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Temperature- and Salinity-Decoupled Overproduction of Hydroxyectoine by *Chromohalobacter salexigens*

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Hydroxyectoine overproduction by the natural producer *Chromohalobacter salexigens* is presented in this study. Genetically engineered strains were constructed that at low salinity coexpressed, in a vector derived from a native plasmid, the ectoine (*ectABC*) and hydroxyectoine (*ectD*) genes under the control of the *ectA* promoter, in a temperature-independent manner. Hydroxyectoine production was further improved by increasing the copies of *ectD* and using a *C. salexigens* genetic background unable to synthesize ectoines.

Ectoine and hydroxyectoine (ectoines) are compatible solutes synthesized and accumulated by halophilic and halotolerant bacteria in response to osmotic and heat stress (1, 2). Ectoines have current applications as biostabilizers of proteins and nucleic acids, as well as a potential role as therapeutics for certain diseases (3, 4). This, together with the complexity of their chemical synthesis, has encouraged recent efforts to improve ectoine production from bacteria. Hydroxyectoine is especially interesting, as it seems to confer additional protections derived from its hydroxylated nature (3, 4). Ectoines are synthesized from aspartate semialdehyde. First, this metabolite is converted into diaminobutyric acid, which is acetylated to Nγ-acetyldiaminobutyric acid and subsequently cycled to ectoine (5, 6). The main route of hydroxyectoine synthesis is via ectoine hydroxylation (2). The enzymes for ectoine synthesis are usually encoded in an *ectABC*-type gene cluster, which is usually well conserved among ectoine-producing microorganisms (7). There are exceptions, such as incomplete operons, gene clusters including the *ask* gene (for the aspartate kinase), gene clusters carrying *ectABC-ectD-ask*, and scattering of the genes within the chromosome, with duplications of *ectC* and *ectD* or even solitary *ectC* (5, 7, 8). Industrial production of hydroxyectoine uses *Halomonas elongata* ATCC 33173^T grown under high-salinity and high-temperature conditions, using the bacterial milking method (9), or a derivative of this technique (10), followed by separation and purification of ectoine and hydroxyectoine (9). This salt and temperature requirement for hydroxyectoine synthesis is a serious drawback of using natural producers, since fermentation under high temperature and salinity increases production costs and corrodes industrial reactors.

Chromohalobacter salexigens is a halophilic gammaproteobacterium which produces ectoine and hydroxyectoine in response to salt and heat stress, respectively (7). It is easy to grow and its genome sequence is available (http://genome.ornl.gov/microbial/csal/). It has been suggested as an alternative to *H. elongata* (11). In *C. salexigens*, the genes encoding ectoine synthesis lay within a 2.8-kb region encoding the diaminobutyric acid acetyltransferase (EctA), diaminobutyric acid transaminase (EctB), and ectoine synthase (EctC) (12). The microorganism has two paralogs of the enzyme ectoine hydroxylase, EctD and EctE, but EctD is the main responsible enzyme for hydroxyectoine production (2). In *C. salexigens*, the gene cluster *ectABC* and the genes *ectD* and *ectE* are at different loci within the chromosome.

Whereas accumulation of both solutes in *C. salexigens* is maximal during stationary phase, the accumulation of hydroxyectoine is upregulated by salinity and temperature, and the accumulation of ectoine is upregulated by salinity and downregulated by temperature. Thus, hydroxyectoine production and accumulation is maximum at 45°C and 14.5% NaCl, while ectoine accumulation reaches its maximum at 37°C and 17.4% NaCl (2). This regulation occurs, at least in part, at the transcriptional level. The ectoine synthesis genes *ectABC* can be expressed from two promoter regions, one located upstream of *ectA* and composed of four putative promoters (P*ectA1* to P*ectA4* [P*ectA1-4*]) and a second internal promoter located upstream of *ectB* (P*ectB*). *In silico* analysis of the −10 and −35 sequences of these regions showed that P*ectA1* and P*ectA2* may be dependent on the main vegetative factor σ^{70} (and therefore constitutively expressed), whereas P*ectA3* and P*ectB* were similar to σ^S- and σ^{32}-dependent promoters, respectively. In agreement with these predictions, expression of a P*ectA1-4*::*lacZ* fusion was osmoregulated and depended in part on the general stress factor σ^S, whereas P*ectB* was induced by continuous growth at a high temperature (13). On the other hand, the promoter region of *ectD* is composed of two promoters (P*ectD1* and P*ectD2*), and *ectD* expression is both osmo- and thermoregulated (M. Reina-Bueno, unpublished data).

In this work, we have metabolically engineered *C. salexigens* to overproduce hydroxyectoine at low salinity, in a temperature-independent manner. In order to maximize hydroxyectoine production and to minimize its temperature and salinity requirements, we designed transcriptional fusions between the ectoine synthesis genes *ectABC* and the main hydroxyectoine synthesis gene *ectD*, so that the second became transcriptionally controlled by the *ectABC* promoter region. To construct a functional *ectABCD* cassette, we first amplified by PCR a 3,384-bp sequence, including the promoter region upstream of *ectA*, the *ectABC* gene cluster, and the rho-independent terminator downstream of *ectC*,

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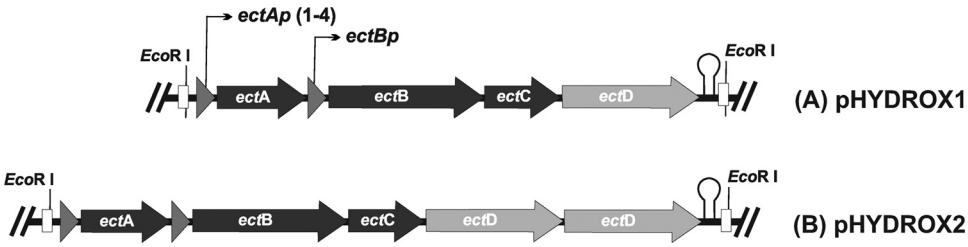

FIG 1 Genetic organization of constructed inserts, cloned in pHS15 to obtain pHYDROX1 (A) and pHYDROX2 (B).

and cloned it into pBluescript SK, resulting in pME2. Then, we inserted a BamHI restriction site between *ectC* and the rho-independent terminator by site-directed mutagenesis using the primer pair ectBam_fw and ectBam_rv (see Table S1 in the supplemental material for a list of primers used in this study). Subsequently, we eliminated the BamHI restriction site from the multicloning site of pBluescript SK using the primers QuitBam_fw and QuitBam_rv, getting pME2.3. Next, we amplified a 1,200-bp sequence from the *C. salexigens* genome, including *ectD*, and inserted it into pBluescript SK, getting the plasmid pECTD. Then, we introduced a BclI restriction site downstream of *ectD* using the primers ectDBcl_fw and ectDBcl_rv, resulting in pECTD2. Subsequently, we excised promoterless *ectD* from pECTD2 by digesting it with BclI and inserted it in BamHI-digested pME2.3, yielding pECTABCD. Finally, the engineered *ectABCD* gene cluster was excised from pECTABCD by digestion with EcoRI and cloned into EcoRI-digested pHS15 (a cloning and expression vector based on a native plasmid from *H. elongata* harboring a streptomycin resistance gene [14]), obtaining pHYDROX1 (Fig. 1A). To increase the *ectD* gene dose, a second functional cassette, *ectABCDD*, was constructed. For this purpose, a second copy of *ectD* was cloned between *ectD* and the rho-independent terminator in the previous *ectABCD*-constructed gene cluster. First, we introduced a BamHI restriction site between *ectD* and the rho-independent terminator in pECTABCD, using the primers ABCDBam_fw and ABCDBam_rv, getting pME2.4. Then, an internal BamHI site of *ectD* was eliminated by introducing a silent mutation with the primers QuitBamD_fw and QuitBamD_rv, resulting in pME2.5. Next, *ectD* was excised from pECTD2 with BclI and cloned in BamHI-digested pME2.5, resulting in pECTABCDD. Subsequently, the *ectABCDD* synthetic gene cluster was excised by digestion with EcoRI from pECTABCDD and cloned in EcoRI-digested pHS15, resulting in pHYDROX2 (Fig. 1B). Both plasmids pHYDROX1 and pHYDROX2 were transformed into *Escherichia coli* DH5α cells, and the resulting strains were used as donors in a conjugation with *C. salexigens* wild type and mutant CHR137 (Δ*ectABC*::Tn*1732*, *ectD*::Ω; unable to synthesize ectoines) (2).

To evaluate hydroxyectoine production in both the natural producer *C. salexigens* and the heterologous host *E. coli* DH5α, we determined the ectoine content in all strains containing pHYDROX1 and pHYDROX2. For this purpose, cells were grown at different temperatures (37°C for *E. coli* and 37, 40, and 45°C for *C. salexigens*) in shaking flasks with minimal medium M63 added with 20 mM glucose and different salinities (1%, 2%, or 3% NaCl for *E. coli* strains and 4.35%, 8.7%, or 14.5% NaCl for *C. salexigens* strains) until early stationary phase. Cellular extracts used for liquid chromatography-mass spectrometry (LC-MS) and supernatants used for high-pressure liquid chromatography with UV detector (HPLC-UV) analysis of ectoine and hydroxyectoine were prepared by using a modified Bligh-Dyer technique described by Kraegeloh and Kunte (15). For cellular extracts, chromatographic separation and HPLC-electrospray ionization was performed as described by Argandoña et al. (16). For supernatants, samples were analyzed as described by García-Estepa et al. (2).

Hydroxyectoine levels observed in the heterologous recombinant strains *E. coli* DH5α/pHYDROX1 and *E. coli* DH5α/pHYDROX2 grown at 1%, 2%, or 3% NaCl were very low, and only traces of ectoine were detected (data not shown). Table 1 summarizes growth rates and ectoine and hydroxyectoine production by the different *C. salexigens* wild-type and recombinant strains, as well as their specific production rates. As production of ectoines is directly related to the biomass produced at a certain salinity (11), the specific production rate (μmol/g bacterial dry matter [BDM] · h) is a simple function reflecting solute content and growth rate (17). As previously reported (2), ectoine accumulation by *C. salexigens* wild type was salinity dependent and, at a given salinity, inversely correlated to increasing temperature. Ectoine reached its maximal accumulation (725 μmol/g BDM) at 37°C with 14.5% of NaCl, with an ectoine/hydroxyectoine ratio of 2.24:1. On the other hand, hydroxyectoine accumulation by the wild type was salinity and temperature dependent, reaching its maximum at 45°C with 14.5% NaCl, with an ectoine/hydroxyectoine ratio of 0.45:1 and a yield of 942 μmol/g BDM.

All *C. salexigens* recombinant strains carrying plasmids pHYDROX1 or pHYDROX2, grown at 37°C or 40°C with 4.35% or 8.7% NaCl, showed much higher hydroxyectoine and much lower ectoine yields, respectively, than the wild-type strain carrying no plasmid under the same conditions. In all cases, the major product was hydroxyectoine, and the higher hydroxyectoine production was accompanied by a decrease of growth rates. This was correlated with the lower ectoine yields observed, confirming that ectoine is necessary for osmoprotection of *C. salexigens*. Otherwise, ectoine production by *C. salexigens* recombinant strains at 14.5% NaCl could not be measured, as they did not grow or showed only residual growth. For a given recombinant strain, growth at 40°C did not result in a significant increase of hydroxyectoine yield, if compared to the same strain grown at 37°C. This finding indicated that the higher hydroxyectoine synthesis in the engineered strains was mostly driven by the P*ectA* promoter region and therefore decoupled from temperature control.

With the exception of cells grown at 40°C with 8.7% NaCl, in the wild-type background the presence of an extra copy of *ectD* (i.e., cells carrying pHYDROX2 versus cells carrying pHYDROX1 grown under the same conditions) did not improve hydroxyecto-

TABLE 1 Ectoine and hydroxyectoine yield and production rates of *C. salexigens* assayed strains[a]

Strain	Temp (°C)	Salinity (%)	Growth rate (μ h^{-1})	Ectoine		Hydroxyectoine	
				Yield (μmol/g BDM)	Specific production rate (μmol/g BDM · h)[b]	Yield (μmol/g BDM)	Specific production rate (μmol/g BDM · h)
C. salexigens DSM3043 wild type	37	4.35	0.24	393 ± 18.5	94.3	59 ± 2.9	14.1
	37	8.7	0.33	654 ± 27.6	215.8	202 ± 7.8	66.6
	37	14.5	0.21	725 ± 22.3	152.2	324 ± 11.3	68
	40	4.35	0.22	180 ± 8.5	39.6	23 ± 0.8	5.06
	40	8.7	0.30	449 ± 9.6	134.7	176 ± 7.8	52.8
	45	14.5	0.15	381 ± 12.2	57.21	942 ± 20	141
Wild type/pHYDROX1	37	4.35	0.14	220 ± 5.4	30.8	483 ± 9.9	67.62
	37	8.7	0.25	156 ± 3.1	39	424 ± 7.5	106
	40	4.35	0.12	48.5 ± 1.2	5.8	468 ± 8.2	56.16
	40	8.7	0.24	20.4 ± 0.4	4.9	255 ± 3.3	61.2
Wild type/pHYDROX2	37	4.35	0.18	68 ± 1.2	12.2	384 ± 6.8	69.12
	37	8.7	0.29	108 ± 2.4	31.3	361 ± 7.3	104.6
	40	4.35	0.17	48.85 ± 0.9	8.3	419 ± 9.3	71.23
	40	8.7	0.24	105 ± 3.4	25.2	354 ± 10.1	84.96
CHR137/pHYDROX1	37	4.35	0.14	81 ± 2.2	11.3	598 ± 12.8	83.72
	37	8.7	0.1	170 ± 4.1	17	632 ± 14.5	63.2
	40	4.35	0.13	36.5 ± 1.4	4.7	478 ± 12.2	62.14
	40	8.7	0.1	90 ± 3.3	9	521 ± 16.4	52.1
CHR137/pHYDROX2	37	4.35	0.14	93 ± 2.9	13	883 ± 18.9	123.62
	37	8.7	0.1	286 ± 5.8	28.6	967 ± 24.1	96.7
	40	4.35	0.12	67.5 ± 3.1	8.1	768 ± 31.2	92.1
	40	8.7	0.11	122.6 ± 4.5	13.48	805 ± 25.2	88.5

[a] Experiments were repeated twice with three independent measurements. The results are averages from the six measurements ± standard deviations.
[b] BDM, bacterial dry matter.

ine yield. In addition, an increase in salinity (i.e., the same recombinant strain grown at 8.7% versus 4.35% NaCl at a given temperature) did not improve hydroxyectoine production in the wild-type background. However, in the ectoine-deficient strain CHR137 (2), incrementing *ectD* dose or salinity did enhanced hydroxyectoine yields. These findings suggest that control mechanisms ruled by endogenous *ectABC* and/or *ectD* genes are somehow repressing hydroxyectoine synthesis in the wild-type background.

The best production results were achieved with strain CHR137 carrying pHYDROX2. This strain grown at 8.7% NaCl reached a hydroxyectoine yield of 967 μmol/g BDM (4.78-fold higher than that achieved by the wild-type strain at this salinity), with a total ectoine yield of 1,253 μmol/g BDM (ectoine/hydroxyectoine ratio of 0.29:1) and a significant decrease of the growth rate (0.1 h^{-1}) that reduced the specific production rate to 96.7 μmol/g BDM · h. However, the same strain grown at 4.35% NaCl showed a hydroxyectoine yield of 883 μmol/g BDM (14.96-fold higher than that observed in the wild-type strain at the same salinity), maintaining a reasonable growth rate (0.14 h^{-1}, with a specific production rate of 123.6 μmol/g BDM · h) and reaching an ectoine/hydroxyectoine ratio of 0.1:1. These production data at low salinity and optimal temperature are very similar to those shown by the wild type grown at high salinity and high temperature (14.5% NaCl and 45°C).

In most bacteria, the responses to osmotic and/or heat stress involve the synthesis of a cocktail of compatible solutes (7). Thus, the presence of by-products other than ectoine was investigated in the most promising strain, CHR137/pHYDROX2, grown at 37°C with low salinity and compared to the cytoplasmic solute pool synthesized by the wild type carrying either pHYDROX2 or no plasmid. As shown in Fig. S1 in the supplemental material, the major compatible solute in *C. salexigens* wild-type cells grown at 37°C with 4.35% NaCl was ectoine, followed by glutamate and minor amounts of glucosylglycerol and hydroxyectoine. Introduction of pHYDROX2 in wild-type cells switched the synthesis to hydroxyectoine, which became the major compatible solute, followed by glutamate, trehalose, and minor amounts of glucosylglycerol and ectoine. The presence of trehalose in the wild type overexpressing hydroxyectoine at 37°C was unexpected, as this sugar is synthesized by *C. salexigens* in response to heat stress or when ectoine is absent (18). This apparent induction of trehalose synthesis by hydroxyectoine will be investigated in a further work. Finally, transfer of pHYDROX2 to the ectoine-deficient strain CHR137 led to a much cleaner compatible solute profile, consisting of mainly hydroxyectoine and reduced amounts of glutamate and trehalose. Ectoine was not detected, although it should be present in minor amounts, as judged by our previous estimations (Table 1).

In general, two alternative biological systems would be suitable to approach hydroxyectoine production: nonhalophilic microorganisms bearing hydroxyectoine synthesis genes and natural (halophilic) producers, either cultured in optimized conditions for solute production or metabolically engineered for hydroxyectoine overproduction (4). Table 2 summarizes most of the so-far-reported hydroxyectoine production systems, including relevant

TABLE 2 Comparison of hydroxyectoine production systems

Strain	Temp (°C)	Salinity (%)	Growth phase	Growth rate ($\mu\ h^{-1}$)	OH-ectoine yield (μmol/g BDM)	Specific OH-ectoine production rate (μmol/g BDM · h)[a]	Byproduct(s)	Reactor system	Product extraction	Reference
Nonhalophilic producers										
E. coli DH5α/pSB01[b]	37	2.0	Late stationary	0.35	500	175	Trehalose ectoine (<5%)	Batch	Methanol-chloroform extraction	17
E. coli FF4169/pNST5[c]	37	1.74	After 16 h of incubation	NR[k]	29.95	NR	Ectoine (10.32 μmol/g BDM), other solutes not tested	Batch	Methanol-chloroform extraction	19
E. coli FF4169/pNST6[d]	37	1.74	After 16 h of incubation	NR	91	NR	Ectoine (107 μmol/g BDM), other solutes not tested	Batch	Methanol-chloroform extraction	19
Natural producers										
Marinococcus sp. strain M52	35	10.0	Stationary	0.25	860	215	Glutamate	Fed-batch-batch[e]	Methanol-chloroform extraction	20
Marinococcus sp. strain M52	37	10.0	Stationary	0.20	670	134		Batch-fed-batch[e]	Thermal permeabilization	21
Marinococcus sp. strain M52	37	10.0	Stationary	0.03	603	18		Batch-fed-batch[e]	Thermal permeabilization	21
Halomonas boliviensis	35	18.5[f]	Stationary	Multistep process	950	169	Ectoine, PHB	Two-step fed-batch	Downshocks	22
Halomonas elongata ATCC 33173^T	25	15	Exponential	0.04	28	NR	Ectoine (1,462 μmol/g BDM), *N*γ-acetyldiamino-butyric acid, glutamate, alanine, other amino acids	Batch-fed-batch	Downshocks	9
Halomonas elongata ATCC 33173^T	30	10.0	Late exponential	0.242	36	8.71	Ectoine (762 μmol/g BDM), glutamate, glucose	Batch	Chloroform extraction	23
Halomonas elongata ATCC 33173^T	40	15	Late exponential	NR	290	NR	Ectoine (740 μmol/g BDM), glutamate, glucose	Batch	Methanol-chloroform extraction	24
Halomonas elongata ATCC 33173^T	40	20	Late exponential	NR	440	NR	Ectoine (860 μmol/g BDM), glutamate, glucose	Batch	Methanol-chloroform extraction	24
Chromohalobacter salexigens DSM 3043^T	37	14.5	Early stationary	0.21	324	68	Ectoine (725 μmol/g BDM), glutamate	Batch	Methanol-chloroform extraction	13; this study
Chromohalobacter salexigens DSM 3043^T	45	14.5	Early stationary	0.15	942	141	Ectoine (381 μmol/g BDM), glutamate, trehalose, *N*γ-acetyldiamino-butyric acid	Batch	Methanol-chloroform extraction	2; this study
Chromohalobacter salexigens DSM 3043^T	37	10.75	Exponential	0.3	2,528	76	Ectoine (3,797 μmol/g BDM), other solutes not determined	Continuous with cell retention[g]	Downshocks	11
Pseudomonas stutzeri DSM5190^T	37	5.0	Exponential	0.16	480	76.8	Ectoine, trehalose, NAGGN	Batch	Methanol-chloroform extraction	17
Pseudomonas stutzeri A1501	37	4.0	Mid-exponential	NR	367	NR	Ectoine, trehalose, NAGGN	Batch	Methanol-chloroform extraction	19
Engineered natural producers										
C. salexigens DSM 3043^T/pJP-2R[h]	37	10	Early stationary	NR	100[i]	NR	0% ectoine, other solutes not determined	Batch	Downshocks	25
C. salexigens DSM 3043^T/pHYDROX2[j]	37	4.35	Early stationary	0.18	384	69.12	Glutamate, trehalose, glycolsylglycerol, ectoine	Batch	Methanol-chloroform extraction	This study
C. salexigens CHR137/pHYDROX2[j]	37	4.35	Early stationary	0.14	883	123.62	Glutamate, trehalose, ectoine (93 μmol/g BDM)	Batch	Methanol-chloroform extraction	This study

[a] Maximum specific hydroxyectoine production rates were calculated on the basis of growth rates and biomass content.
[b] Carrying the *ectABCDask* genes from *P. tsutzeri* DSM 5190^T.
[c] Trehalose-deficient *E. coli* carrying the *ectABCD* genes from *P. stutzeri* A1501.
[d] Trehalose-deficient *E. coli* carrying the *ectABCDasK* genes from *P. stutzeri* A1501.
[e] With medium exchange once, during fed-batch.
[f] Cells were first grown during 24 h at optimal salinity for biomass production (4.5% NaCl) and then transferred to a high-salinity medium (with 18.5% NaCl) for ectoines production.
[g] Calculations for a maximum biomass of 61 g/liter. Fermentation was optimized for a simultaneous production of ectoine and hydroxyectoine.
[h] Carrying the *thpD* (*ectD*) gene, encoding the ectoine hydroxylase from *Streptomyces crysomallus*.
[i] Percentage of ectoine conversion to hydroxyectoine; absolute yield not reported.
[j] Carrying the *ectABCDD* cassette from *C. salexigens* DSM 3043^T.
[k] NR, not reported.

parameters such as growth conditions, hydroxyectoine yield and production rate, by-products, reactor systems, and product extraction procedures used. In this work, attempts to overproduce the *C. salexigens ectABCD* hydroxyectoine synthesis genes in *E. coli* DH5α were unsuccessful. It is possible that, as observed by Bestvater et al. (26) for the heterologous production of the *Marinococcus halophilus* ectoine synthesis genes, our heterologous production system might be improved by coexpressing a feedback-insensitive aspartate kinase. This metabolic bottleneck was not found in the two reports so far describing successful hydroxyectoine production by *E. coli* carrying the hydroxyectoine synthesis genes from *Pseudomonas stutzeri*. However, differences in the osmotolerance conferred to the host strain, hydroxyectoine yield, presence of by-products, and the dependence of coexpresion of the *ask* gene for enhanced ectoine/hydroxyectoine production were found (17, 19) (Table 2), Thus, the trehalose-deficient *E. coli* strain FF4169 bearing the *P. stutzeri* A1501 *ectABCD* genes accumulated moderate levels of ectoine and hydroxyectoine, and the introduction of the *ask* gene (encoding an aspartate kinase specialized for ectoine/hydroxyectoine synthesis) led to a very strong increase in the contents of both solutes (Table 2). In both cases, host cells were osmoprotected (19). In contrast, *E. coli* DH5α carrying the *ectABCDask* gene cluster from *P. stutzeri* DSM 5190^T synthesized almost exclusively hydroxyectoine as compatible solute (with about 5% of ectoine and trehalose), although conversion of ectoine into hydroxyectoine was delayed until late stationary phase. Surprisingly, this strain was not osmotolerant, and enhanced hydroxyectoine production was not dependent on coexpression of the aspartate kinase (17). Despite all this, hydroxyectoine content was much higher than that observed in the natural producer, with a yield of 500 μmol/g BDM (at stationary phase) and a specific production rate of 175 μmol/g BDM · h at 37°C and 2% NaCl. Compared to this strain, the specific hydroxyectoine production rate of *C. salexigens* CHR137/pHYDROX2 grown at the same temperature with 4.35% NaCl was slightly lower [123.6 μmol/g BDM · h], but the hydroxyectoine yield was much higher (883 μmol/g BDM) (Table 2).

For the industrial production of hydroxyectoine, robust microorganisms with a broad salt tolerance are favored to perform the bacterial milking process (3, 4). Methods based on the Gram-positive *Marinococcus* sp. strain M52 (20, 21) yielded considerable hydroxyectoine amounts at 37°C (Table 2) but had two disadvantages: (i) they cannot be milked by simple dilution of the medium, and (ii) they produce growth-inhibiting components such as acetate, which impede their use in batch and batch fermentation processes, unless complex techniques are utilized. Among Gram-negative bacteria of the *Halomonadaceae* family, *C. salexigens* (2), *H. boliviensis* (22), and *H. elongata* (9, 24) naturally produce more ectoine than hydroxyectoine, and increasing the hydroxyectoine content implies high-temperature and -salinity growth conditions (Table 2), with the disadvantages that these extreme conditions have for any industrial production system. In contrast, in *P. stutzeri*, hydroxyectoine is the predominant compatible solute at normal temperature (Table 2), and this microorganism has been suggested as an interesting candidate for the biotechnological production of hydroxyectoine (19).

At present, hydroxyectoine is produced on an industrial scale with *H. elongata* ATCC 33173^T (same as *H. elongata* DSM 2581^T). Both *C. salexigens* DSM 3043^T (strain 1H11; formerly named *H. elongata* DSM 3043) and *H. elongata* ATCC 33173^T (strain1H9) were isolated by Vreeland et al. from a solar salt facility at Bonaire Island and initially assigned to *H. elongata* (27). On the basis of their phenotypic differences and their phylogenetic distance, strain *H. elongata* DSM 3043 was proposed as a new species of the genus *Chromohalobacter* and designated *C. salexigens* (7). Although both species show a similar temperature range (from 15 to 45°C, with optimum at 37°C) and optimal salinity (8.7 to 11.6% NaCl) for growth (28, 29), *C. salexigens* DSM 3043 seems to have more stringent requirements for salt. Thus, while the *H. elongata* type strain grew well with 0.3% NaCl in a minimal medium which is similar to M63 (29), *C. salexigens* could not grow at all in M63 unless it contained 2.9% NaCl (28). There are few reports describing hydroxyectoine production by *H. elongata*. Early studies by Wohlfarth et al. (24) and Severin et al. (23) showed increasing hydroxyectoine content in *H. elongata* in response to salinity and temperature, with 290 μmol/g BDM of hydroxyectoine (and 740 μmol/g BDM of ectoine) in cells grown in glucose mineral medium at 40°C with 15% NaCl (Table 2). This yield is lower than the hydroxyectoine accumulated by the *C. salexigens* wild-type strain at 37 or 45°C with a similar salinity (Table 2). Unfortunately, hydroxyectoine production by *H. elongata* at temperatures higher than 40°C or specific hydroxyectoine production rates at 40°C or higher were not reported, making it difficult to compare production data among the two wild-type strains.

In this study, we describe hydroxyectoine overproduction by using recombinant strains of the natural producer *C. salexigens* genetically engineered (i) to coexpress, in a native-plasmid-based vector, the ectoine and hydroxyectoine genes under the control of the *ectA* promoter region, (ii) to improve the first approach by increasing the copies of *ectD*, and (iii) to further improve the two first designs by using an ectoine-deficient mutant as the genetic background. As stated above, transcriptional regulation of the *C. salexigens ectABC* genes for ectoine synthesis is rather complex, with a total of four promoters regulating *ectABC* transcription (two putative σ^{70}-dependent promoters, one σ^S-controlled promoter, and a fourth promoter of unknown specificity) and one putative σ^{32}-dependent promoter driving *ectBC* expression. This multiplicity of promoters allows the cells to respond to many environmental stimuli such as high salinity and temperature and the presence of iron, external osmoprotectants, or the DNA gyrase inhibitor nalidixic acid (13). In *H. elongata* ATCC 33173^T, Schwibbert et al. (30) found a different but also complex promoter assembly, with two transcriptional initiation sites upstream of *ectA* (corresponding to putative σ^{70}- and σ^S-dependent promoters), and a third one mapped immediately upstream of *ectC*, resembling σ^{54}-controlled promoters. Based on these findings, the authors suggested that ectoine synthesis in *H. elongata* could be regulated not only by salinity but also by nitrogen supply. In addition, in the presence of the external osmoprotectant betaine, ectoine accumulation is totally abolished in *C. salexigens* (28) but not in *H. elongata* (24), reflecting an apparently different regulation of ectoine production in both organisms. In summary, with the available data, it is difficult to predict if the same strategy followed in this study would efficiently work in *H. elongata*.

As stated above, the presence of by-products in any strain devoted to industrial production of hydroxyectoine is undesirable, as it would increase production costs (4). All strains depicted in Table 2 that have been tested for the presence of other solutes show from trace (i.e., *E. coli* DH5α/pSB01) to moderate (i.e., *H. elongata*, *C. salexigens*, or *P. stutzeri* wild-type strains) amounts of

other contaminating solutes. Therefore, downstream processes involving a certain purification step are mostly unavoidable. For the most promising strain, CHR137/pHYDROX2, hydroxyectoine was the main product, which would be useful to reduce separation and purification costs. In any case, separation of hydroxyectoine from trehalose and glutamate is easy by simple chromatographic techniques (3, 9).

Our findings indicate the superiority of the *C. salexigens* ectoine-minus strains over the wild-type strain for hydroxyectoine production, including much better yields and specific production rates than the wild type. Production achieved with strain CHR137/pHYDROX2 growing at a relatively low salinity, 4.35% NaCl, exceeds that of most other strains reported elsewhere (Table 2). Its specific hydroxyectoine production yield is of the same order as that of heterologous production by *E. coli* DH5α/pSB01 at 2% NaCl (17), and the absolute yield is much higher. In addition, conversion of ectoine to hydroxyectoine by *E. coli*/pSB01 was much delayed until late stationary phase, whereas in *C. salexigens* production was maximal at early stationary phase. Nevertheless, *E. coli* DH5α/pSB01 has the advantage of yielding purer hydroxyectoine. In addition, due to its natural ability to cope with strong changes in medium osmolarity, *C. salexigens* is a much more robust strain than *E. coli* for the industrial bacterial milking process, and therefore there is much room for improvement of hydroxyectoine production, for instance by continuous fermentation coupled to product extraction by osmotic downshocks. Thus, considering all relevant yield parameters, the *C. salexigens* recombinant strains are promising candidates for the biotechnological production of hydroxyectoine.

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REFERENCES

1. **Bursy J, Kuhlmann AU, Pittelkow M, Hartmann H, Jebbar M, Pierik AJ, Bremer E.** 2008. Synthesis and uptake of the compatible solutes ectoine and 5-hydroxyectoine by *Streptomyces coelicolor* A3(2) in response to salt and heat stresses. Appl. Environ. Microbiol. **74**:7286–7296.
2. **García-Estepa R, Argandoña M, Reina-Bueno M, Capote N, Iglesias-Guerra F, Nieto JJ, Vargas C.** 2006. The *ectD* gene, which is involved in the synthesis of the compatible solute hydroxyectoine, is essential for thermoprotection of the halophilic bacterium *Chromohalobacter salexigens*. J. Bacteriol. **188**:3774–3784.
3. **Lentzen G, Schwartz T.** 2006a. Extremolytes: natural compounds from extremophiles for versatile applications. Appl. Microbiol. Biotechnol. **72**:623–634.
4. **Pastor JM, Salvador M, Argandoña M, Bernal V, Reina-Bueno M, Csonka LN, Iborra JL, Vargas C, Nieto JJ, Cánovas M.** 2010. Ectoines in cell stress protection: uses and biotechnological production. Biotechnol. Adv. **6**:782–801.
5. **Louis P, Galinski EA.** 1997. Characterization of genes for the biosynthesis of the compatible solute ectoine from *Marinococcus halophilus* and osmoregulated expression in *Escherichia coli*. Microbiology **143**:1141–1149.
6. **Peters P, Galinski EA, Trüper H.** 1990. The biosynthesis of ectoine. FEMS Microbiol. Lett. **71**:157–162.
7. **Vargas C, Argandoña M, Reina-Bueno M, Rodríguez-Moya J, Fernández-Aunión C, Nieto JJ.** 2008. Unravelling the adaptation responses to osmotic and temperature stress in *Chromohalobacter salexigens*, a bacterium with broad salinity tolerance. Saline Syst. **4**:14.
8. **Kurz M, Burch AY, Seip B, Lindow SE, Gross H.** 2010. Genome-driven investigation of compatible solute biosynthesis pathways of *Pseudomonas syringae* pv. syringae and their contribution to water stress tolerance. Appl. Environ. Microbiol. **76**:5452–5462.
9. **Sauer T, Galinski EA.** 1998. Bacterial milking: a novel bioprocess for production of compatible solutes. Biotechnol. Bioeng. **57**:306–313.
10. **Lentzen G, Schwartz T.** 2006b. Kompatible solute: mikrobielle herstellung und anwendung, p 355–371. *In* Antranikian G (ed), Angewandte mikrobiologie. Springer-Verlag, Berlin, Germany.
11. **Fallet C, Rohe P, Franco-Lara E.** 2010. Process optimization of the integrated synthesis and secretion of ectoine and hydroxyectoine under hyper/hypo-osmotic stress. Biotechnol. Bioeng. **107**:124–133.
12. **Cánovas D, Vargas C, Calderón MI, Ventosa A, Nieto JJ.** 1998. Characterization of the genes for the biosynthesis of the compatible solute ectoine in the moderately halophilic bacterium *Halomonas elongata* DSM 3043. Syst. Appl. Microbiol. **21**:487–497.
13. **Calderón MI, Vargas C, Rojo F, Iglesias-Guerra F, Csonka LN, Ventosa A, Nieto JJ.** 2004. Complex regulation of the synthesis of the compatible solute ectoine in the halophilic bacterium *Chromohalobacter salexigens* DSM 3043T. Microbiology **150**:3051–3063.
14. **Vargas C, Fernández-Castillo R, Cánovas D, Ventosa A, Nieto JJ.** 1995. Isolation of cryptic plasmids from moderately halophilic eubacteria of the genus *Halomonas*. Characterization of a small plasmid from *H. elongata* and its use for shuttle vector construction. Mol. Gen. Genet. **246**:411–418.
15. **Kraegeloh A, Kunte HJ.** 2002. Novel insights into the role of potassium for osmoregulation in *Halomonas elongata*. Extremophiles **6**:453–462.
16. **Argandoña M, Nieto JJ, Iglesias-Guerra F, Calderón MI, García-Estepa R, Vargas C.** 2010. Interplay between iron homeostasis and the osmotic stress response in the halophilic bacterium *Chromohalobacter salexigens*. Appl. Environ. Microbiol. **76**:3575–3589.
17. **Seip B, Galinski EA, Kurz M.** 2011. Natural and engineered hydroxyectoine production based on the *Pseudomonas stutzeri ectABCD-ask* gene cluster. Appl. Environ. Microbiol. **77**:1368–1374.
18. **Reina-Bueno M, Argandoña M, Salvador M, Rodríguez-Moya J, Iglesias-Guerra F, Csonka LN, Nieto JJ, Vargas C.** 2012. Role of trehalose in salinity and temperature tolerance in the model halophilic bacterium *Chromohalobacter salexigens*. PLoS One **7**:e33587. doi:10.1371/journal.pone.0033587.
19. **Stöveken N, Pittelkow M, Sinner T, Jensen RA, Heider J, Bremer E.** 2011. A specialized aspartokinase enhances the biosynthesis of the osmoprotectants ectoine and hydroxyectoine in *Pseudomonas stutzeri* A1501. J. Bacteriol. **193**:4456–4468.
20. **Frings E, Sauer T, Galinski EA.** 1995. Production of hydroxyectoine: high cell-density cultivation and osmotic downshock of *Marinococcus* strain M52. J. Biotechnol. **43**:56–63.
21. **Schiraldi C, Maresca C, Catapano A, Galinski EA, De Rosa M.** 2006. High-yield cultivation of *Marinococcus* M52 for production and recovery of hydroxyectoine. Res. Microbiol. **157**:693–699.
22. **Van-Thuoc D, Guzman H, Guillaguaman J, Hatti-Kaul R.** 2010. High productivity of ectoines by *Halomonas boliviensis* using a combined two-step fed-batch culture and milking process. J. Biotechnol. **147**:46–51.
23. **Severin J, Wohlfarth A, Galinski EA.** 1992. The predominant role of recently discovered tetrahydropyrimidines for the osmoadaptation of halophilic eubacteria. J. Gen. Microbiol. **138**:1629–1638.
24. **Wohlfarth A, Severin J, Galinski EA.** 1990. The spectrum of compatible solutes in heterotrophic halophilic eubacteria of the family *Halomonadaceae*. J. Gen. Microbiol. **136**:705–712.
25. **Prabhu J, Schauwecker F, Grammel N, Keller U, Bernhard M.** 2004. Functional expression of the ectoine hydroxylase gene (*thpD*) from *Streptomyces chrysomallus* in *Halomonas elongata*. Appl. Environ. Microbiol. **70**:3130–3132.
26. **Bestvater T, Louis P, Galinski EA.** 2008. Heterologous ectoine production in *Escherichia coli*: by-passing the metabolic bottle-neck. Saline Syst. **4**:12.
27. **Vreeland RH, Litchfield CD, Martin EL, Elliot E.** 1980. Halomonas elongata, a new genus and species of extremely salt-tolerant bacteria. Int. J. Syst. Bacteriol. **30**:485–495.
28. **Cánovas D, Vargas C, Csonka LN, Ventosa A, Nieto JJ.** 1996. Osmoprotectants in Halomonas elongata: high-affinity betaine transport system and choline-betaine pathway. J. Bacteriol. **178**:7221–7226.
29. **Vreeland RH, Martin EL.** 1980. Growth characteristics, effects of temperature, and ion specificity of the halotolerant bacterium *Halomonas elongata*. Can. J. Microbiol. **26**:746–752.
30. **Schwibbert K, Marin-Sanguino A, Bagyan I, Heidrich G, Lentzen G, Seitz H, Rampp M, Schuster SC, Klenk HP, Pfeiffer F, Oesterhelt D, Kunte HJ.** 2011. A blueprint of ectoine metabolism from the genome of the industrial producer *Halomonas elongata* DSM 2581^T. Environ. Microbiol. **13**:1973–1994.