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Functional Expression of the Ectoine Hydroxylase Gene (*thpD*) from *Streptomyces chrysomallus* in *Halomonas elongata*

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The formation of hydroxyectoine in the industrial ectoine producer *Halomonas elongata* was improved by the heterologous expression of the ectoine hydroxylase gene, *thpD*, from *Streptomyces chrysomallus*. The efficient conversion of ectoine to hydroxyectoine was achieved by the concerted regulation of *thpD* by the *H. elongata ectA* promoter.

Under hyperosmotic stress, a variety of microorganisms accumulate organic low-molecular-weight compounds such as polyols, amino acids, sugars, betaines, and ectoine. These so-called compatible solutes enable the organisms to survive under conditions of high osmotic pressure (2). Since it has been demonstrated that compatible solutes, especially ectoine, are able to protect enzymes, membranes, and whole cells against stresses caused by exposure to salt, heating, freezing, and desiccation, there is an increasing interest in the use of compatible solutes for various applications in biotechnology (8, 12, 21, 20). Today, ectoine is already used as a moisturizer in cosmetics and skin care products (RonaCare Ectoin; Merck KGaA, Darmstadt, Germany) (12). In addition, ectoine's hydroxyl derivative, hydroxyectoine, has gained attention as a protein-protecting agent which, in a number of applications, has properties superior to those of ectoine (8, 4). Moreover, hydroxyectoine has also been investigated as a protectant of healthy cells during chemotherapy (1). Thus, the development of hydroxyectoine as a by-product of ectoine is foreseeable.

The biotechnological production of ectoine, a process called bacterial milking, is carried out with the halophilic gram-negative bacterium *Halomonas elongata*, which responds to a hypoosmotic shock by the rapid release of the accumulated solutes (15). After growth at 25°C, the typical product solution consists of more than 93% ectoine, 4% of the ectoine precursor *N*-acetyl-diaminobutyric acid, and only traces of glutamate, alanine, and hydroxyectoine. However, the relative proportion of hydroxyectoine can be increased to 50% by using considerably higher salinities or elevated temperatures, factors increasing costs for both the equipment and the process (15). Moreover, the costly separation of ectoine and hydroxyectoine is still necessary. Therefore, the biotechnological production of hydroxyectoine is carried out with the gram-positive *Marinococcus* sp. strain M52, which produces predominantly hydroxyectoine but is less amenable to the milking process than *H. elongata*.

The in vivo formation of hydroxyectoine has been described for a variety of bacteria (7, 10, 18). The in vitro conversion of ectoine to hydroxyectoine by a specific hydroxylase from *Streptomyces chrysomallus* ATCC 11523 (Fig. 1) has been shown previously (5, 6). The corresponding gene (*thpD*) has been shown to be part of the ectoine-hydroxyectoine gene cluster as shown in Fig. 2. Unlike the streptomycetes, the moderately halophilic bacteria *H. elongata* and *Marinococcus halophilus* lack a *thpD*-like gene in the *ect* gene cluster (3, 9).

For the industrial production of hydroxyectoine, a microorganism with a broad salt tolerance is favored to perform the bacterial milking process (15). Since *H. elongata* is already used for the industrial production of ectoine, there would be commercial interest in broadening its industrial application. We therefore expressed the ectoine hydroxylase gene of *S. chrysomallus*, *thpD*, in *H. elongata* to improve this industrially used ectoine producer so that it may serve as a hydroxyectoine producer.

Construction of *H. elongata* expression plasmids. For expression of the *thpD* gene, we assembled an autonomous replicating plasmid for *H. elongata* by combining the *Escherichia coli* pUC derivative pSP72 (Promega) with the *H. elongata* plasmid pHE1 (17) by the insertion of a spectinomycin resistance cassette for selection in *Halomonas* and an *oriT* gene for plasmid transfer. First, *oriT* of *Pseudomonas aeruginosa* was PCR amplified as an 0.8-kb PstI cassette from plasmid pHM10a (11) with oligonucleotides prim1a (5′-ATT ACT GCA GTC GGT CTT GCC TTG CTC GTC GG-3′) and prim1b (5′-TTT CTG CAG TGC ATA ACC CTG CTT CGG GG-3′), subcloned into plasmid pLITMUS28 (New England Biolabs) cleaved with NsiI, reisolated as a BglII-XhoI fragment, and cloned into pSP72 cleaved with BamHI and XhoI. The next step was insertion of the spectinomycin cassette, which was obtained as an EcoRI fragment from plasmid pHP45omega (14), and insertion of the BglII-linearized plasmid pHE1 into the respective sites of the pSP72 backbone. The resultant plasmid, pJP-0, harbors a single PstI site located in the pHE1 region, which was used for inserting two different *thpD* gene cassettes (Fig. 2B). A promoterless *thpD* gene cassette was generated by PCR from a genomic *thpD* clone (plasmid pAF1sub10) (unpublished data) with oligonucleotide prim2a (5′-GCC GAA TTC CAT ATG ACC ACC GAA GTA

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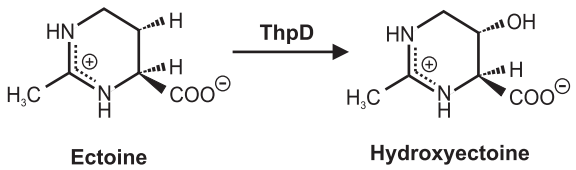

FIG. 1. Reaction catalyzed by ThpD, the ectoine hydroxylase from *S. chrysomallus*.

CGC GCC GAT-3′), introducing an NdeI site encompassing the ATG translation start codon of the *thpD* stop codon, and with oligonucleotide prim2b (5′-AGC GAA TTC CCC TGC AGG GGC CGG GAC GGC GTA CCC GTC CCG G-3′), introducing a PstI and EcoRI site about 50 bp downstream of the *thpD* gene. The NdeI-EcoRI fragment was subcloned into pSL1180 (Amersham Biosciences) cleaved with NdeI and EcoRI, thus maintaining untouched the PCR-introduced PstI site downstream of *thpD* and generating a second PstI site upstream of *thpD* located in the pSL1180 polylinker. We also generated a promoter-driven variant of the *thpD* gene cassette by inserting the *ectA* promoter of *H. elongata* DSM 3043 as a 250-bp NotI-NdeI fragment immediately upstream of *thpD* by using a single NotI site of the pSL1180 polylinker. The *ectA* promoter was PCR amplified from genomic DNA with oligonucleotides prim3a (5′-CGG GGA TCC GCG CCG ACG AGC GCT CGA TCG-3′) and prim3b (5′-AGC GAA TTC CCC TGC AGG GGC CGG GAC GGC GTA CCC GT-3′) to generate restriction sites. All PCR-generated fragments were sequenced to confirm the entire DNA sequence. Insertion of these *thpD* gene cassettes into the PstI site of plasmid pJP-0 generated plasmids pJP-2 (*thpD* with *ectA* promoter) and pJP-1 (promoterless *thpD* gene) (Fig. 2B). In both plasmids, the orientation of the inserted *thpD* gene was chosen in such a way that a known pHE1 promoter region (16) providing basal gene expression was always located upstream of the *thpD* gene cassette. Thus, we also expected the promoterless *thpD* gene in pJP-1 to be expressed to some extent.

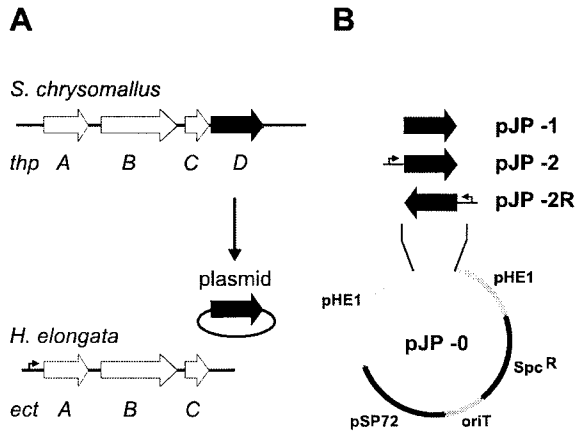

FIG. 2. (A) Map of the ectoine and hydroxyectoine biosynthesis genes of *H. elongata* (*ect*) and *S. chrysomallus* (*thp*). The *thpD* gene coding for the *S. chrysomallus* ectoine hydroxylase, which was transferred to *H. elongata*, is shown in black. (B) Mobilizable pHE1-derived shuttle vectors for the expression of the *S. chrysomallus thpD* gene in *H. elongata*. The *ectA* promoter region is indicated by small arrows.

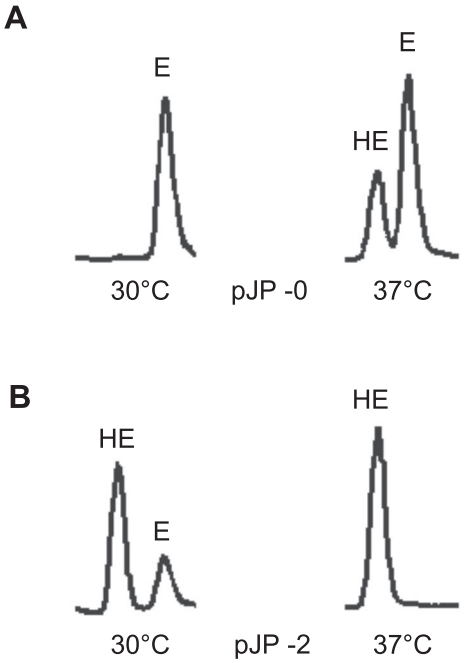

FIG. 3. HPLC detection of ectoine (E) and hydroxyectoine (HE) produced by *H. elongata* strains containing plasmid pJP-0 (A) and pJP-2 (B). After growth of *H. elongata* at the indicated temperatures, ectoine and hydroxyectoine were released by hypoosmotic shock and subjected to HPLC analysis.

Plasmids were transferred from *E. coli* S17-1 to *H. elongata* by conjugation. To eliminate *E. coli* cells after plasmid transfer, a spontaneous rifampin-resistant mutant of *H. elongata* DSM 304 was isolated by serial plating on solid medium saline (SWYE) containing rifampin (25 μl/ml) (17, 13). Comparison of the resulting rifampin-resistant strain *H. elongata* AD-98 with the wild-type strain *H. elongata* DSM 3043 did not reveal any dissimilarities regarding the production of ectoine or hydroxyectoine, even in the presence of plasmid pJP-D-0 (data not shown). We therefore routinely used *H. elongata* AD-98 for studying the effect of the introduction of *thpD* on the formation of hydroxyectoine in that strain.

ThpD-dependent hydroxyectoine production in *H. elongata*. To determine the effect of the *thpD* gene on the formation of hydroxyectoine, *H. elongata*/pJP-2 (*thpD* with *ectA* promoter) and *H. elongata*/pJP-1 (promoterless *thpD*) were cultivated at 30°C in 10 ml of SWYE medium in 100-ml flasks, with shaking at 200 rpm, to an optical density at 600 nm of 2.4 to 2.6. Bacterial milking was carried out by lowering the salt concentration from 10 to 2% (wt/vol) with distilled water. An aliquot of the cell-free supernatant was incubated at 95°C for 15 min, cleared by centrifugation, and subjected to high-pressure liquid chromatography (HPLC) analysis (LiChroCART 250-4, LiChrospher 100 NH_2; 5-μm particle size with 70% acetonitrile as the eluent). Ectoine and hydroxyectoine were quantified by using commercial ectoine and hydroxyectoine standards (Biomol). At 30°C and with 10% (wt/vol) salts, *H. elongata*/pJP-2 converted 76% of the ectoine into hydroxyectoine with a yield of 340 nmol of hydroxyectoine per ml (Fig. 3B, left chromatogram). In contrast, the control strain *H. elongata*/pJP-0 (plasmid without insert) did not convert significant amounts of

TABLE 1. Hydroxyectoine formation by *H. elongata* strains at 37°C

Plasmid	Characteristics	Relative proportion[a]	
		Hydroxyectoine	Ectoine
pJP-0	$P_{pHE1,}$ no insert	37.2	62.8
pJP-1	$P_{pHE1,}$ *thpD*	80.4	19.6
pJP-2	$P_{pHE1,}$ P_{ect}, *thpD*	100	ND
pJP-2R	P_{ectA}, *thpD*	100	ND

[a] Values are given as percentages of the total amount of ectoine and hydroxyectoine and are the means of results for at least three independent cultures. ND, not detectable.

ectoine into hydroxyectoine under these conditions (Fig. 3A, left chromatogram). Also, in the case of *H. elongata*/pJP-1, none or only traces (<3%) of the ectoine were converted into hydroxyectoine. These results show that the *thpD* gene of *S. chrysomallus* was actively expressed in *H. elongata* in the presence of a suitable promoter.

Increased yields of hydroxyectoine have been described for *H. elongata* at elevated temperatures and higher salinities (19). We therefore measured the hydroxyectoine formation in the transformed strains at 37°C (instead of the 30°C described above) and 10% (wt/vol) salts. As expected, the control strain *H. elongata*/pJP-0 produced significantly higher amounts of hydroxyectoine (37%) but still less than the ectoine produced (63%) (Fig. 3A, right chromatogram). In contrast, strain *H. elongata*/pJP-2 (*thpD* with *ectA* promoter) produced exclusively hydroxyectoine (100%) (Fig. 3B, right chromatogram). No ectoine could be detected under these growth conditions. Remarkably, the introduction of the promoterless *thpD* gene (by plasmid pJP-1), driven only by an intrinsic pHE1 promoter (16), also resulted in an enhanced formation of hydroxyectoine (80%). Ectoine (20%), however, was still present under these conditions (Table 1). Complete conversion of ectoine to hydroxyectoine was also observed when the P_{ectA}-*thpD* cassette was in the opposite orientation (pJP-2R) (Table 1). This clearly indicates that the P_{ectA}-*thpD* cassette alone is well suited for the industrial production of hydroxyectoine.

The economic benefit of the bacterial milking process, compared to fed-batch cultures, implies that at least two cycles of compatible solute extraction have to be applied (15). We therefore studied the formation of hydroxyectoine in the absence of the selection marker streptomycin at 37°C. Cultures were subjected to hypoosmotic shocks and used as seed culture every 24 h over a period of 9 days. After three cycles of bacterial milking, the amount of hydroxyectoine dropped from 100 to 96%. Prolonged cultivation led to wild-type levels of hydroxyectoine (36%) after nine cycles. In parallel, aliquots of cultures were used to assay the amount of plasmid still present. The results of the plasmid quantification indicate that pJP-2 is not stable under nonselective conditions. However, since the milking process can be repeated at least two times without the detectable appearance of ectoine, the genetically engineered *H. elongata* strain may be valuable for future biotechnological applications.

In this study, we showed the functional expression of the ectoine hydroxylase gene of the gram-positive bacterium *S. chrysomallus*, *thpD*, in the gram-negative host *H. elongata*. Introduction of the P_{ectA}-*thpD* gene cassette greatly stimulates production of hydroxyectoine from ectoine at 30°C and completely converts all ectoine to hydroxyectoine at 37°C. The efficient conversion of ectoine to hydroxyectoine is achieved by the concerted regulation of *thpD* via the *H. elongata ectA* promoter. Although the *thpD* expression plasmid is not stable under nonselective conditions, the results suggest that the bacterial milking process may be applicable for the industrial production of hydroxyectoine. To this end, we plan to transfer the *thpD* expression cassette stably into the genome of *H. elongata*.

Nucleotide sequence accession number. The nucleotide sequence of the *thp* genes from *S. chrysomallus* (ATCC 11523) has been deposited in the GenBank nucleotide sequence database under accession number AY524544.

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