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

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David Cánovas‡‡, Carmen Vargas‡, Fernando Iglesias-Guerra**, Laszlo N. Csonka§, David Rhodes§, Antonio Ventura‡, and Joaquín J. Nieto‡‡

From the ‡Department of Microbiology and Parasitology, **Department of Organic Chemistry and Pharmaceutical Chemistry, Faculty of Pharmacy, University of Seville, 41012 Seville, Spain and the ‡‡Department of Biological Sciences and the ‡‡‡Department of Horticulture, Purdue University, West Lafayette, Indiana 47907

The moderate halophile Halomonas elongata Deutschesammlung für Mikroorganismen 3043 accumulates ectoine, hydroxyectoine, glutamate, and glutamine in response to osmotic stress (3 mM 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 mM NaCl, and CHR63 did not grow above 1.5 mM 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-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 mM 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 mM 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 mM 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 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 1. H. elongata strains were routinely grown in SW-10 medium, which contained 19% (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 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).

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.

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.

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region flanking the Tn732 insertion in mutant CHR63, genomic DNA of CHR63 was digested with BglII, ligated to BamIII-digested pKS, 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, not accounted for in the published restriction map of CHR63, was cloned in pBR322. A 1.5-kb HindIII fragment was inserted in the broad host range cosmids 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 library 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’ 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 Tn732 insertion in mutant CHR63. In fact, one of the EcoRI sites used to generate the probe lies in the right inverted repeat of Tn732 (see Fig. 7B). Therefore, the 370-bp EcoRI region should contain part of the H. elongata ectoine synthase gene (ect). Plasmids isolated after library screening were digested from E. coli to H. elongata by tripleparental matings on SW-2 by using pKf600 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. To achieve inotheplexal 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 deduced from the spectrum of DA signals. Ectoine was identified by comparison of chemical shifts with published values (25, 26).

**Ectoine, Neutral, and Basic Amino Acids—**Wild-type and mutants CHR63 and CHR62 were grown in 1-liter cultures of M63 minimal medium, plus 5 ml of 2 M NaCl, and the composition of its internal solutes was determined by GC-MS analysis (see below). Ectoine did not produce a volatile derivative in this procedure, as determined by GC-MS. An authentic standard of N,N,N-trimethylglycine was used as internal standard.

**RESULTS**

**13C 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 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 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
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 NaCl for CHR63 or at >1.5 M NaCl 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 Tn1732 as a probe against genomic DNA of the mutants digested with the restriction enzymes SuII 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.

**TABLE II**

<table>
<thead>
<tr>
<th>Mutant strain</th>
<th>NaCl 0.5</th>
<th>NaCl 0.75</th>
<th>NaCl 1.5</th>
<th>NaCl 2.0</th>
<th>NaCl 2.7</th>
<th>NaCl 2.7 + betaine*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHR62</td>
<td>+</td>
<td>+</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CHR63</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>M</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CHR64</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>M</td>
<td>+</td>
</tr>
<tr>
<td>CHR65</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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

**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).

**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: □, wild-type *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 SuII-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 SuII (lanes 2–5) or BgII (lanes 6–9) to a derivative of DA. Signals at 174 and 175 ppm could be attributed to the carboxonyl moiety of the carboxylac 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
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).
Ectoine Synthesis in H. elongata

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Osmoprotectant</th>
<th>NaCl 1.5</th>
<th>NaCl 2.0</th>
<th>NaCl 2.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>None</td>
<td>$9.80 \times 10^{-2}$</td>
<td>$1.33 \times 10^{-1}$</td>
<td>$7.92 \times 10^{-2}$</td>
</tr>
<tr>
<td>CHR62</td>
<td>None</td>
<td>$2.90 \times 10^{-3}$</td>
<td>$5.00 \times 10^{-5}$</td>
<td>$1.00 \times 10^{-4}$</td>
</tr>
<tr>
<td>Wild-type extract</td>
<td></td>
<td>$1.95 \times 10^{-2}$</td>
<td>$1.90 \times 10^{-2}$</td>
<td>$1.30 \times 10^{-2}$</td>
</tr>
<tr>
<td>CHR63</td>
<td>None</td>
<td>$1.87 \times 10^{-2}$</td>
<td>$1.80 \times 10^{-2}$</td>
<td>$1.95 \times 10^{-2}$</td>
</tr>
<tr>
<td>CHR63</td>
<td>Wild-type extract</td>
<td>$4.31 \times 10^{-2}$</td>
<td>$5.10 \times 10^{-2}$</td>
<td>$1.50 \times 10^{-2}$</td>
</tr>
<tr>
<td>CHR63</td>
<td>Wild-type extract</td>
<td>$2.80 \times 10^{-2}$</td>
<td>$2.45 \times 10^{-2}$</td>
<td>$2.00 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

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 stimulus 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 (in 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 (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 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
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. 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\(^{\text{v}}\)-acetylornithine, and ectoine, which is a cyclic derivative of NADA, are accumulated at >500 mM concentrations (9). N\(^{\text{v}}\)-Acetylornithine (28) and N\(^{\text{v}}\)-acetyllysine (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\(^{\text{v}}\)-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

\[ \text{D. Cánovas, C. Vargas, L. N. Csonka, A. Ventosa, and J. J. Nieto, unpublished results.} \]
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.

**Acknowledgment**—We thank Dr. E. A. Galinski for SM10 (pSUP102-Gm::Tn1732) and ectoine.

**REFERENCES**

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