APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2008, p. 4560–4563 Vol. 74, No. 14
0099-2240/08/$08.00+0 doi:10.1128/AEM.00492-08

Synthesis of the Compatible Solute Ectoine in *Virgibacillus pantothenticus* Is Triggered by High Salinity and Low Growth Temperature▽

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Received 29 February 2008/Accepted 10 May 2008

The quantification of the intracellular concentration of ectoine in *Virgibacillus pantothenticus* revealed that the production of this compatible solute is triggered either by an increase in the external salinity or by a reduction in the growth temperature. This finding reflects increased transcription of the ectoine biosynthetic operon (*ectABC*) under both environmental conditions.

Increases in the salinity of the environment impose a considerable strain on the water balance of microorganisms (3, 11, 17). To counteract the efflux of water from the cells when they are challenged by high salinity, many microorganisms accumulate large quantities of a particular group of organic compounds, the so-called compatible solutes (3, 11, 17). This accumulation can be achieved either through de novo biosynthesis or through uptake from the environment via dedicated and osmotically controlled transport systems (3, 17). In addition to their osmoprotective function, compatible solutes affect the stability and correct folding of proteins under unfavorable conditions (2, 10, 15). The stabilizing effect of compatible solutes on proteins is generally explained by the preferential exclusion model (27). The protein-stabilizing property of compatible solutes is likely to contribute to the function of these chemical chaperons as heat and chill stress protectants for various microbial species (1, 4, 6, 8, 10, 12, 14, 16, 18, 21, 24).

Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is one of the most widely found compatible solutes in the domain of the *Bacteria* (5, 11, 19, 25). Molecular analyses of its biosynthesis in various gram-negative and gram-positive microorganisms have shown that ectoine production depends on the evolutionarily highly conserved *ectABC* gene cluster (5, 9, 13, 19, 20, 22, 25, 26). Ectoine synthesis relies on the sequential reactions of the diaminobutyric acid transaminase (EctB), the diaminobutyric acid acetyltransferase (EctA), and the ectoine synthase (EctC) that together convert the precursor L-aspartate-β-semialdehyde into ectoine. Some microorganisms also produce a hydroxylation derivative of ectoine, 5-hydroxyectoine, via an evolutionarily conserved ectoine hydroxylase (5, 12, 23).

Consistent with the proposed function of ectoine as a microbial osmoprotectant, substantial ectoine production occurs in cells that are osmotically challenged. For instance, in *Bacillus pasteurii* and *Salibacillus salexigens*, an osmotically mediated induction of the transcription of the *ectABC* gene cluster occurs and leads to the accumulation of ectoine in accordance with the external salinity (5, 19). However, in certain microorganisms, ectoine biosynthesis seems to be triggered not only by osmotic stress, but also by other environmental cues. For instance, in *Streptomyces griseus*, increased levels of this compatible solute were found previously when the cells were subjected to high growth temperatures (21), and the *ectABC* gene cluster of *Chromohalobacter salexigens* (DSM 3043) is induced when the cells are cultivated at high growth temperatures (7). These findings suggest a role for ectoine as a protectant against the detrimental effects of high temperatures.

By natural-abundance ^{13}C nuclear magnetic resonance spectroscopy, it was recently shown that the gram-positive soil bacterium *Virgibacillus pantothenticus* produces both proline and ectoine when it is exposed to high salinity (19). Here, we have characterized the ectoine biosynthetic genes from *V. pantothenticus* and demonstrated that their transcription is triggered by two environmental cues: high salinity and a low growth temperature. Chill-induced expression of an *ectABC* gene cluster has never been observed before.

Ectoine synthesis in *V. pantothenticus* increases at high salinity. To monitor proline and ectoine production in *V. pantothenticus* (DSM 26^T) in response to high salinity, we grew this bacterium at its optimal growth temperature (37°C) to an optical density at 578 nm (OD_{578}) of 1 in a chemically defined minimal medium (19) with salinities ranging from 0 M NaCl to 1.9 M NaCl. Cells were then harvested by centrifugation, and the compatible solutes were extracted using a modified Blight and Dyer technique (19). Subsequently, the amounts of proline and ectoine produced were determined by high-performance liquid chromatography (HPLC) analysis (19). There was no proline or ectoine detectable in *V. pantothenticus* cultures when the cells were grown in a minimal medium without added NaCl (Fig. 1A). As the salinity was raised, there was a concomitant increase in both the proline and ectoine contents of the cells (Fig. 1A). At external salinities of up to 0.7 M NaCl, the proline level rose in proportion to the increase in the external salinity, but then there was no further increase in the proline content despite the fact that the salinity of the medium was raised up to a level of 1.9 M NaCl (Fig. 1A). The ectoine level was initially lower than that of proline (at up to 0.7 M NaCl) but then progressively increased when the salinity of the growth medium was raised up to 1.3 M NaCl (Fig. 1A). A

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▽ Published ahead of print on 16 May 2008.

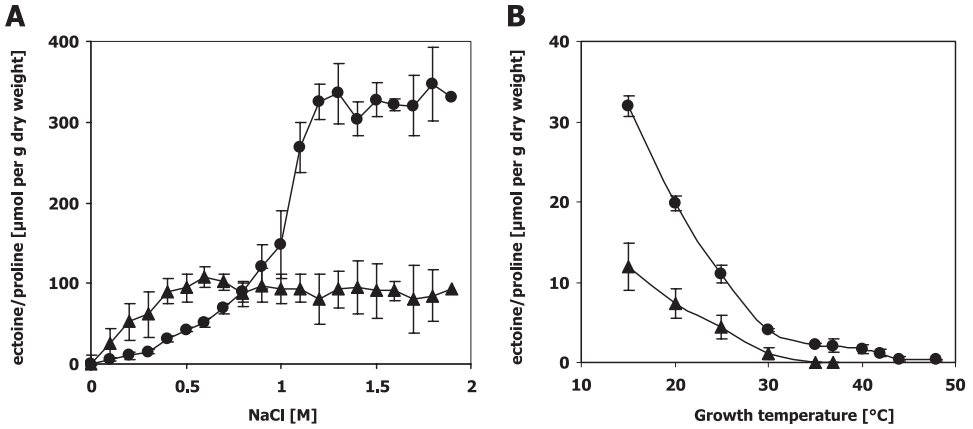

FIG. 1. Ectoine and proline biosynthesis in *V. pantothenticus* in response to salt and cold stress. (A) Cultures of *V. pantothenticus* were grown in minimal medium at 37°C with the indicated concentrations of NaCl to an OD_{578} of approximately 1, and the produced proline (▲) and ectoine (●) were quantitated by HPLC analysis. (B) Cultures of *V. pantothenticus* were grown in a minimal medium at the indicated temperatures in the absence of salt stress to an OD_{578} of approximately 1, and the proline (▲) and (●) ectoine produced by the cells were quantitated by HPLC analysis. The values given for the ectoine and proline contents are the means of results of two independent measurements each of two independently grown *V. pantothenticus* cultures.

further increase in salinity did not lead to the further enhancement of ectoine production. Consequently, both proline and ectoine seem to serve an osmoregulatory function in *V. pantothenticus*, but ectoine is clearly the physiologically more important compatible solute (Fig. 1A). It should be noted, however, that the ectoine content of *V. pantothenticus* cells under high-salinity conditions is about fivefold lower than that of *Bacillus pasteurii* cells (19). In *B. pasteurii* cells, the ectoine content is linearly correlated with the external salinity (19), whereas this is clearly not the case for *V. pantothenticus* (Fig. 1A).

Ectoine biosynthesis in *V. pantothenticus* is triggered by low but not by high growth temperatures. We also monitored both ectoine and proline production in cells of *V. pantothenticus* that were grown at various temperatures in the absence of salt stress. A reduction of the growth temperature from the optimal growth temperature (37°C) of *V. pantothenticus* led to a progressive increase in both the ectoine and proline contents of the cells (Fig. 1B). Consequently, for *V. pantothenticus*, both ectoine and proline biosynthesis can be triggered in the absence of salt stress simply by growing the cells at a low temperature. Cells propagated at 15°C, the lowest growth temperature tested in this series of experiments, exhibited the biosynthesis of approximately 35 μmol of ectoine per g (dry weight) of cells (Fig. 1B). This value is comparable to the ectoine content of *V. pantothenticus* cells that were grown at 37°C in the presence of 0.3 M NaCl (Fig. 1A). Growth at a low temperature thus triggers a moderate increase in the ectoine content of *V. pantothenticus* cells in comparison to the ectoine levels found in severely salt-stressed cells (approximately 330 μmol of ectoine per g [dry weight] of cells that were grown in minimal medium with 1.3 M NaCl) (Fig. 1A). While proline production was also triggered by the growth of *V. pantothenticus* at low temperatures, the level of proline production was considerably lower than that of ectoine production under the same cultivation conditions.

We also tested whether an increase in the growth temperature would trigger ectoine production in *V. pantothenticus*. However, this was not the case, since there was no ectoine detectable in cells that were continuously grown at 48°C (Fig. 1B), a temperature close to the upper limit for the growth of *V. pantothenticus* (data not shown). We did not determine the proline content of *V. pantothenticus* cells at high growth temperatures.

Molecular analysis of the ectoine biosynthetic genes in *V. pantothenticus*. We assumed that in *V. pantothenticus*, ectoine biosynthesis is mediated by the typical *ectABC* gene cluster and found that this was indeed the case. We initially used a PCR approach to recover a DNA fragment that contained part of the *ectB* gene and part of the *ectC* gene. This DNA segment was then used to complete the DNA sequence of the *ectABC* genes by using chromosomal DNA of *V. pantothenticus* as a sequencing template (data not shown). We determined in total the sequence of a 2,670-bp DNA segment covering the entire *ectABC* gene cluster and its 5′ and 3′ regions. The EctABC proteins deduced from the genomic sequence of the *V. pantothenticus ectABC* gene cluster show a considerable degree of amino acid sequence identity (between 27 and 65%) to the corresponding and functionally characterized EctABC proteins from *B. pasteurii* (19), *Marinococcus halophilus* (20), *Halomonas elongata* (13), *C. salexigens* (9), *S. salexigens* (5), "*Halobacillus halophilus*" (26), *Methylomicrobium alcaliphilum* (25), and *Vibrio cholerae* (22) (data not shown). Furthermore, many *ectABC* genes related to the *V. pantothenticus ectABC* genes can be found by searching databases containing full or partial sequences of microbial genomes (data not shown). The EctABC proteins from the various microorganisms are all closely related, and their features have already been described in considerable detail (9, 13, 19, 20, 22, 25).

The transcription of the *ectABC* gene cluster is osmotically controlled. An intergenic region of 43 bp separates the *V. pantothenticus ectA* and *ectB* genes, and the *ectB* gene is separated from *ectC* by 30 bp. This tight spacing of the three *ect* genes suggested that in *V. pantothenticus* the *ectABC* gene cluster might be organized as an operon, as it is in *B. pasteurii* (19), *S. salexigens* (5), and *H. halophilus* (26). We used Northern blot analysis to investigate the transcriptional organization

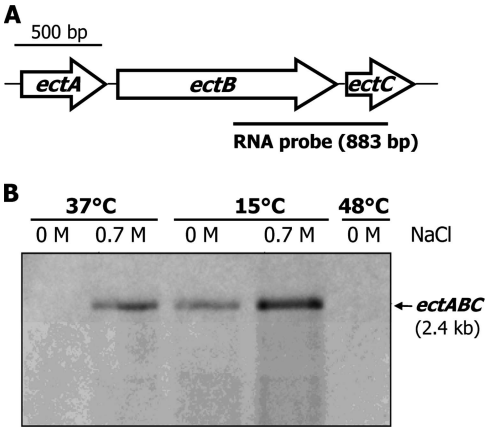

FIG. 2. Northern blot analysis of the transcription of the *V. pantothenticus ectABC* genes in response to high salinity and cold stress. (A) Genetic organization of the *ectABC* genes in *V. pantothenticus* and position of the RNA antisense probe used for the transcriptional analysis of the *ectABC* gene cluster. (B) Northern blot analysis of the *ectABC* operon. Total RNA was isolated from *V. pantothenticus* cells grown in a minimal medium under the indicated environmental conditions and hybridized to the *ectBC*-specific antisense RNA probe.

of the *V. pantothenticus ectABC* genes experimentally by using a single-stranded RNA probe (883 bp) that carried part of the *ectB* gene and part of the *ectC* gene (Fig. 2A). The isolation of total RNA from *V. pantothenticus*, the preparation of the single-stranded antisense RNA probe, hybridization conditions, and signal detection were as described previously for the Northern blot analysis of the *B. pasteurii ectABC* operon (19). No *ect*-specific RNA signal was detected in the RNA sample isolated from *V. pantothenticus* cells grown in minimal medium in the absence of salt stress (Fig. 2B). However, a 2.4-kb *ect*-specific transcript was readily detectable when the *V. pantothenticus* cells were continuously propagated in a minimal medium that contained 0.7 M NaCl (Fig. 2B). The size of the detected *ect* transcript matched the calculated distance (2,188 bp) between the start codon of the *V. pantothenticus ectA* gene and the termination codon of *ectC*. Hence, the *ectABC* gene cluster of *V. pantothenticus* is transcribed as an osmotically regulated operon. This pattern of *ectABC* transcription was reflected by the amount of ectoine synthesized under both the low- and high-salinity growth conditions that were used for the RNA isolation. There was an increase of about 15-fold in the ectoine content of *V. pantothenticus* cells that were grown in a minimal medium with 0.7 M NaCl compared to that of the cells that were grown in the absence of NaCl (Table 1).

Induction of the transcription of the *ectABC* operon by cold stress. The quantification of the ectoine contents of cells that were propagated at suboptimal growth temperatures by HPLC analysis revealed that the *V. pantothenticus* cells responded to such cold stress with increased ectoine formation (Fig. 1B). To test whether this response was caused by the increased transcription of the *ectABC* operon, we looked for the *ectABC* transcript by Northern blot analysis of cells that were grown at 15°C in the absence of salt stress. *ectABC* transcription in the cold-stressed cells was clearly induced in comparison to that in cells that were propagated at the optimal growth temperature of *V. pantothenticus* of 37°C (Fig. 2B). Under these growth conditions, there was an increase in the ectoine content of the cold-stressed *V. pantothenticus* cells of about fivefold in comparison to that of the cells that were grown at 37°C (Table 1).

TABLE 1. Biosynthesis of ectoine in *V. pantothenticus* in response to salt and temperature stress

Growth temp (°C)	External salinity (M NaCl)	Ectoine content (μmol per g [dry wt] of cells)[a]
37	0	6.3 ± 0.2
37	0.7	92.7 ± 2.3
15	0	33.0 ± 1.0
15	0.7	108.4 ± 2.9
48	0	0.3 ± 0.0

[a] The values given are the means ± the standard deviations of results of two independent measurements each of two independently grown *V. pantothenticus* cultures.

The highest level of the *V. pantothenticus ectABC* transcript was detected in cells that were subjected simultaneously to chilling (15°C) and salt stress (0.7 M NaCl) (Fig. 2B), and the highest levels of ectoine were found in the cells under these conditions. No *ectABC* transcript was detectable in *V. pantothenticus* cells that were subjected to heat stress (growth at 48°C), and as expected, these cells did not produce any significant amounts of ectoine (Table 1).

Conclusions. There are two major findings with respect to the data presented in this communication. First, the ectoine content of salt-stressed *V. pantothenticus* cells gradually increased in response to the amount of NaCl added to the growth medium (Fig. 1A), indicating that *V. pantothenticus*, like *B. pasteurii* (19) and *S. salexigens* (5), can sensitively detect gradual changes in the external salinity. How this detection can be accomplished by the cell at the molecular level is currently unclear. Increased ectoine biosynthesis in salt-stressed *V. pantothenticus* cells has an obvious function in osmoadaptation.

The most interesting result of our study is the finding that ectoine biosynthesis in *V. pantothenticus* can be induced at the transcriptional level in the absence of salt stress in cells that are subjected to continued growth at a low temperature (15°C) (Fig. 2B). To the best of our knowledge, such chill stress induction of *ectABC* transcription in any ectoine-synthesizing microorganism has not been observed before. We consider it likely that the cold-induced ectoine biosynthesis in *V. pantothenticus* serves a chill protection function for this microorganism. This proposal is fully consistent with previous observations of the protection of the human pathogen *Listeria monocytogenes* (1, 18) and the soil bacterium *Bacillus subtilis* (4) from cold stress by exogenously provided compatible solutes (e.g., glycine betaine). Indeed, we have recently found that exogenously provided ectoine or 5-hydroxyectoine serves as an effective chill protectant for *V. pantothenticus* (J. Bursy, A. Kuhlmann, and E. Bremer, unpublished results).

Nucleotide sequence accession number. The sequence obtained in this study has been deposited in GenBank under accession number AY585263.

We thank V. Koogle for her help in editing the manuscript and appreciate the expert technical assistance of J. Gade during part of this project.

Financial support for this study was provided by the Deutsche Forschungsgemeinschaft through the SFB-395, the Bundesministerium für

Bildung und Forschung through the "Genomnetzwerk Göttingen," the Fonds der Chemischen Industrie, and the Max Planck Institute for Terrestrial Microbiology (Marburg, Germany).

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