were first subjected to 5 min of centrifugation at 3,000 × *g* in a Sorvall RC-2 centrifuge to remove the debris. Samples of the culture were then mixed with the solution of low density and the density gradient was prepared. The sample was then centrifuged for 30 min at 14,000 rev/min in an SW25 rotor at 4 C. After centrifugation, a hole was punctured in the bottom of the tubes and the fractions were collected. The number of bacteria per fraction was obtained by plating the appropriate dilution of each fraction on solid medium and counting the colonies which appeared after 10 days of incubation at 37 C.

DNA preparation. DNA samples were prepared from exponential or early stationary-phase cultures by the method of Marmur et al. (26), with an additional incubation of 1 hr at 37 C with 100 μg of self-digested Pronase (Calbiochem, Los Angeles, Calif.) per ml after ribonuclease treatment.

CsCl density gradient centrifugation. Analytical CsCl equilibrium density gradients were centifuged at 25 C for a minimum of 20 hr at 42,040 rev/min in a Spinco model E ultracentrifuge with an AN-F 4 cell rotor. Ultraviolet photographs, made on Kodak commercial film, were analyzed by using a Beckman Analytrol densitometer. The buoyant density of DNA was determined as described by Sueoka (40). The density of *Proteus rettgeri* DNA the marker DNA employed in this study was found to be 1.7018 g/ml by using *E. coli* DNA (1.7100 g/ml) as the standard. The base compositions of DNA were calculated from the relationship between per cent guanine plus cytosine (GC) and the buoyant density given by Schildkraut et al. (35).

RESULTS

Demonstration of satellite DNA in extremely halophilic bacteria. To determine the extent to which satellite DNA occurs among bacteria capable of growing in high salt concentrations, a number of strains were obtained from other laboratories (Table 1) or isolated by the procedure described above (16). DNA was extracted and purified from these bacteria and selected strains of nonhalophilic bacteria and analyzed by CsCl density gradient centrifugation in a Spinco model E ultracentrifuge (Table 2).

DNA from the extremely halophilic bacteria belonging to the genus *Halobacterium*, *H. salinarium*, *H. halobium*, *H. cutirubrum*, isolate II, and isolate III have major DNA components with a base composition ranging from 66 to 68%

TABLE 1. *Bacterial strains used and their sources*

Organism	Source
Halobacterium salinarium strain 1	Helge Larsen The Technical Institute of Norway
Halobacterium cutirubrum CCNRLO 9	Helge Larsen
Halobacterium halobium strain Delft	Helge Larsen
Halococcus morrhuae strain 24	Helge Larsen
Rhodopseudomonas spheroides ATCC 14690	Jane Gibson Cornell University
Rhodopseudomonas spheroides strains L and L-57	W. Garrard Univ. of California at Los Angeles
Rhodopseudomonas spheroides strains 241 and UV 33	W. R. Sistrom Univ. of Oregon
SL-1	W. R. Sistrom
Escherichia coli strain B	Univ. of Washington Culture Collection
Pseudomonas aeruginosa	Univ. of Washington Culture Collection

TABLE 2. *Analysis of bacterial DNA by CsCl equilibrium centrifugation*[a]

Organism	Buoyant density (g/ml)		Per cent GC		Approx per cent minor component
	Major component	Minor component	Major component	Minor component	
Halobacterium salinarium	1.7253	1.7179	67	59	19
H. halobium	1.7253	1.7180	67	59	30
H. cutirubrum	1.7248	1.7158	66	57	11
Isolate I	1.7224		64		0
Isolate II	1.7272	1.7192	68	60	29
Isolate III	1.7260	1.7163	67	57	36
Isolate V	1.7216		63		0
Halococcus morrhuae	1.7260	1.7179	67	59	31
R. spheroides	1.7302		72		0
SL-1	1.7288		70		0

[a] A 4-μg amount of the sample DNA and 1 μg of *P. rettgeri* marker DNA (density 1.7018 g/ml) were centrifuged to equilibrium in 57% CsCl at 42,040 rev/min and 25 C for a minimum of 20 hr in a Spinco model E ultracentrifuge. The buoyant density and % GC of the DNA were determined as described by Schildkraut, Marmur, and Doty (35). The percentage of the minor DNA component was approximated by weighing the area under the curves obtained by microdensitometer tracings of ultraviolet photographs.

GC and a minor DNA component ranging in base composition from 57 to 60%GC (Table 2). These values are in the range expected for bacteria belonging to the family *Pseudomonadaceae* (19, 29). The overall base compositions of these DNA types agree well with those determined from the T_m of the denaturation profiles (27) which were obtained in the renaturation experiments described in the accompanying paper (32). It was, however, more difficult to detect the presence of the minor DNA component in the denaturation curves. The base compositions of *H. salinarium* and *H. cutirubrum* DNA and the relative proportion of minor components are in good agreement with those reported by Joshi et al. (22). These authors were unable to demonstrate the presence of a satellite in the four strains of moderate halophiles tested. This finding is consistent with the results obtained in the present study for the moderately halophilic strains, isolates I and V.

Although differences were found in the amount of the minor DNA component among the various strains, the quantity for any one strain was found to be reproducible in several DNA preparations isolated from cultures at various stages of the growth cycle. Moreover, the quantity of satellite present in a particular DNA was the same, whether determined from the distribution of optical density peaks in analytical CsCl gradients as in Table 2 or by measuring the areas under the curves given by radioactively labeled DNA in preparative CsCl gradients.

A notable result obtained in this analysis was the observation of a satellite in *Halococcus morrhuae*, an extreme halophilic coccus belonging to the *Sarcina-Micrococcus* group (23). The base composition of the major component (67%GC) and the minor component (59%GC), and the relative proportion of the minor component are almost identical to those observed in *Halobacterium halobium*.

The spirillum, SL-1, isolated by Raymond and Sistrom (33), is described as a strictly anaerobic, photoautotrophic, extreme halophile. The DNA from this bacterium exhibits only a single DNA component with a base composition of 70%GC.

Although the presence of a satellite DNA in *R. spheroides* has been reported by Suyama and Gibson (42), only a single DNA component with base composition of 72%GC was observed in the strains of *R. spheroides* (Table 1) analyzed in the present study.

Examination of culture purity. In a study of this kind, extreme caution must be exercised to ensure that the satellite DNA is indeed a component of the DNA of the organism under study rather than a symptom of a mixed culture. For this reason, the stock halophile cultures used in this investigation were subjected to seven successive single-colony isolations by standard plate-streaking techniques. Furthermore, all cultures used for the preparation of DNA were first examined microscopically for the presence of contaminants. These techniques, however, do not rule out the possibility of a symbiotic relationship involving a morphologically similar organism. In such a case serial dilutions plated for colony counting would indicate a lower value for the bacterial concentration of the culture at the high dilutions than at the lower dilutions. Plate counts of dilutions of 10^{-5}, 10^{-6}, and 10^{-7} from a culture

of isolate III, however, were found to be essentially the same and to be in agreement with direct counts made with a Petroff-Hauser counting chamber. Thus, the presence of a symbiont of this type appears unlikely.

Purification of bacterial strains in a salt density gradient. Density gradients have been extremely useful in separating certain types of cells which differ only in physiology, such as competent and incompetent *Bacillus subtilis* (10, 18). There is also some indication that *B. subtilis* exhibits slightly different densities at different rates of growth (C. Hadden, *personal communication*). Other examples of cells separable on the basis of buoyant density are offered by *Saccharomyces cerevisiae* cells capable of undergoing sporulation and those which are incapable (G. Darland, *personal communication*), and certain ribosomal ribonucleic acid mutants of *E. coli* and the parent strain (25). This technique may, therefore, be valuable in detecting contaminants present in a culture which vary even slightly in density from the main population of cells. Separation would be expected even in the case that the contaminant is physically attached during normal growth by some means such as contact with the capsule or slime layer.

In view of the potential power of the density gradient method in detecting small numbers of contaminants, various cultures of halophilic bacteria were analyzed in this manner. The distribution of cells in a mixture of cultures of isolate I and isolate III, in preformed CsCl density gradients, is illustrated in Fig. 1. The cells of each type were distributed in a single peak. A mean density of approximately 1.29 g/ml for isolate III, and 1.23 g/ml for isolate I was observed. No other minor peaks diagnostic of contaminants were apparent. Moreover, there was no evidence for the presence of cells displaying the density of the moderate halophiles in a culture of isolate III analyzed separately.

It should be noted that the 0.06 g/ml difference in density observed between isolate I and isolate III is consistent with the view that the intracellular salt concentration of the extremely halophilic bacteria is substantially higher than that of the moderately halophilic bacteria. Measurements of the intracellular salt concentration in the moderate halophile, *Micrococcus halodenitrificans* gave a maximum value of 5% (17), although the maximum value for the extreme halophile, *Sarcina litoralis* (now *Halococcus morrhuae*) was found to be 19%. Thus, it is likely that most of the observed density difference between isolate I and isolate III is a direct result of the disparity in intracellular salt concentrations.

Figure 2 shows the results of an experiment in

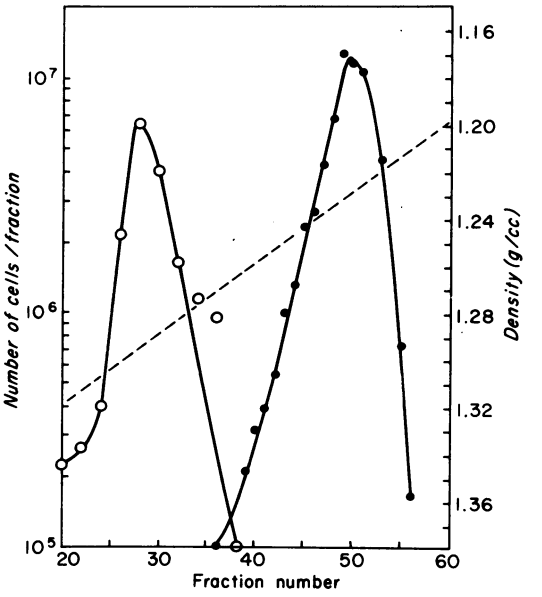

FIG. 1. *Density gradient centrifugation of halophilic bacteria. Approximately 10^8 cells from log-phase cultures of isolate I and isolate III were centrifuged in 25 ml of preformed density gradient (0 to 32 g of CsCl/100 ml of high-salt broth minus tryptone and yeast extract). Centrifugation was for 30 min at 14,000 rev/min at 4 C. Sixty-eight 10-drop fractions were collected and plated in duplicate at appropriate dilutions on high-salt solid medium containing 10 or 25% NaCl. The white colonies of isolate I (●) were counted on the 10% NaCl medium where isolate III is unable to grow. Colony counts for isolate III (○) were made on the 25% NaCl medium. The broken line represents the gradient density.*

which the DNA from cells of isolate III recovered from various positions in the density gradient was analyzed to determine the percentage of the satellite DNA component. The values obtained for the three samples were essentially the same within experimental error. Thus it seems reasonable to conclude that the population of cells is homogeneous with respect to the amount of the minor DNA component.

In preparing the lysates of these samples, advantage was taken of the fact that members of the genus *Halobacterium* lyse in salt solutions of less than 5 to 10% (1, 2, 39). The proportion of the satellite DNA component in crude lysates made by this method was essentially the same as that found in highly purified DNA prepared by the Marmur (26) method from either exponential or stationary-phase cells.

Effects of various inhibitors on the presence of satellite DNA. One obvious explanation for satellite DNA would be the presence of an episomal element. Many other bacteria have been cured of

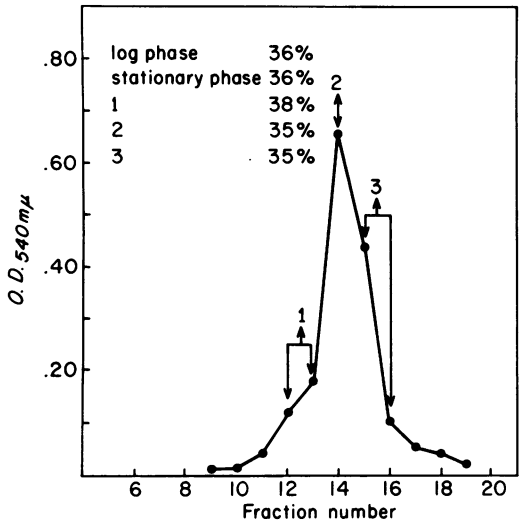

FIG. 2. *Relative percentages of the minor DNA component in various samples of isolate III. Approximately 2 × 10⁹ cells from a log-phase culture were centrifuged in a preformed 25-ml CsCl density gradient as described in the legend for Fig. 1. Fractions (0.7 ml) were collected and the cells in the designated fractions were lysed by dialyzing against 0.1 × SSC (0.15 M NaCl plus 0.015 M sodium citrate) containing 0.5% sodium lauryl sulfate. The lysate was then dialyzed against 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8) and brought to 57% CsCl for banding by CsCl density gradient centrifugation in a Spinco model E ultracentrifuge. Values for DNA purified according to the method of Marmur (26) from log- and stationary-phase cells are included for comparison.*

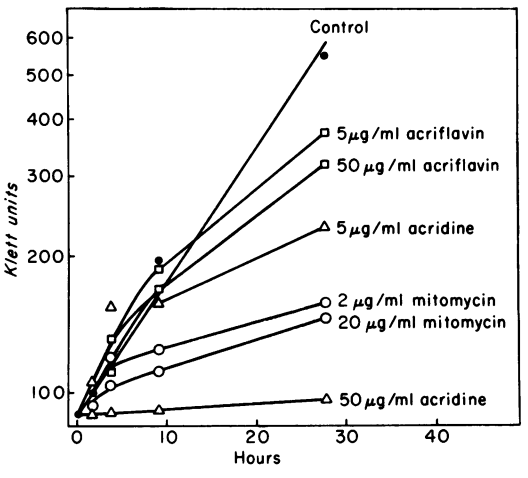

FIG. 3. *Effect of various inhibitors on the growth of isolate III. At time zero cells from a culture in log phase were transferred to screw-cap tubes containing 5 ml of culture medium with the designated concentration of the various inhibitors. The cultures were incubated with shaking at 37 C, and growth was observed by periodic readings in a Klett-Somerson colorimeter fitted with a green filter.*

episomal DNA by agents such as acridine orange or acriflavine. This technique has been successful with the F factor (20) and the R factor (30) of *E. coli*. The effects of acridine orange, acriflavine, and mitomycin on halophilic bacteria were examined by studying the kinetics of growth and the nature of the DNA extracted from treated cultures. Mitomycin appears to act by inhibition of DNA replication through binding to preexisting DNA (21, 24, 38). The induction of pigment production in *R. spheroides* is inhibited by mitomycin (19a). Most standard antibiotics fail to inhibit the growth of halophiles, presumably because of the high-salt environment (R. L. Moore, Ph.D. Thesis, Univ. of Washington, Seattle).

Figure 3 illustrates the effect of these substances at two different concentrations on the rate of growth of isolate III. All three were bacteriostatic at both concentrations. Solid media containing acriflavine (50 g/ml), acridine orange (50 g/ml), or mitomycin (20 g/ml) were inoculated with samples of the appropriate culture after 40 hr of growth. No resistant colonies or colonies lacking pigmentation appeared after 2 weeks of incubation at 37 C.

After growth in the presence of various drugs for 40 hr (approximately four generations), DNA was extracted from each culture and analyzed by CsCl density gradient centrifugation. Each sample contained satellite DNA in the same proportion as the untreated control. This result would argue against the episomal nature of satellite DNA. Such evidence is hardly conclusive, since not all episomes are cured by acridines. The possible existence of episomal DNA is tested by other methods in the accompanying paper (32).

DISCUSSION

All of the extremely halophilic bacteria examined, except the photosynthetic extreme halophile, SL-1, appear to share the common property of possessing a minor DNA component of lower buoyant density in CsCl equilibrium density gradients. The minor component is from 7 to 10%GC lower than the major component and comprises from 11 to 36% of the total DNA. No satellite DNA was demonstrable in DNA prepared from the moderate halophiles or nonhalophiles. Although Marmur et al. (29) list six strains under the genus *Halobacterium*, Larsen (23) contends that four of these, which do not show the presence of a satellite DNA, are probably not members of the genus *Halobacterium* or even extreme halophiles. Joshi et al. (22) state

that none of these four strains required the high-salt media.

Conventional techniques such as microscopic examination of cultures, restreaking of isolated colonies, and plating of serial dilutions of the culture failed to reveal the presence of a contaminant in these cultures. These techniques were also used by Joshi et al. (22) for cultures of *H. salinarium* and *H. cutirubrum* with identical results. In addition, these workers found no indication for the presence of intracellular parasites or viruses in electron micrographs of the cells.

Centrifugation of samples from cultures of the moderate halophile, isolate I, and the extreme halophile, isolate III, on preformed CsCl density gradients (Fig. 1) also failed to reveal the presence of a contaminant possessing a different density. Further support for the homogeneity of the cultures was obtained from the demonstration that the relative proportion of satellite DNA in isolate III is essentially the same in cells sampled from various parts of the density profile. Although these samples were lysed by dialysis against a solution of low-salt concentration, which would selectively lyse only extreme halophiles of the genus *Halobacterium*, the proportion of satellite DNA was found to be approximately the same as that observed in DNA purified according to the method of Marmur (26) from log-phase or stationary-phase cells. Therefore an assignment of the minor DNA component to a contaminant requires that it be an extreme halophile with a DNA base composition of approximately 58% GC, having exactly the same cell density as isolate III. Since there are consistent and reproducible differences in the amount of the minor component among the various strains (Table 2), it would also be necessary to assume that the contaminant be present in the same relative proportion of total cells in isolate III throughout the growth cycle. Furthermore, it must be present in a different but constant proportion in cultures of other extreme halophiles.

The presence of certain polysaccharides which can appear as satellite bands in CsCl density gradients has been observed in DNA preparations of *E. coli* (36), photosynthetic bacteria (42), blue-green algae (15), *Drosophila* (34), and HeLa cells (37). Another possible source of error in the interpretation of these results would derive from the presence of an uncommon base in a portion of the DNA molecule, thus leading to a difference in buoyant density (35).

Strong evidence may be marshalled against both of these possibilities. Denaturation of the DNA from the extremely halophilic bacteria examined results in a hyperchromic shift varying from 36 to 39% and a shift in density of 0.015 g/ml. These are the values expected for highly purified DNA (27, 41).

In addition, the percentage of satellite material in the DNA of isolate III was the same, whether determined by ultraviolet absorbance or radioactivity. Thus, the satellite cannot be attributed to the presence of a polysaccharide in the DNA preparations. Since measurements of the base composition by thermal denaturation techniques are less affected by unusual bases than measurements made by CsCl density gradient techniques (28), the agreement of these values in the present investigation suggests that there are few if any unusual bases present. This conclusion is supported by the chromatographic analysis of DNA of extreme halophiles reported by Joshi et al. (22).

Therefore the results described in the present communication, together with those of Joshi et al. (22), suggest that the presence of a minor DNA component of different density is characteristic of all of the extremely halophilic bacteria so far isolated. Such a DNA component was observed in several strains of *Halobacterium* and *Halococcus*, although its base composition and absolute amount vary from one strain to another. The question of origin of this unique genetic component and its possible functional relationship to halophilism are discussed in more detail in the accompanying paper (32).

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