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* Deutscher Sammlung für Mikroorganismen 3043 accumulated ectoine, hydroxyectoine, glutamate, and glutamine in response to osmotic stress (3 m NaCl). Two Th1732-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 13C NMR and mass spectroscopy. Mutant CHR62 accumulated low levels of diaminobutyric acid, and mutant CHR63 accumulated high concentrations of *N*-acyetyl-diaminobutyric 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*-acyetyl-diaminobutyric acid. This is the first evidence for the role of *N*-acyetyl-diaminobutyric acid as osmoprotectant. Finally, a cosmid from the *H. elongata* genome 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 generate 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 than 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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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, 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::Tn1732 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, as described by Sambrook et al. (21).

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

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**TABLE I**

**Bacterial strains and plasmids used in this study**

| Strain/plasmids | Relevant features | Ref. or source |
|-----------------|-------------------|----------------|
| H. elongata     | Wild-type         | 1              |
| DSM 3043        | Spontaneous Rf † mutant of DSM 3043 | This study |
| CHR61           | Mutant of CHR61 defective in the synthesis of ectoine | This study |
| CHR62           | Mutant of CHR61 defective in the synthesis of ectoine | This study |
| CHR63           | Salt-sensitive mutant of CHR61 | This study |
| CHR64           | Salt-sensitive mutant of CHR61 | This study |
| CHR65           | Salt-sensitive mutant of CHR61 | This study |
| E. coli SM10    | Δpap-proA62 leuB thi-1 lacY187395::Gm rpsL20 (Str r) ara-14 galKZ | 31 |
| DH5感受体 | thi thr leu tonA lac Y sup E recK Mu † | 18 |
| Plasmids        | pSUP102 Gm::Tn1732 | 18, 19 |
| pVK102          | Gm', Km', vector for transposon mutagenesis | 22 |
| pKS(–)          | Te †, Km †, cosmid vector, Inc P | 22 |
| pRK600          | Ap † | Stratagene |
| pDE1            | Cm †, helper plasmid, ColE1 | 32 |
| pDE3            | 8.6-kb SalI fragment from CHR63 DNA carried 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 |

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1 The abbreviations used are: Ap, ampicillin; Km, kanamycin; NAD, N-γ-acetyldiaminobutyric acid; DA, diaminobutyric acid; bp, base pair(s); kb, kilobase pair(s); GC-MS, gas chromatography-mass spectrometry.
region flanking the Tn1732 insertion in mutant CHR63, genomic DNA of CHR63 was digested with BglII, ligated to BamHI-digested pKS1, and transformed into DH5α. Transformants were selected on LB + Km + Ap. From one Km+ Ap colony, the plasmid pDE1, containing a 23-kb BglII fragment from CHR63 DNA, was isolated. An 8.6-kb SalI fragment of a strain of E. coli and treated in pK1202, a H. elongata plasmid, was banked 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 SalI, and DNA fragments in the size range of 23–30 kb were separated in sucrose gradients and cloned in the pVK102 vector, which had been linearized with SalI and treated with alkaline phosphatase. The ligated DNA was packed into vitro into bacteriophage lambda lambda heads by using a kit from Amersham Corp. and transduced into E. coli HB101. Out of 3,000 Km+ transductants, 30 colonies were analyzed, and all proved to have insertions 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 EcoRI fragment from the 1.9-kb region carried in pDE3. This fragment, gel-isolated after EcoRI digestion of pDE3, was selected because it is adjacent to the Tn1732 insertion in mutant CHR63. In fact, one of the EcoRI sites used to generate the probe lies in the right inverted repeat of Tn1732 (see Fig. 7B). Therefore, the 370-bp EcoRI region should contain an integral 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.

**13C NMR Spectroscopy—**Cell extracts were lyophilized and resuspended in 0.5 ml of D2O. Natural abundance 13C NMR spectra were recorded on a Bruker 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 deducted 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 grow, and the supernatant was filtered through a 0.65-μm pore membrane filter. To 3-mL samples, 250 nmol of internal standards, α-amino-n-butylate and α-aminoacidic acid, were added. Samples were applied to 3.5 × 1-cm columns of Dowex-1-acetate (100–200 mesh), equilibrated with H2O, and washed with 5 ml of H2O. The acidic amino acids, glutamate, glutamine, and aspartate, were eluted from the 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 H2O. The neutral amino acids were then applied to 4 × 1.5-cm columns of Dowex-50W-H+ (100–200 mesh), washed with 24 ml of H2O, and the neutral amino acids eluted with 18 ml of 6 M NH4OH. The basic amino acids were eluted from the Dowex-50W-NH4+ with 18 ml of 6 M NH4OH. Fractions containing the desired amino acids were evaporated to dryness under a stream of air. The samples were redissolved in 0.8 ml of freshly prepared isobutyl 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-HFBF) 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-HBF 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**

**13C NMR Analysis of the Compatible Solutes in H. elongata DSM 3043—**H. elongata DSM 3043, formerly named strain H111 (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 13C 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+ colonies screened, four showed this phenotype (Table II). Each was able to grow on M63 + 2.7 M NaCl + 1 μM 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
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 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 all salinities. The plasmid pSUP102-Gm::Tn1732 was used as a probe against genomic DNA of the mutants digested with the restriction enzymes SaII or BgII, which do not have any recognition site in Tn1732. 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.

**Ectoine Synthesis in H. elongata**

Mutants in Minimal Medium—

| Mutant strain | NaCl (M) | Growth rate
|---------------|----------|-------------
|               | 0.5      | 0.75        | 1          | 1.5        | 2          | 2.7        | 2.7 + betaine* |
| CHR62         | +        | +          | -          | -          | -          | +          |             |
| CHR63         | +        | +          | +          | +          | -          | -          | +          |
| CHR64         | +        | +          | +          | +          | +          | -          | +          |
| CHR65         | +        | +          | +          | +          | +          | +          | +          |

* Growth in M63 + 2.7 M NaCl + 1 mM betaine.

**TABLE II**

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

**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 HindIII fragment from the Tn1732 was used as a probe (see "Experimental Procedures"). The plasmid pSUP102-Gm::Tn1732 digested with EcoRI was used as a positive control (lane 1), and SaII-digested chromosomal DNA from the wild-type strain *H. elongata* 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 SaII (lanes 2–5) or BgII (lanes 6–9).

**Fig. 2.** Natural abundance 13C 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 13C NMR. Signals are as follows: ectoine (E), hydroxyectoine (H), glutamate (G), and glutamine (Q).
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-acetylnornithine 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).
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 Tn1732 insertion in mutant CHR63 was isolated as a 23-kb *BglII* fragment in plasmid pDE1 (Fig. 7A) as described under “Experimental Procedures.” A restriction analysis of an 8.6-kb *SalI* fragment (pDE3) indicated that it contained the transposon Tn1732 plus a 1.9-kb region from mutant CHR63 flanking the transposon (Fig. 7B).

The wild-type genes responsible for the synthesis of ectoine were isolated by colony hybridization, using a 370-bp chromosomal fragment flanking the Tn1732 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 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 *SalI* fragment which was lacking from the *SalI*-digested DNA from the mutants (Fig. 8). When the 370-bp *EcoRI* probe was hybridized with the three plasmids, only the common 3-kb *SalI* fragment gave a positive hybridization signal (not shown). This demonstrated that the Tn1732 insertions in mutants CHR63 and CHR62 were located in the same 3-kb *SalI* fragment (Fig. 7C), 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 Tn1732 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 Tn1732-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.

13C 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 13C 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-NH2 and not to the α-NH2 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 can 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
subcloned in pDE3 belongs to the cloning vector and therefore is not present in the wild-type DNA. The 370-bp EcoC as an disappeared in the construction of pDE1.

Eco that hybridized with the 370-bp ment mutants CHR62 or CHR63. All DNAs were digested with 7 correspond to two other plamids from the 1732 (ectoine synthase) and part of ectB (diaminobutyric acid acetyltransferase) genes of H. elongata. The insertion site of Tn1732 in CHR62 (EctB) lies in a 1.1-kb SalI-BglII region, but it has not been precisely mapped. Restriction sites are as follows: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; P, PstI; S, SalI; X, XhoI. Sites within brackets disappeared in the construction of pDE1.

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-acetylornithine, and ectoine, which is a cyclic derivative of NADA, are accumulated at >500 mM concentrations (9). N-Acetylspermine (28) and N-acetylspermidine (14) have been documented to be involved in the osmoadaptive 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-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 SalI 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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REFERENCES

1. Vreeland, R. H., Litchfield, C. D., Martin, E. L., and Elliot, E. (1980) *Int. J. Syst. Bacteriol.* 30, 485–495
2. Ventosa, A. (1984) in *Bacterial Diversity and Systematics* (Priest, F. G., Ramos-Cormenzana, A., and Tindall, B. J., eds) pp. 231–242, Plenum Publishing Corp., New York
3. Ventosa, A., and Nieto, J. J. (1995) *World J. Microbiol. & Biotechnol.* 11, 85–94
4. Fernández-Castillo, R., Vargas, C., Nieto, J. J., Ventosa, A., and Ruiz-Berraquero, F. (1992) *J. Gen. Microbiol.* 138, 1133–1137
5. Kunte, H. J., and Galinski, E. A. (1995) *FEMS Microbiol. Lett.* 128, 293–299
6. Mellado, E., Asturias, J. A., Nieto, J. J., Timmis, K. N., and Ventosa, A. (1995) *J. Bacteriol.* 177, 3443–3450
7. Vargas, C., Fernández-Castillo, R., Cánovas, D., Ventosa, A., and Nieto, J. J. (1995) *Med. Gen. Genet.* 246, 411–418
8. Kushner, D. J., and Kamekura, M. (1988) in *Halophilic Bacteria* (Rodríguez-Valera, F., ed) Vol. I, pp. 109–140, CRC Press, Inc., Boca Raton, FL
9. Galinski, E. A. (1995) *Adv. Microb. Physiol.* 37, 273–328
10. Cánovas, D., Vargas, C., Csonka, L. N., Ventosa, A., and Nieto, J. J. (1996) *J. Bacteriol.* 178, 7221–7226
11. Csonka, L. N., and Hansen, A. D. (1991) *Annu. Rev. Microbiol.* 45, 569–606
12. Galinski, E. A., Pfeiffer, H. P., and Truper, H. G. (1985) *Eur. J. Biochem.* 149, 135–139
13. Peters, P., Galinski, E. A., and Truper, H. G. (1990) *FEMS Microbiol. Lett.* 71, 157–162
14. Severin, J., Wohlfarth, A., and Galinski, E. A. (1992) *J. Gen. Microbiol.* 138, 1629–1638
15. Nieto, J. J., Fernández-Castillo, R., Marquez, M. C., Ventosa, A., and Ruiz-Berraquero, F. (1988) *Appl. Environ. Microbiol.* 55, 2385–2390
16. Davis, R. W., Botstein, D., and Roth, J. R. (1980) *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Cohen, G. N., and Rickenberg, R. H. (1956) *Ann. Inst. Pasteur* (Paris) 91, 695–720
18. Simon, R., Priefer, U., and Huhler, A. (1983) *Bio/Technology* 1, 784–791
19. Ubben, D., and Schmitt, R. (1986) *Gene* (Amst.) 41, 145–152
20. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. R., and Struhl, K. (1989) *Current Protocols in Molecular Biology*, Greene Associates/Wiley Interscience, New York
21. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Knauf, V. C., and Nester, E. W. (1982) *Plasmid* 8, 45–54
23. Vargas, C., Coronado, M. J., Ventosa, A., and Nieto, J. J. (1997) *Syst. Appl. Microbiol.* 20, 173–181
24. Clark, L., and Carbon, J. (1979) *Methods Enzymol.* 68, 396–408
25. Fernández-Linares, L., Faure, R., Bertrand, J. C., and Gauthier, M. (1996) *Lett. Appl. Microbiol.* 22, 169–172
26. Wohlfarth, A., Severin, J., and Galinski, E. A. (1990) *J. Gen. Microbiol.* 136, 705–712
27. Rhodes, D., Hogan, A. L., Deal, L., Jamieson, G. C., and Haworth, P. (1987) *Plant Physiol. (Bethesda)* 84, 775–780
28. Wohlfarth, A., Severin, J., and Galinski, E. A. (1993) *Appl. Microbiol. Biotechnol.* 39, 568–573
29. Liss, I. (1962) *Phytochemistry* 1, 87–88
30. Fowden, L. (1972) *Phytochemistry* 11, 2271–2276
31. Boyer, H. W., and Roulland-Dussiau, D. (1969) *J. Mol. Biol.* 41, 459–472
32. Kessler, B., de Lorenzo, V., and Timmis, K. N. (1992) *Mol. Gen. Genet.* 233, 293–301

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