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Isolation and Characterization of Salt-sensitive Mutants of the Moderate Halophile *Halomonas elongata* and Cloning of the Ectoine Synthesis Genes*

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The moderate halophile *Halomonas elongata* Deustche Sammlung für Mikroorganismen 3043 accumulated ectoine, hydroxyectoine, glutamate, and glutamine in response to osmotic stress (3 M NaCl). Two Tn*1732*-induced mutants, CHR62 and CHR63, that were severely affected in their salt tolerance were isolated. Mutant CHR62 could not grow above 0.75 M NaCl, and CHR63 did not grow above 1.5 M NaCl. These mutants did not synthesize ectoine but accumulated ectoine precursors, as shown by ^{13}C NMR and mass spectroscopy. Mutant CHR62 accumulated low levels of diaminobutyric acid, and mutant CHR63 accumulated high concentrations of *N*-γ-acetyldiaminobutyric acid. These results suggest that strain CHR62 could be defective in the gene for diaminobutyric acid acetyltransferase (*ectB*), and strain CHR63 could be defective in the gene for the ectoine synthase (*ectC*). Salt sensitivity of the mutants at 1.5–2.5 M NaCl could be partially corrected by cytoplasmic extracts of the wild-type strain, containing ectoine, and salt sensitivity of strain CHR62 could be partially repaired by the addition of extracts of strain CHR63, which contained *N*-γ-acetyldiaminobutyric acid. This is the first evidence for the role of *N*-γ-acetyldiaminobutyric acid as osmoprotectant. Finally, a cosmid from the *H. elongata* genomic library was isolated which complemented the Ect$^-$ phenotype of both mutants, indicating that it carried at least the genes *ectB* and *ectC* of the biosynthetic pathway of ectoine.

Halomonas elongata is a moderately halophilic bacterium that can grow over a wide range of salinity, from ~0.1 to ~4 M NaCl (1, 2). This property makes this halophile an excellent model to study the osmoregulatory mechanisms in this group of extremophilic organisms. Moreover, *H. elongata* has recently received considerable interest because of its potential for use in biotechnology. Thus, it is a good source for halophilic enzymes as well as the compatible solutes ectoine and hydroxyectoine that can be used as protecting agents for enzymes and whole cells (3). Although genetic tools for moderate halophiles have been developed recently (4–7), the genetic basis of the osmoregulatory mechanisms in these bacteria remains unclear. As most other bacteria, moderate halophiles maintain their internal osmolality and generates turgor in media of high salinity by accumulating organic compatible solutes (8). When grown in media lacking osmoprotectants, *H. elongata* synthesizes ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylate) as its major compatible solute (9). It can also accumulate glycine betaine and related osmoprotectants by transport from the medium (9, 10). Glycine betaine has been shown to suppress the accumulation of ectoine partially or completely in *H. elongata*, depending on the NaCl concentration and the strain (9, 10).

Although *H. elongata* is typical among bacteria in that it accumulates glycine betaine in response to high salinity stress, the biochemical basis for its unusual NaCl tolerance is not clear. *Escherichia coli* and *Salmonella typhimurium*, which served as model organisms for the elucidation of basic principles of osmoregulation, also use glycine betaine as the preferred osmoprotectant (11). However, although glycine betaine can elicit dramatic stimulation of growth in media of inhibitory osmolality in these organisms, it can support growth of the latter organisms only to about 1.2 M NaCl, considerably less that the maximum salinity that can be tolerated by *H. elongata*.

Ectoine has been discovered as a compatible solute in the extremely halophilic bacteria *Ectothiorhodospira halochloris* (12) and subsequently shown in *H. elongata* (13). The biosynthetic pathway of this compound is shown in Fig. 1 (13). Because the organisms that can synthesize ectoine are generally halophilic or marine bacteria (14), it has been suggested that high salinity tolerance could be connected with the ability to synthesize this compatible solute. To test whether there is such a causal connection between halotolerance and the synthesis of ectoine and to identify the ectoine biosynthetic genes, we isolated mutants of *H. elongata* DSM 3043 that are blocked in the synthesis of this compound. This work describes the isolation and characterization of these mutants as well as the isolation of the genes involved in the biosynthesis of ectoine in *H. elongata*.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, and Growth Conditions—Bacterial strains and plasmids used in this study are listed in Table I. *H. elongata* strains were routinely grown in SW-10 medium, which contained 10% (w/v) total salts (15) and 0.5% (w/v) yeast extract (Difco). Salt-sensitive mutants were isolated on a modified version of this medium, which contained 2% total salts (designated SW-2 medium). The complex LB medium was used for the growth of *E. coli* (16). M63 (17), containing 20 mM glucose as the sole carbon source, was used as the minimal medium.

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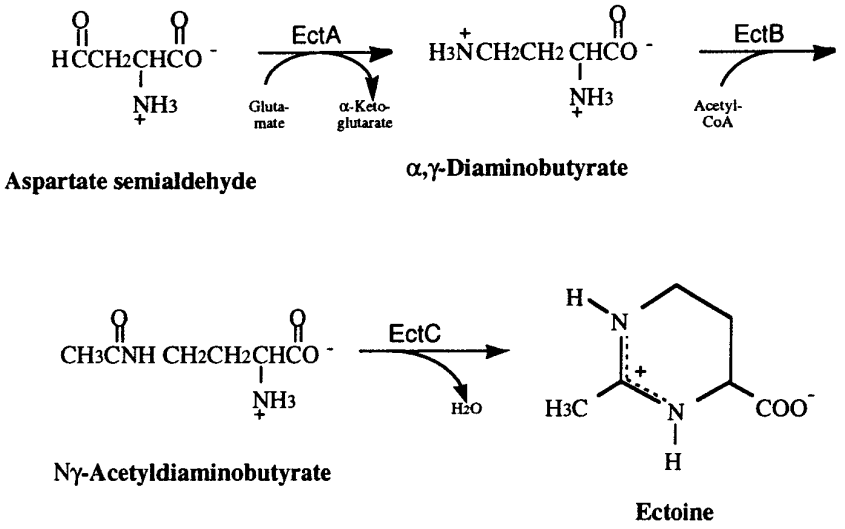

FIG. 1. **The ectoine biosynthetic pathway (13).** The loci encoding the three enzymes of ectoine synthesis, L-diaminobutyric acid transaminase, diaminobutyric acid acetyltransferase, and ectoine synthase, have been provisionally designated as *ectA*, *ectB*, and *ectC*, respectively.

TABLE I
Bacterial strains and plasmids used in this study

Strain/plasmids	Relevant features	Ref. or source
H. elongata		
DSM 3043	Wild-type	1
CHR61	Spontaneous Rf^r mutant of DSM 3043	This study
CHR62	Mutant of CHR61 defective in the synthesis of ectoine	This study
CHR63	Mutant of CHR61 defective in the synthesis of ectoine	This study
CHR64	Salt-sensitive mutant of CHR61	This study
CHR65	Salt-sensitive mutant of CHR61	This study
E. coli		
HB101	Δ*(gpt-proA)62 leuB6 thi-1 lacY1hsdS*$_B$*20 recA rpsL20 (Str*^{r}*) ara-14 galKZ dxyl-5 mtl-1 supE44 mcrB*$_B$	31
SM10	*thi thr leu tonA lac* Y *sup* E *rec*A Mu_c^+	18
DH5α	*sup*E44Δ*lac*U169 (ϕ80*lac*ZΔM15)*hsd*R17 *rec*A1 *endA1 gyr*A96 *thi*-1 *rel*A1	21
Plasmids		
pSUP102−Gm::Tn*1732*	Gm^r, Km^r, vector for transposon mutagenesis	18, 19
pVK102	Tc^r, Km^r, cosmid vector, Inc P	22
pKS(−)	Ap^r	Stratagene
pRK600	Cm^r, helper plasmid, ColE1	32
pDE1	23-kb *Sal*I fragment from CHR63 DNA carried in pKS(−)	This study
pDE3	8.6-kb *Sal*I fragment from pDE1 cloned in pKS(−)	This study
pDE9	pVK102-derivative carrying the *H. elongata* genes encoding the ectoine synthase and diaminobutyric acid acetyltransferase	This study
pDE10	pVK102 derivative carrying the *H. elongata* gene for the ectoine synthase	This study
pDE11	Same as pDE10	This study

The osmotic strength of M63 was increased by the addition of 0.5 to 4 M final concentrations of NaCl. When used, glycine betaine (Aldrich) was added to a final concentration of 1 mM. The pH of all media was adjusted to 7.2 with KOH. Solid medium contained 20 g/liter Bacto-agar (Difco). When used, filter-sterilized antibiotics were at the following final concentrations (μg/ml): Ap,[1] 100; chloramphenicol, 25; Km, 75; rifampicin, 25; tetracycline, 15. Liquid cultures were incubated at 37 °C in an orbital shaker at 200 rpm. Growth was monitored as the optical density of the culture at 600 nm with a Perkin-Elmer 551S UV/VIS spectrophotometer.

Transposon Mutagenesis and Isolation of Mutants—Transposon mutagenesis was performed by conjugal transfer of pSUP102-Gm::Tn*1732* from *E. coli* SM10 (18, 19) to *H. elongata* strain CHR61. Matings were carried out by mixing the donor and recipient cultures at a ratio of 1:4 (100 μl of donor, 400 μl of recipient). The mixed cultures were washed with sterile SW-2 medium to eliminate the antibiotics. The pellet was resuspended in 100 μl of SW-2 and placed on a 0.45-μm pore filter on SW-2 solid media (which allows the growth of *E. coli* and the putative salt-sensitive mutants of *H. elongata*). After overnight incubation at 30 °C, cells were resuspended in 20% (v/v) sterile glycerol and, after appropriate dilutions, inoculated on SW-2 + rifampicin + Km plates at a density resulting in about 100–200 colonies per plate. Colonies from these master plates were transferred with sterile toothpicks to duplicate M63 plates, one contained 2.7 M NaCl and the other contained 0.5 M NaCl. Plates were incubated at 37 °C and inspected for colonies that had grown at 0.5 M but not at 2.7 M NaCl.

DNA Manipulation—Chromosomal DNA from *H. elongata* was isolated as described by Ausubel *et al.* (20). Plasmid DNA was isolated from *E. coli* with the alkaline lysis method (21). Restriction enzyme digestion and ligations were performed as recommended by the manufacturers. Probes used for plasmid, genomic DNA, and colony hybridization were generated by using the non-radioactive digoxigenin DNA labeling and detection kit from Boehringer Mannheim. For genomic DNA hybridization, genomic DNA was isolated from the wild-type strain and salt-sensitive mutants of *H. elongata*, digested with restriction enzymes, separated by agarose gel electrophoresis and transferred to nylon filters (Amersham Corp.) as described by Sambrook *et al.* (21). An internal 1-kb *Hin*dIII fragment of the transposon Tn*1732* was used as a probe. For colony hybridization, 3,000 single colonies of the *H. elongata* genomic library were allowed to grow 12 h at 37 °C on LB + Km plates. After growth, plates were chilled for 1 h at 4 °C and transferred to nylon filters, as described by Sambrook *et al.* (21). For colony hybridization, cellular debris was removed before hybridization to avoid background. Hybridization was in 3 × SSC, 0.1% SDS with shaking for 2 h at 68 °C. Hybridization, washes, and detection were done according to the instructions of the kit provided by Boehringer Mannheim.

Construction of Plasmids and a Gene Bank—To clone the DNA

[1] The abbreviations used are: Ap, ampicillin; Km, kanamycin; NADA, *N*-γ-acetyldiaminobutyric acid; DA, diaminobutyric acid; bp, base pair(s); kb, kilobase pair(s); GC-MS, gas chromatography-mass spectrometry.

region flanking the Tn*1732* insertion in mutant CHR63, genomic DNA of CHR63 was digested with *Bgl*II, ligated to *Bam*HI-digested pKS−, and transformed into DH5α. Transformants were selected on LB + Km + Ap. From one Km^r Ap^r colony, the plasmid pDE1, containing a 23-kb *Bgl*II fragment from CHR63 DNA, was isolated. An 8.6-kb *Sal*I fragment of pDE1 was subcloned in pKS−, giving pDE3. A *H. elongata* gene bank was constructed in the broad host range cosmid pVK102 (22), a low copy number cosmid that can replicate in both *E. coli* and *Halomonas* (23). *H. elongata* DNA was partially digested with *Sal*I, and DNA fragments in the size range of 23–30 kb were separated in sucrose gradients and cloned into the pVK102 vector, which had been linearized with *Sal*I and treated with alkaline phosphatase. The ligation mix was packed *in vitro* into bacteriophage lambda heads by using a kit from Amersham Corp. and transduced into *E. coli* HB101. Out of 3,000 Km^r transductants, 30 colonies were analyzed, and all proved to have inserts of an average size of 27 kb, which guarantees a 99.8% probability of finding a given sequence in the bank (24). To isolate the genes responsible for the synthesis of ectoine, a total of 3,000 colonies of the *H. elongata* genomic library were screened by using as a probe a 370-bp *Eco*RI fragment from the 1.9-kb region carried in pDE3. This fragment, gel-isolated after *Eco*RI digestion of pDE3, was selected because it is adjacent to the Tn*1732* insertion in mutant CHR63. In fact, one of the *Eco*RI sites used to generate the probe lies in the right inverted repeat of Tn*1732* (see Fig. 7*B*). Therefore, the 370-bp *Eco*RI region should contain part of the *H. elongata* ectoine synthase gene (*ectC*). Plasmids isolated after library screening were conjugated from *E. coli* to *H. elongata* by triparental matings on SW-2 by using pRK600 as helper plasmid.

Extraction of Intracellular Osmolytes—Wild-type and salt-sensitive mutants of *H. elongata* were grown in M63 containing the maximal NaCl concentration that they could tolerate. At mid-exponential phase, cells were harvested and washed twice with the growth medium without any carbon source. To extract the cytoplasmic solutes, cells were resuspended in 10 ml of double distilled water and incubated for 5 h at room temperature. Cell debris was removed by centrifugation, and the supernatant was filtered through a 0.65-μm pore membrane filter.

^{13}C NMR Spectroscopy—Cell extracts were lyophilized and resuspended in 0.5 ml of D_2O. Natural abundance ^{13}C NMR spectra were recorded on a Brucker ac200 spectrometer at 50 MHz with probe temperature of 20–22 °C. Signals due to glutamate, glutamine, and diaminobutyrate were identified by comparison with the spectra of each of these pure compounds. Signals generated by *N*-acetyldiaminobutyrate were deduced from the spectrum of DA signals. Ectoine was identified by comparison of chemical shifts with published values (25, 26).

Purification of Acidic, Neutral, and Basic Amino Acids—Wild-type and mutants CHR63 and CHR62 were grown in 1-liter cultures of M63 plus the maximal NaCl that they can tolerate to A_{600} of 1.8, 0.6, and 0.8, respectively, and extracted in 20 ml of distilled H_2O, as described above. To 3-ml samples, 250 nmol of internal standards, α-amino-*n*-butyrate and α-aminoadipic acid, were added. Samples were applied to 3.5 × 1-cm columns of Dowex-1-acetate (100–200 mesh), equilibrated with H_2O, and washed with 5 ml of H_2O. The acidic amino acids, glutamate and aspartate, retained on this column were eluted with 6 ml of 2 M acetic acid. The water wash that contained the neutral and basic amino acids was applied to 3.5 × 1-cm columns of Dowex-50W-H^+ (100–200 mesh) equilibrated with H_2O. The latter columns were washed with 8 ml of H_2O, and neutral and basic amino acids were eluted with 8 ml of 6 M NH_4OH. Fractions were evaporated to dryness under a stream of air and redissolved in 0.8 ml of 30% methanol, and aliquots were analyzed by TLC on Whatman Silica Gel AL-SIL-G (aluminum-backed) plates (20 × 20-cm; 250-μm layer), developed in *n*-butyl alcohol/acetic acid/water (60:20:20, v/v/v). After development and drying, the plates were sprayed with ninhydrin (0.15% w/v in ethanol) and amino acids visualized by heating in a drying oven (140 °C for 1–2 min). The above protocol was first tested with an aqueous extract from mutant CHR63, without internal standards; analyses of the acidic and neutral + basic amino acid fractions by GC-MS (below) revealed that these extracts were devoid of α-amino-*n*-butyrate and α-aminoadipic acid, hence the choice of these compounds as internal standards for the neutral + basic and acidic amino acid fractions, respectively.

NADA was purified free of DA from the neutral amino acid fraction obtained from extracts of the mutant CHR63 by the following procedure. Aqueous extract was applied to a 4 × 1.5-cm column of Dowex-1-acetate, equilibrated with H_2O, to first remove acidic amino acids. Columns were washed with 10 ml of H_2O, and the water wash was applied to 4 × 1.5-cm columns of Dowex-50W-NH_4^+ (100–200 mesh) equilibrated with H_2O. This effectively removed basic amino acids, including DA. The water wash from the latter column, containing neutral amino acids, was then applied to 4 × 1.5-cm columns of Dowex-50-W-H^+ (100–200 mesh), washed with 24 ml of H_2O, and the neutral amino acids eluted with 18 ml of 6 M NH_4OH. The basic amino acids were eluted from the Dowex-50W-NH_4^+ with 18 ml of 6 M NH_4OH. Fractions containing the desired amino acids were evaporated to dryness under a stream of air. The neutral amino acid fraction was dissolved in a small volume of 60% methanol and applied to the origin of a preparative 20 × 20-cm Whatman Silica Gel 150A glass-backed TLC plate (100-μm layer). The plate was developed in *n*-butyl alcohol/acetic acid/water (60:20:20, v/v/v). After development and drying, the central portion of the TLC plate was covered with a glass plate, and the plate edges were sprayed with ninhydrin (0.15% w/v in ethanol) and amino acids visualized by heating the edges of the plate with a hot air gun. The zone corresponding to NADA (the most abundant amino acid in the neutral fraction of mutant CHR63) was scraped from the central portion of the plate, and the NADA was then eluted from the silica gel with water. A small aliquot (2 μl) of this material was then re-analyzed by analytical TLC (above) before and after acid hydrolysis (1.25 N HCl, 110 °C, 2 h).

Derivatization of Amino Acids—Amino acids purified by ion exchange chromatography and preparative TLC were derivatized to *N*(*O*,*S*)-heptafluorobutyryl (*N*-HFBI) amino acid derivatives, essentially as described by Rhodes *et al.* (27). Briefly, this procedure entails reaction of the dried amino acid sample with 200 μl of freshly prepared isobutyl alcohol/acetyl chloride (5:1, v/v) at 120 °C for 20 min, evaporation to dryness, followed by reaction with 100 μl of heptafluorobutyric anhydride at 120 °C for 10 min, and evaporation to incipient dryness. The samples are finally redissolved in 100 μl of ethyl acetate/acetic anhydride (1:1, v/v) for GC-MS analysis (see below). Authentic ectoine did not produce a volatile derivative in this procedure, as determined by GC-MS. An authentic standard of NADA was not available. However, tests with authentic *N*-acetylornithine indicated that substantial hydrolysis to ornithine occurred during derivatization, suggesting that hydrolysis of NADA to DA is likely to occur in this protocol.

GC-MS of N-HFBI Esters of Amino Acids—Electron ionization and chemical ionization GC-MS of the amino acid derivatives were performed as described previously (27), except that the column used was a DB-1 fused silica capillary column (30 m × 0. 25 mm inner diameter) and the oven temperature program was 100 °C for 4 min to 280 °C at 12 °C/min.

RESULTS

^{13}C NMR Analysis of the Compatible Solutes in H. elongata DSM 3043—H. elongata DSM 3043, formerly named strain 1H11 (1), has a broad salinity range in M63 minimal medium, being able to grow from 0.5 to 3 M NaCl (10). The type strain of *H. elongata* ATCC 33173 has been shown to synthesize both ectoine and hydroxyectoine as compatible solutes in response to osmotic stress (26). To test whether this is also true for *H. elongata* DSM 3043, the latter organism was grown in M63 plus 3 M NaCl, and the composition of its internal solutes was analyzed by ^{13}C NMR. Major signals corresponded to ectoine and hydroxyectoine, and glutamate and glutamine were also detectable at lower levels (Fig. 2).

Isolation of Mutants of H. elongata Defective in the Synthesis of Ectoine—Salt-sensitive mutants of *H. elongata* were isolated by transposon mutagenesis, as described under "Experimental Procedures." Putative salt-sensitive mutants were identified as those that were unable to grow on M63 plus 2.7 M NaCl plates but still able to grow on M63 containing 0.5 M NaCl. Out of ~4,000 Km^r colonies screened, four showed this phenotype (Table II). Each was able to grow on M63 + 2.7 M NaCl + 1 mM betaine. This result indicated that the mutations did not cause a general NaCl sensitivity and suggested that the mutants might be defective in the synthesis of a compatible solute. Strain CHR63 could not grow above 1.5 M NaCl in the absence of betaine. Strain CHR62 was affected more severely, being unable to grow above 0.75 M NaCl without betaine. Strains CHR64 and CHR65 could not grow above 2.0 M NaCl. We showed (see below) that strains CHR62 and CHR63 were blocked in the synthesis of ectoine; these two strains have been designated as Ect^-. The target site of the mutations in the

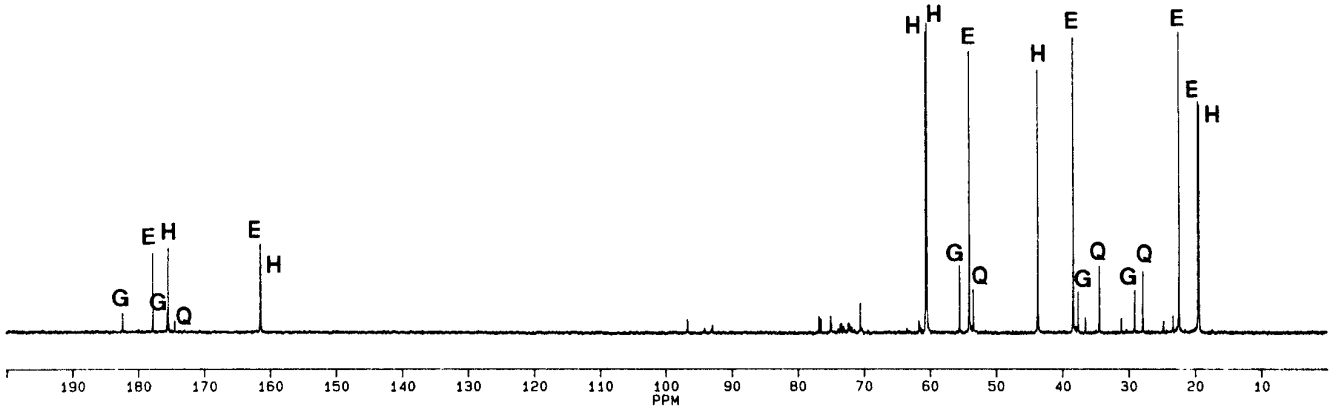

FIG. 2. **Natural abundance ^{13}C NMR spectrum of major cytosolic solutes of *H. elongata* DSM 3043.** Cells grown in M63 + 3.0 M NaCl were extracted in double distilled water, and cytosolic solutes were analyzed by ^{13}C NMR. Signals are as follows: ectoine (*E*), hydroxyectoine (*H*), glutamate (*G*), and glutamine (*Q*).

TABLE II
Growth of H. elongata salt-sensitive mutants in M63 with different NaCl concentrations

Mutant strain	NaCl						
	0.5	0.75	1	1.5	2	2.7	2.7 + betaine[a]
				M			
CHR62	+	+	−	−	−	−	+
CHR63	+	+	+	+	−	−	+
CHR64	+	+	+	+	+	−	+
CHR65	+	+	+	+	+	−	+

[a] Growth in M63 + 2.7 M NaCl + 1 mM betaine.

other two strains, CHR64 and CHR65, has not yet been identified.

The phenotypes of the highly NaCl-sensitive mutants CHR62 and CHR63 were characterized more extensively in liquid cultures. Fig. 3 shows the growth rates of these mutants in M63 with different salinities, in the presence or absence of exogenous betaine. Both grew more slowly than the wild-type at any salinity. Partial growth at high salinity was restored by betaine for both mutants, although this osmoprotectant could not restore wild-type growth rate at ≥2.5 M for CHR63 or at ≥1.5 M for CHR62.

To check that the mutant phenotype was due to a single transposition event in each of the mutants, hybridization analysis was performed with an internal fragment of the transposon Tn*1732* as a probe against genomic DNA of the mutants digested with the restriction enzymes *Sal*I or *Bgl*II, which do not have any recognition site in Tn*1732*. As shown in Fig. 4, unique hybridization signals were detected in the mutant DNAs, confirming that the salt-sensitive phenotype was due to single insertions of the transposon.

^{13}C NMR Analysis of the Cytoplasmic Solutes Accumulated by the Ect$^-$ Mutants in Minimal Medium—Mutants CHR62 and CHR63 were grown in M63 glucose minimal medium containing the maximal NaCl concentration that they could tolerate (0.75 M for CHR62 and 1.5 M for CHR63), and their major cytoplasmic solutes were analyzed by ^{13}C NMR (Fig. 5). Signals corresponding to ectoine were absent from the extracts of both mutants, indicating that they were defective in the synthesis of this compatible solute. Hydroxyectoine could not be detected either, suggesting a common biosynthetic pathway for both solutes. Signals corresponding to DA at 29, 37, 51, and 174 ppm were found in the CHR62 extract (Fig. 5*A*), suggesting that this mutant accumulated this compound. The spectrum of the CHR63 extract showed major signals around 25, 32, 37, 53, 174, and 175 ppm (Fig. 5*B*). A comparison with the chemical shifts of DA data suggested that those signals could correspond

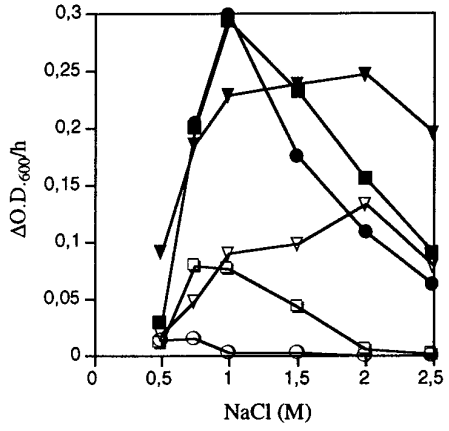

FIG. 3. **Salinity range of the salt-sensitive mutants.** Growth rates of the *H. elongata* wild-type strain DSM 3043 and the salt-sensitive mutants CHR62 and CHR63 were calculated in M63 with different salinities, in the presence (*solid symbols*) or absence (*open symbols*) of exogenous betaine. Symbols are as follows: ▽/▼, *H. elongata* DSM 3043; ○/●, CHR62; □/■, CHR63.

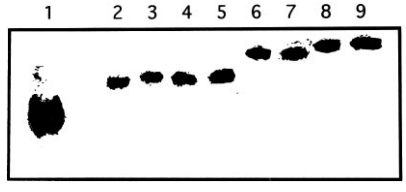

FIG. 4. **Hybridization analysis of the transposon insertions in *H. elongata* salt-sensitive mutants.** An internal 1-kb *Hin*dIII fragment from the Tn*1732* was used as a probe (see "Experimental Procedures"). The plasmid pSUP102-Gm::Tn*1732* digested with *Eco*RI was used as a positive control (*lane 1*), and *Sal*I-digested chromosomal DNA from the wild-type strain *H. elongata* was used as a negative control. Chromosomal DNA from the mutants strains CHR62 (*lanes 2* and *6*), CHR63 (*lanes 3* and *7*), CHR64 (*lanes 4* and *8*), and CHR65 (*lanes 5* and *9*) were digested with *Sal*I (*lanes 2–5*) or *Bgl*II (*lanes 6–9*).

to a derivative of DA. Signals at 174 and 175 ppm could be attributed to the carbonylic moiety of the carboxylic and acetyl groups, and a signal at 25 ppm was typical of the methyl moiety of an acetyl group. These data were consistent with those expected for NADA and suggested the accumulation of this compound by CHR63.

GC-MS of the N-HFBI Derivatives of the Amino Acids Accumulated by the Ect$^-$ Mutants—Electron ionization and chemical ionization GC-MS analyses of *N*-HFBI amino acid derivatives confirmed that the major amino acid constituents of mutant CHR62 was DA (HFBI derivative molecular weight = 566 (Fig. 6); CI protonated molecular ion = *m/z* 567; major EI

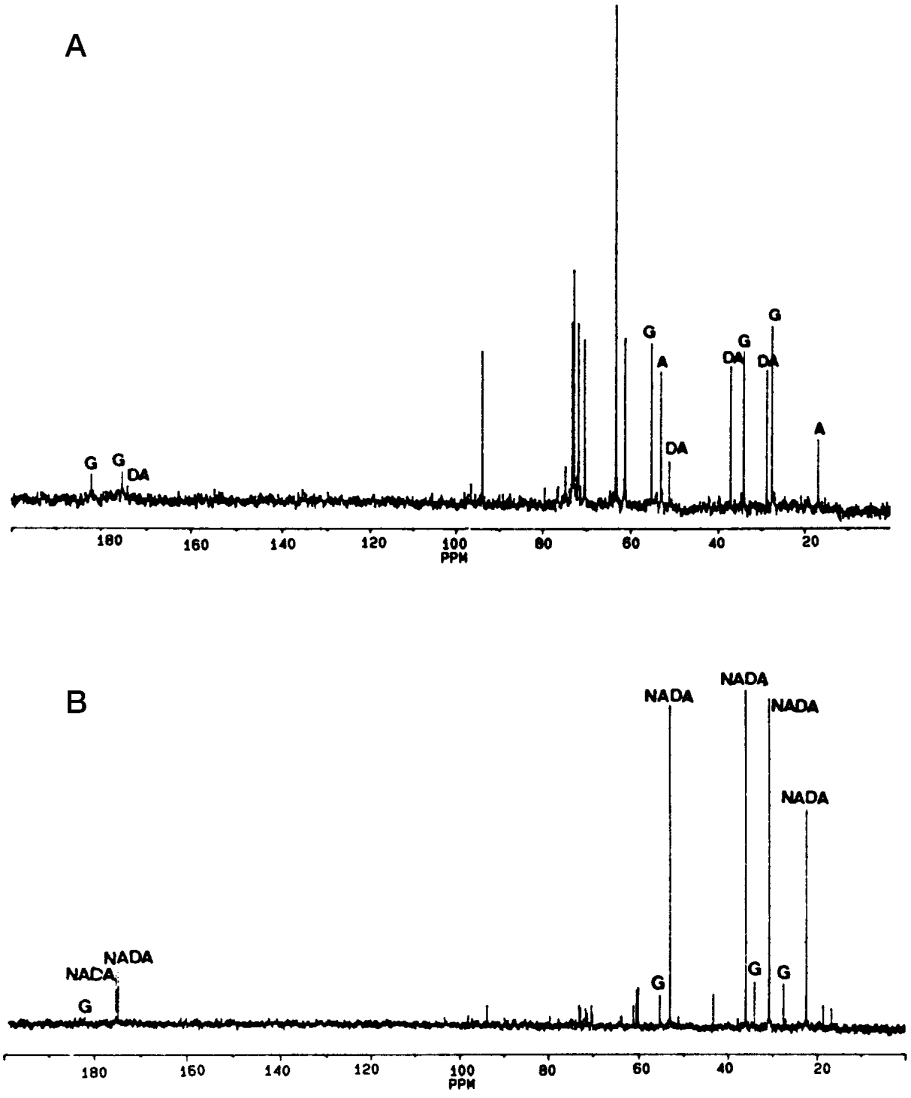

FIG. 5. **Natural abundance ^{13}C NMR spectrum of major cytosolic solutes of the salt-sensitive mutants.** *H. elongata* mutant strains CHR62 (*A*) and CHR63 (*B*) were grown in M63 + 0.75 M NaCl and in M63 + 1.5 M NaCl, respectively. Cells were extracted in double distilled water, and the cytosolic solutes were analyzed by ^{13}C NMR. Signals used were: *N*-γ-acetyldiaminobutyric acid (*NADA*), diaminobutyric acid (*DA*), glutamate (*G*), and alanine (*A*).

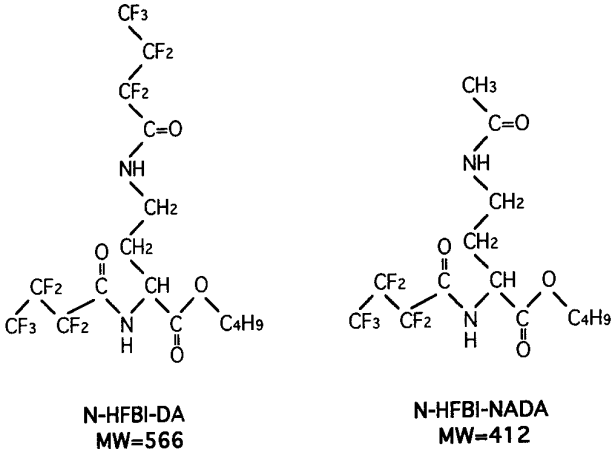

FIG. 6. **Predicted structures of the *N*-HFBI derivatives of DA and NADA.** Amino acids purified by ion exchange chromatography and preparative TLC from water-soluble extracts of CHR62 or CHR63 were derivatized to *N*(*O,S*)-heptafluorobutyryl amino acid derivatives, essentially as described by Rhodes *et al.* (27). The molecular weights (*MW*) of the derivatived DA and NADA are shown.

fragment ions = *m/z* 252 and 240), whereas that of mutant CHR63 was NADA (HFBI derivative molecular weight = 412; CI protonated molecular ion = *m/z* 413; major EI fragment ions = *m/z* 269 and 311) (Fig. 6). Initial analyses of the neutral + basic amino acid fraction of mutant CHR63 revealed high levels of DA in addition to NADA. However, the vast majority of this DA probably originated from hydrolysis of NADA during derivatization. This was confirmed by purifying NADA free of DA from extracts of CHR63 by ion exchange chromatography and preparative TLC. Approximately 80% of the purified NADA was converted to DA during derivatization. Similarly, *N*-α-acetylornithine was largely converted to ornithine during derivatization. By separating NADA from DA by ion exchange chromatography prior to derivatization, it was determined that NADA was about 10 times more abundant than DA in mutant CHR63. In contrast, NADA was not detectable in mutant CHR62. The wild-type contained trace levels of both DA and NADA.

Restoration of the Growth of Mutants CHR62 and CHR63 with Cell Extracts from the Wild-type Strain—We found that the addition of cytoplasmic extracts of wild-type *H. elongata* DSM 3043 to cultures of strains CHR62 and CHR63 could restore growth to these mutants in M63 containing 1.5–2.5 M NaCl (Table III). Because the most prominent low molecular solute in the wild-type strain is ectoine (10, 26), and because ectoine can be taken up by *H. elongata* (10), the growth stimulation of the two mutants at high salinity by wild-type extracts was presumably due to this compatible solute. In support of this hypothesis, we noted that purified ectoine could restore growth to the mutants at 1.5–2.5 M NaCl (data not shown).

TABLE III

Restoration of the growth of mutants CHR62 (with extract from the wild-type and CHR63) and CHR63 (with extract from the wild-type

Numbers indicate growth rates (ΔA_{600}/h).

Strain	Osmoprotectant	NaCl		
		1.5	2.0	2.5
			M	
Wild-type	None	9.80×10^{-2}	1.33×10^{-1}	7.92×10^{-2}
CHR62	None	2.90×10^{-3}	5.00×10^{-5}	1.00×10^{-4}
	Wild-type extract	1.95×10^{-2}	1.90×10^{-2}	1.30×10^{-2}
	CHR63 extract	1.87×10^{-2}	1.80×10^{-2}	1.95×10^{-2}
CHR63	None	4.31×10^{-2}	5.10×10^{-3}	1.50×10^{-3}
	Wild-type extract	2.80×10^{-2}	2.45×10^{-2}	2.00×10^{-2}

Although wild-type extracts increased the salinity range of the mutants, their growth rates at 1.5–2.5 M NaCl in the presence of this osmoprotectant were considerably lower than that of the wild type.

Growth could also be restored to strain CHR62 at 1.5–2.5 M NaCl by extracts of CHR63, which contained NADA (Table III). This result indicates that *H. elongata* is able to take up NADA and use it as an osmoprotectant. However, because strain CHR62 is blocked in the conversion of DA to NADA but might be able to convert the latter compound to ectoine, we cannot infer whether the stimulation is due to NADA itself or to the ectoine formed from it.

Isolation of the Ectoine Synthesis Genes of H. elongata—The region flanking the Tn*1732* insertion in mutant CHR63 was isolated as a 23-kb *Bgl*II fragment in plasmid pDE1 (Fig. 7*A*) as described under "Experimental Procedures." A restriction analysis of an 8.6-kb *Sal*I fragment (in pDE3) indicated that it contained the transposon Tn*1732* plus a 1.9-kb region from mutant CHR63 flanking the transposon (Fig. 7*B*).

The wild-type genes responsible for the synthesis of ectoine were isolated by colony hybridization, using a 370-bp chromosomal fragment flanking the Tn*1732* in pDE3, as described under "Experimental Procedures." Eight plasmids identified in this manner were introduced by conjugation from *E. coli* to the Ect$^-$ mutant strains CHR63 and CHR62. Transconjugants were selected on M63 minimal medium containing 2 M NaCl. One plasmid, pDE9, was able to complement the mutations in both CHR62 and CHR63, indicating that it carried the wild-type genes encoding the enzymes for the synthesis of NADA (diaminobutyric acid acetyltransferase) and ectoine (ectoine synthase). Two other plasmids, pDE10 and pDE11, were able to complement only the mutations in CHR63, indicating that they carried only the ectoine synthase gene. Using the plasmid pDE9 as a probe against genomic DNA of the wild-type and mutant strains, and against pDE10 and pDE11, we showed that the inserts in these three plasmids carried overlapping sequences, including a 3-kb *Sal*I fragment which was lacking from the *Sal*I-digested DNA from the mutants (Fig. 8). When the 370-bp *Eco*RI probe was hybridized with the three plasmids, only the common 3-kb *Sal*I fragment gave a positive hybridization signal (not shown). This demonstrated that the Tn*1732* insertions in mutants CHR63 and CHR62 were located in the same 3-kb *Sal*I fragment (Fig. 7*C*), suggesting that the ectoine synthesis genes are closely linked, and thus, they may be organized into a single operon. However, this fragment must not contain the complete gene for the diaminobutyric acid acetyltransferase (*ectB*), as judged by the fact that pDE10 and pDE11, carrying this region, were not able to complement CHR62.

DISCUSSION

The type strain of *H. elongata* ATCC 33173 adapts to high salinity by synthesizing ectoine and hydroxyectoine (26). We found that another isolate of *H. elongata*, strain DSM 3043, likewise accumulates these two compounds, but in addition, it also synthesizes considerable amounts of glutamate and glutamine.

To identify genes of the ectoine biosynthetic pathway, we carried out a Tn*1732* mutagenesis of strain DSM 3043 and obtained four derivatives that showed impaired growth in media containing high concentrations of NaCl. Two of these, strains CHR62 and CHR63, were dramatically affected in their salt tolerance, whereas the other two, CHR64 and CHR65, exhibited only moderate NaCl sensitivity. These mutations were due to single insertion events, as shown by Southern analysis. We focused our studies on the characterization of the mutations in strains CHR62 and CHR63. A number of Tn*1732*-induced salt-sensitive mutants of *H. elongata* ATCC 33173 have been isolated previously by Kunte and Galinski (5). However, none of these mutants have been further characterized.

^{13}C NMR analysis of cytoplasmic extracts of strains CHR62 and CHR63 indicated that they did not synthesize ectoine, but rather they accumulated two amino acids that were identified by a combination of ^{13}C NMR and MS as intermediates in the ectoine pathway. The fact that hydroxyectoine was not detected in the mutants is good evidence that this compound is synthesized from ectoine. Mutant strain CHR63, which was more NaCl-tolerant than CHR62, accumulated high concentrations of NADA, and strain CHR62 accumulated lower levels of DA. Analysis of *N*-HFBI derivatives of NADA suggested that in *H. elongata* DSM 3043 the *N*-acetyl moiety of the ectoine precursor is attached to the γ-NH_2 and not to the α-NH_2 moiety.

The observations that the salt sensitivity of the Ect$^-$ mutants, CHR62 and CHR63, could be partially corrected by exogenous ectoine, that DA is accumulated in strain CHR62 and NADA is accumulated in strain CHR63 and that extracts of strain CHR63 could impart increased NaCl tolerance to CHR62, suggest that the mutation in strain CHR62 blocked the conversion of DA to NADA and the mutation in CHR63 blocked the conversion of NADA to ectoine. We provisionally designate the genes that are inactivated by these two mutations as *ectB* and *ectC*; these could be the structural genes for diaminobutyric acid acetyltransferase and ectoine synthase, respectively, but we cannot rule out that one or the other might encode some positively acting protein that is required either for the synthesis or the activity of the deficient enzyme.

Although exogenous extracts of the wild-type strain increased the NaCl tolerance of the two Ect$^-$ mutants, growth rate of these mutants at high NaCl in the presence of these supplements was lower than that of the wild-type in the absence of any osmoprotectant. This result may be due to the fact that ectoine cannot be accumulated to the same level by transport as by internal synthesis. We do not know, however, whether exogenous ectoine stops the synthesis of the intermediates of the ectoine pathway and, consequently, cannot rule out that the residual NaCl sensitivity of the mutants in the presence of ectoine might be due to deleterious effects of the

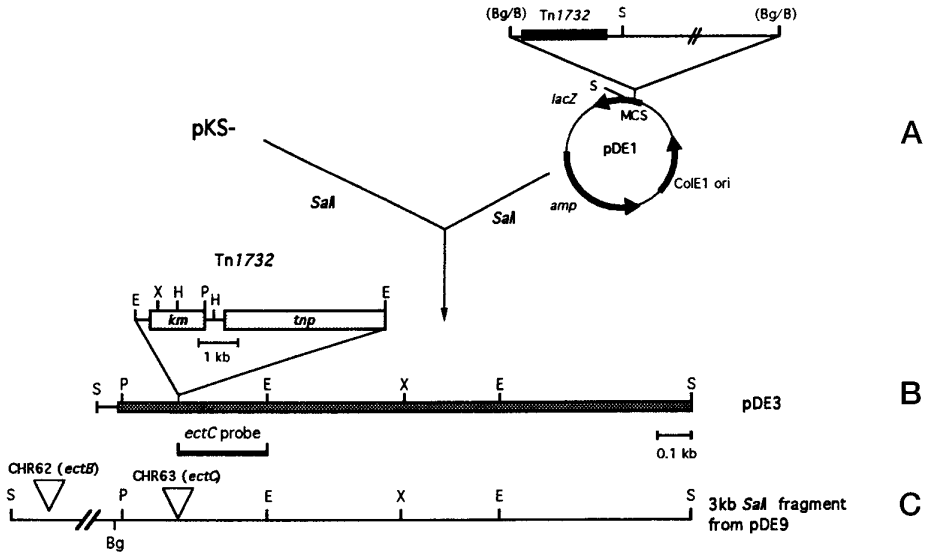

FIG. 7. **Isolation the *H. elongata* genes for the biosynthesis of ectoine.** *A*, plasmid pDE1, carrying a 23-kb *Bgl*II fragment from the Ect$^-$ mutant CHR63 DNA, including the transposon Tn*1732*. *B*, restriction map of pDE3, carrying a 8.6-kb *Sal*I fragment from pDE1, including the transposon. Only the *Sal*I site flanking the 3′ end of the region subcloned in pDE3 is shown in pDE1. The *Sal*I site flanking the 5′ end of the region subcloned in pDE3 belongs to the cloning vector and therefore is not present in the wild-type DNA. The 370-bp *Eco*RI fragment from pDE3, used as an *ectC* probe, is shown. *C*, 3-kb *Sal*I region from pDE9 containing the entire *ectC* (ectoine synthase) and part of *ectB* (diaminobutyric acid acetyltransferase) genes of *H. elongata*. The insertion site of Tn*1732* in CHR62 (EctB$^-$) lies in a 1.1-kb *Sal*I-*Bgl*II region, but it has not been precisely mapped. Restriction sites are as follows: *B*, *Bam*HI; *Bg*, *Bgl*II; *E*, *Eco*RI; *H*, *Hin*dIII; *P*, *Pst*I; *S*, *Sal*I; *X*, *Xho*I. Sites within *brackets* disappeared in the construction of pDE1.

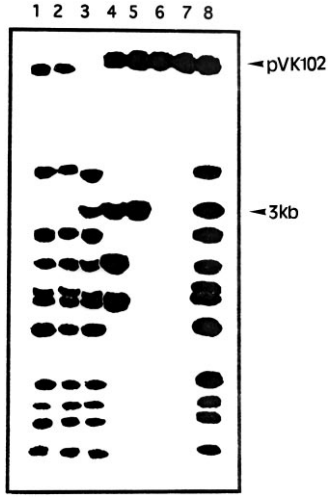

FIG. 8. **Location of the DNA region carrying the ectoine synthesis genes of *H. elongata*.** Plasmid pDE9 (*lane 8*), which complemented both Ect$^-$ mutants, was used as a probe against genomic DNA of mutants CHR62 (*lane 1*), CHR63 (*lane 2*), and the wild-type strain (*lane 3*), or plasmids pDE10 (*lane 4*), and pDE11 (*lane 5*). *Lanes 6* and *7* correspond to two other plamids from the *H. elongata* genomic library that hybridized with the 370-bp *Eco*RI probe (*ectC*) but did not complement mutants CHR62 or CHR63. All DNAs were digested with *Sal*I.

accumulation of these intermediates. Betaine also enhanced the growth rates of the *ectB* and *ectC* mutants, but like ectoine, it resulted in only a partial restoration of growth rate in these mutants at high salinity (≥2.0 M NaCl). We conclude from these results that although ectoine is not necessary for *H. elongata* DSM 3043 for growth at 0.5-~2 M NaCl in the presence of betaine, it might be required for optimal growth at higher salinities.

The result that strain CHR63, which accumulates NADA, is more salt tolerant than strain CHR62, which accumulates DA, provides the first direct evidence that NADA itself can function as an osmoprotectant. This conclusion was further supported by the observation that extracts of CHR63 containing high concentrations of NADA stimulated the growth of *S. typhimurium* and *E. coli* in media of high salinity.[2] The heightened NaCl sensitivity of strain CHR62 could be due to the fact that DA is inherently not as potent an osmoprotectant as NADA, but we cannot rule out the possibility that the reason for this phenotype is that DA is not accumulated to sufficiently high concentrations to act as an osmoprotectant.

The conclusion that NADA can act as a compatible solute is in agreement with the proposal that the compatible solutes generally do not carry a net electrical charge, because they are less disruptive to macromolecules at high concentrations than ionic molecules (9, 11). Accordingly, charged solutes, for example glutamate, glutamate betaine, and DA (in our *H. elongata* mutant) are usually not accumulated to very high concentrations (above ~400 mM). Although DA and ornithine are positively charged at physiological pH, their *N*-acetylated derivatives do not carry a net charge and are highly soluble. Hence, NADA, N^{δ}-acetylornithine, and ectoine, which is a cyclic derivative of NADA, are accumulated at >500 mM concentrations (9). N^{δ}-Acetylornithine (28) and N^{ϵ}-acetyllysine (14) have been documented to be involved in the osmoadaptative processes in *Bacillus* species and other organisms. NADA has been identified as an intracellular solute in *Euphorbia pulcherrima* (29) and also has been found, along with minor quantities of other N^{ω}-acetylated derivatives of DA, in sugar beets (30), although these compounds so far have not been connected with osmotic functions. The result that strain CHR63 was more NaCl-sensitive than the wild-type indicates that NADA cannot replace ectoine in its ability to confer NaCl tolerance, but whether the increased NaCl sensitivity of this mutant is due to the fact that NADA is present at lower intracellular levels than ectoine or to the fact that ectoine is inherently more potent as an osmoprotectant than NADA needs to be resolved.

Finally, genes complementing the Ect$^-$ phenotype of mutants CHR62 (*ectB*) and CHR63 (*ectC*) have been isolated from a genomic library of the wild-type DNA. The fact that both

[2] D. Cánovas, C. Vargas, L. N. Csonka, A. Ventosa, and J. J, Nieto, unpublished results.

mutations were located in the same 3-kb *Sal*I fragment suggests that the ectoine synthesis genes might be organized in one operon. Sequencing of the isolated region is in progress to elucidate the number and role of the genes involved in this biosynthetic pathway in *H. elongata*. The results presented herein would be helpful for the understanding of the molecular basis of osmoregulation in moderately halophilic bacteria.

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Isolation and Characterization of Salt-sensitive Mutants of the Moderate Halophile *Halomonas elongata* and Cloning of the Ectoine Synthesis Genes

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