APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 2007, p. 3343–3347 Vol. 73, No. 10
0099-2240/07/$08.00+0 doi:10.1128/AEM.02482-06

Continuous Synthesis and Excretion of the Compatible Solute Ectoine by a Transgenic, Nonhalophilic Bacterium▽

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Received 24 October 2006/Accepted 12 March 2007

The compatible solute 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid (ectoine) acts in microorganisms as an osmotic counterweight against halostress and has attracted commercial attention as a protecting agent. Its production and application are restricted by the drawbacks of the discontinuous harvesting procedure involving salt shocks, which reduces volumetric yield, increases reactor corrosion, and complicates downstream processing. In order to synthesize ectoine continuously in less-aggressive media, we introduced the ectoine genes *ectABC* of the halophilic bacterium *Chromohalobacter salexigens* into an *Escherichia coli* strain using the expression vector pASK-IBA7. Under the control of a *tet* promoter, the transgenic *E. coli* synthesized 6 g liter^{-1} ectoine with a space-time yield of 40 mg liter^{-1} h^{-1}, with the vast majority of the ectoine being excreted.

Halophilic microorganisms live in highly saline environments. There are two major strategies of adaptation to these hostile conditions. Extreme halophiles such as *Halobacterium salinarum* accumulate salt in the cytosol to maintain the osmotic balance (the salt-in strategy). Other halophiles synthesize and accumulate small organic molecules as osmotic counterweights (the organic-osmolyte strategy). Unlike intracellular salt, the small organic compounds do not affect the metabolism of the organism and are thus called compatible solutes. The enzymes of organic-osmolyte strategists do not need to be haloadapted, allowing these organisms to cope with strong salinity fluctuations. Non-salt-tolerant bacteria like *Escherichia coli* are usually unable to synthesize large amounts of compatible solutes but may resist halostress to a certain extent by taking up and accumulating compatible solutes (8, 9, 12, 27).

Different classes of chemical compounds, including polyols, sugars, methylamines, and linear and cyclic amino acids and betaines, have been found to act as compatible solutes. Besides functioning as osmotic counterweights, compatible solutes were shown to protect biomolecules and whole cells against denaturation caused by heating, freezing, desiccation, or chemical agents (15, 19, 22). This property has attracted commercial attention. Compatible solutes can be used as chemical chaperones for protein folding, enhancers of PCR performance, cryoprotectants of microorganisms, cosmeceuticals, and pharmaceuticals (20, 32). A potentially promising future application could be the enhancement of drought tolerance or salt tolerance of transgenic plants (1, 37).

The best-investigated compatible solute, 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid (ectoine), is biotechnologically produced with the halophilic bacterium *Halomonas elongata* (34). The physiology and genetics of ectoine biosynthesis in this bacterium have been studied in detail (7, 23, 24, 25, 26, 29). The technical bioprocess for the synthesis of ectoine exploits the salt adaptation strategy of *H. elongata* and is called "bacterial milking" (34). When the medium has a high salt concentration, the bacterium synthesizes and accumulates ectoine. After an osmotic down-shock, the cells counteract bursting by the sudden ejection of the ectoine. The technical process involves the cyclic increase and decrease of the salt concentration for ectoine production and ectoine milking, respectively. The weaknesses of the process are its high demand for the stability of the bioreactor materials and the difficult downstream processing of the product due to the discontinuous production scheme and high concentrations of salt. These lead to a relatively high price for ectoine, preventing some potential applications as a protector molecule.

To reduce the salt requirement, attempts were made to introduce the ectoine genes of halophiles into a nonhalophilic bacterium (*E. coli*). This recombinant strain synthesizes ectoine as a result of modest salt stress (21). The accumulation of ectoine in nonhalophilic strains by IPTG (isopropyl-β-D-thiogalactopyranoside)-induced gene expression has also been reported (10, 11, 16, 17). However, the heterologous synthesis of ectoine occurred only at such a low level that ectoine was undetectable extracellularly (10, 11, 16, 17, 21). In contrast, ectoine produced at higher rates should be ejected to maintain the osmotic equilibrium in low-saline medium via unspecific mechanosensitive channels. Mechanosensitive channels are known to act instantly upon osmotic down-shocks. These channels are found in the cell membranes of most microorganisms and are well known to extrude different intracellular solutes, e.g., potassium, amino acids, saccharides, and polyols (3, 5, 30, 35). Our reasoning was thus that a more powerful externally induced expression system should modify *E. coli* in such a way that the strain synthesizes high levels of ectoine, which is continuously excreted into the medium to prevent cell bursting.

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▽ Published ahead of print on 16 March 2007.

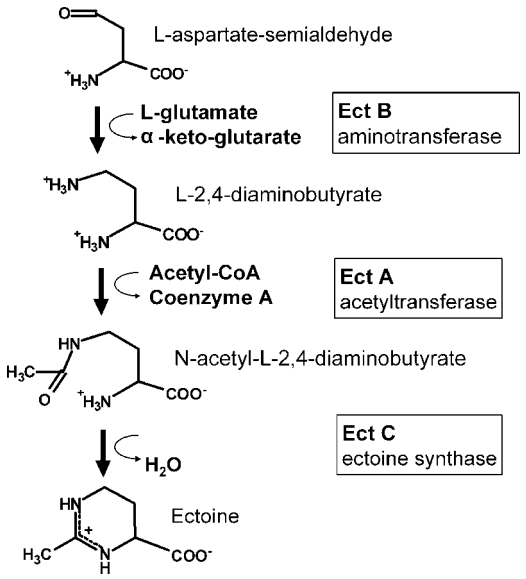

FIG. 1. Biosynthesis of ectoine in *Chromohalobacter salixigens*. The genes *ectB*, *ectA*, and *ectC* encoding aminotransferase, acetyltransferase, and ectoine synthase, respectively, were introduced into *E. coli*.

The goal of our study was to engineer such a strain and to investigate its potential benefits for the continuous production of ectoine under low-salinity conditions.

MATERIALS AND METHODS

Strains and media. *E. coli* DH5α [F^- ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*], as the host strain for bacterial transformation and plasmid propagation, was cultivated in Luria-Bertani medium or in a defined medium (DM) composed of the following (concentrations in mg $liter^{-1}$): $(NH_4)_2SO_4$ (6,000), K_2HPO_4 (4,400); KH_2PO_4 (3,400), $CaCl_2 \cdot 6H_2O$ (90), $MgCl_2 \cdot 6H_2O$ (1,100), glucose (10,000), ZnCl (3.5), $MnCl_2$ (0.46), $CuCl_2$ (7), $Na_2MoO_4 \cdot 2H_2O$ (4.2), $FeCl_2$ (38). The antibiotic ampicillin (100 mg $liter^{-1}$) was used to maintain the heterologous plasmid in the genetically modified strain.

Molecular biological methods. The *ectABC* gene cassette from *Chromohalobacter salexigens* DSM 3043 was used for the construction of the expression vector. *ectA* encodes diaminobutyrate acetylase, *ectB* encodes diaminobutyrate transaminase, and *ectC* encodes ectoine synthase (Fig. 1).

The *ectABC* gene cassette was amplified by PCR using the oligonucleotides 5′-ATG ACG CCT ACA ACC GAG AAC TTC A-3′ and 5′-TCA ATC GAC CGG TGC GTA-3′ and chromosomal DNA of *C. salexigens* as templates. The complete operon containing the genes *ectA*, *ectB*, and *ectC* was amplified without any terminator, operator, or promoter regions. The PCR fragments were cloned into cloning vector pCR2.1 by using the TA cloning kit (Invitrogen, Karlsruhe, Germany). The recombinant vector was transformed into *E. coli* DH5α. Plasmid DNA of the *E. coli* transformants was prepared as described by Birnboim and Doly (4). The BamHI-XbaI fragment from the recombinant cloning vector containing the *ectABC* gene cassette was recloned in the expression plasmid pASK-IBA7 (Fig. 2). The gene cassette was inserted downstream of the *tet* promoter. The resulting vector, pASK-*ectABC*, was transformed in *E. coli* DH5α. The transformation of *E. coli* followed the procedure described by Hanahan (13).

Bioreactor cultivation. Precultures of *E. coli* DH5α(pASK-*ectABC*) were prepared in shaking flasks (30°C, 120 rpm) with DM containing ampicillin. The bioreactor was inoculated with exponentially growing bacteria. Batch cultivations were performed in a Biostat MD bioreactor (Sartorius BBI System GmbH, Melsungen, Germany) with a 1.5-liter working volume at 30°C and a pH of 7.0 ± 0.05, maintained by the automated addition of either 2.7 M NH_4OH or 0.1 M H_2SO_4 as appropriate. The stirrer speed was 1,000 rpm and the aeration rate 3.0 liters min^{-1} (at a standard ambient temperature of 298.15 K and a standard ambient pressure of 10^5 Pa).

After the culture had reached a biomass concentration of 20 g $liter^{-1}$, anhydrotetracycline was added (final concentration, 2 mg $liter^{-1}$) to induce the expression of the ectoine genes. Glucose was added periodically after depletion.

Analyses. The biomass was measured spectrophotometrically at 700 nm after calibration to bacterial dry mass. The concentration of glucose was measured by high-performance liquid chromatography (HPLC) using a Nucleosil carbohydrate column (Nucleogel 300 OA; Macherey-Nagel, Düren, Germany) with 0.01 N sulfuric acid in an isocratic eluent at 70°C (flow rate, 0.6 ml min^{-1}). The refraction index detection system was used. To determine the ectoine concentration, isocratic HPLC using an NH_2 column (Nucleosil 100-5 NH_2; Macherey-Nagel) at 70°C with an acetonitrile-water (85%, vol/vol) solution as the mobile phase at a flow rate of 2.0 ml min^{-1} was applied. UV detection at 225 nm was used. Amino acids as well as a few other compatible solutes were quantified, and the ectoine measurements were confirmed by an HPLC analysis with pulsed amperometric detection as described by Riis et al. (31). The protein concentrations of the supernatant were measured by the method of Bradford (6), using bovine serum albumin as the standard.

Protein separation and identification. Cell extracts of *E. coli* were prepared as described previously (2). Fifty micrograms of acetone-precipitated protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18). Gels were stained with colloidal Coomassie brilliant blue and dried in a stream of unheated air. For mass spectrometric protein identification, bands of interest were excised, digested in gels with trypsin, and prepared for mass spectrometry (MS) (33). The samples were analyzed using an atmospheric pressure (AP) MALDI/TRAP-XCT mass spectrometer (Agilent Technologies, Palo Alto, CA) in automatic tandem MS (MS-MS) mode. The resulting MS-MS data were used for a database search with Mascot (Matrix Science) (28) against the NCBI database.

The assay for the acylation activity of EctA uses a coupled spectrometric test at 412 nm and contains 0.3 mM Ellman's reagent [5,5′-dithiobis (2-nitrobenzoic acid)] in 60 mM Tris-HCl (pH 8.5), 0.4 mM NaCl, 2 mM acetyl-coenzyme A, and 30 mM diaminobutyrate.

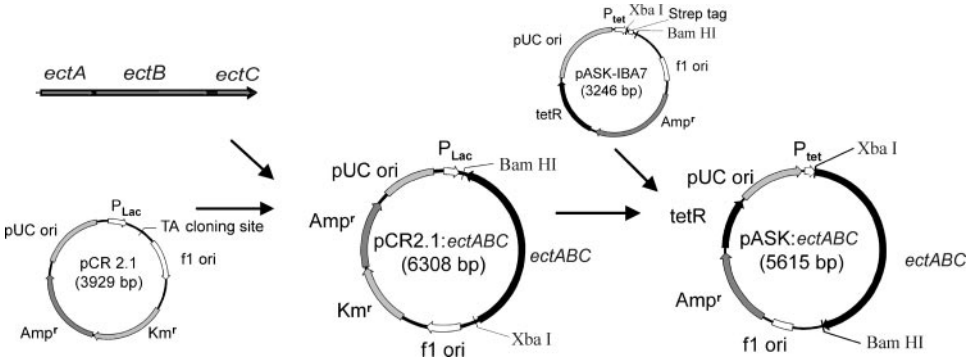

FIG. 2. Design of the expression vector pASK-*ectABC*. The *ectABC* gene cassette from *Chromohalobacter salexigens* was initially inserted into pCR2.1 by PCR cloning using the TA cloning kit (Invitrogen) and then subcloned into the expression vector pASK-IBA7 with Amp^r as the ampicillin resistance gene and *tetR* as the repressor gene of the *tet* promoter P_{tet}.

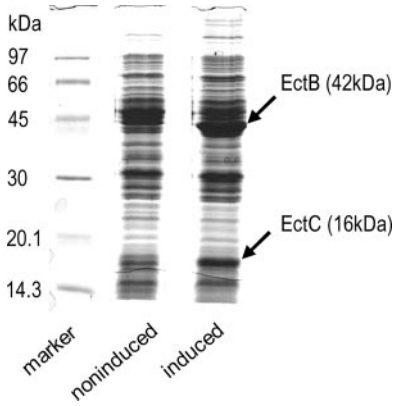

FIG. 3. SDS-PAGE of noninduced and induced cells of *E. coli*(pASK-*ectABC*). Arrows mark amplified bands after 24 h of induction. After the amplified bands were analyzed by MS, the bands were identified by a data bank search as the EctB and EctC proteins of *C. salexigens*.

RESULTS AND DISCUSSION

Expression of the ectoine genes in *E. coli* and product synthesis. The expression vector pASK-IBA7 carrying the *ectABC* gene cassette of *C. salexigens* downstream of the inducible promoter (Fig. 2) was transformed in *E. coli* DH5α. To verify the expression of the ectoine genes in the transgenic *E. coli*, they were induced with anhydrotetracycline and the activity of EctA was measured. The catalytic activity of EctA in lysates of the induced cells was 8 mU mg^{-1}, in contrast to 2 mU mg^{-1} in lysates of noninduced cells. Due to the lack of appropriate assays, the specific activities of EctB and EctC could not be measured.

In order to compare the expression levels of *ectB* and *ectC* genes, extracts of induced and noninduced *E. coli* DH5α cells were used to visualize the respective proteins by SDS-PAGE. Lysates of induced cells showed two clearly amplified bands with molecular masses (42 kDa and 16 kDa) similar to those published for EctB and EctC (44 kDa and 19 kDa, respectively) (26). MS of tryptic digests of the 42-kDa band and the 16-kDa band identified EctB of *Chromohalobacter salexigens* DSM 3034 (five peptides, 20% sequence coverage; gi¦67676419) and EctC of *C. salexigens* DSM 3034 (six peptides, 65% sequence coverage; gi¦67519532), respectively. In each case, *C. salexigens* best matched the obtained peptide sequence in database searches. The heterologous expression of the ectoine genes *ectB* and *ectC* in *E. coli* was thus successful, and protein synthesis was amplified upon gene induction.

Due to the very similar masses, it is possible that EctA (19 kDa) comigrates with EctC in the 19-kDa band, and so no peptide matching EctA was detected by MS analysis. It is conceivable that the instability of EctA, already reported by Ono et al. (26), was responsible for the failure to identify EctA in the protein band containing EctC (Fig. 3).

The successful expression of *ectABC* in *E. coli* and the catalytic function of the three ectoine enzymes were also confirmed by the detection of ectoine by two different HPLC detection methods.

Growth and ectoine synthesis in recombinant *E. coli*. The use of an inducible promoter permitted the separation of an initial phase of biocatalyst production from a subsequent phase

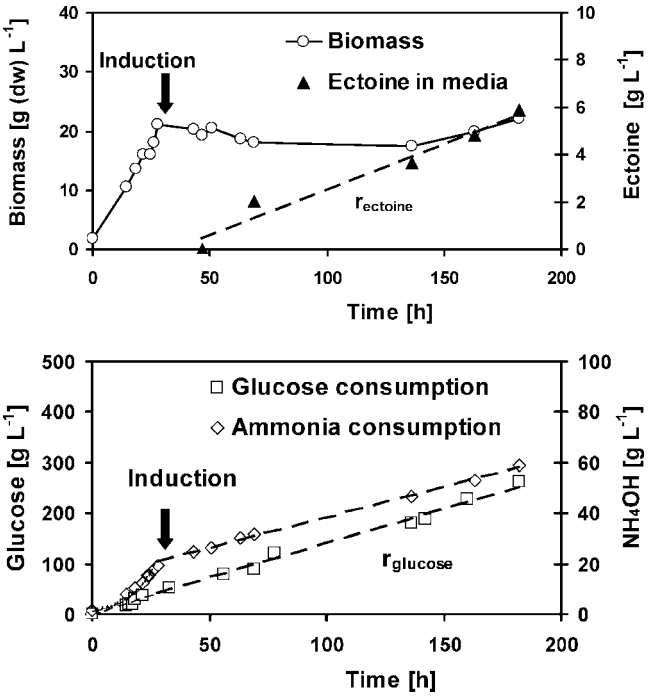

FIG. 4. Cultivation and ectoine synthesis in *E. coli* (pASK-*ectABC*) in a bioreactor (1.5-liter working volume) with DM at 30°C and pH 7.0. After 22 h (marked by an arrow), anhydrotetracycline was added to induce the expression of the ectoine genes. Metabolic fluxes in the induction phase are determined for ectoine synthesis ($r_{ectoine}$ = 0.040 g $liter^{-1}$ h^{-1}) and for the corresponding glucose consumption ($r_{glucose}$ = 1.4 g $liter^{-1}$ h^{-1}) (curves are approximated to a linear rate).

of ectoine synthesis. In the absence of the inducer in the first phase, the bacterium grew exponentially at a rate of 0.09 h^{-1} up to a biomass concentration of 22 g $liter^{-1}$ (Fig. 4). No ectoine was detected during this phase. The second phase was initiated by adding anhydrotetracyline as an inducer. Growth ceased immediately, and ectoine was synthesized and excreted at a rate of 40 mg $liter^{-1}$ h^{-1}. After an induction time of 160 h, the concentrations of ectoine and biomass were 6.0 and 22 g $liter^{-1}$, respectively. There was no indication of declining ectoine excretion up to this time. The ratio of extracellular ectoine to biomass of 0.27 exceeded the maximum ratio of intracellular ectoine to biomass of 0.2 obtained by halophilic strains, e.g., *H. elongata* (23), at this time. The specific ectoine synthesis rate of our recombinant *E. coli* strain (2 mg g^{-1} h^{-1}) was of the same magnitude as the rate of the production strain *H. elongata* (7.1 mg g^{-1} h^{-1}) (34). The cellular concentrations of ectoine during expression and overproduction were both 5 mg per g (dry weight).

Mechanism of ectoine excretion. As the heterologous ectoine synthesis has no underlying physiological control, the risks of rising internal osmotic pressure and bursting of the bacterial cells exist. Bursting would lead to the discharge of intracellular protein and thus to an increase in the extracellular protein concentration. Before induction of ectoine synthesis, 0.4% (wt/wt) of the total protein (assuming 55% protein content of the biomass [36]) was found extracellularly, in contrast to the 5 to 7% extracellular protein found during the ectoine synthesis after induction with anhydrotetracyline. Thus, it seems that

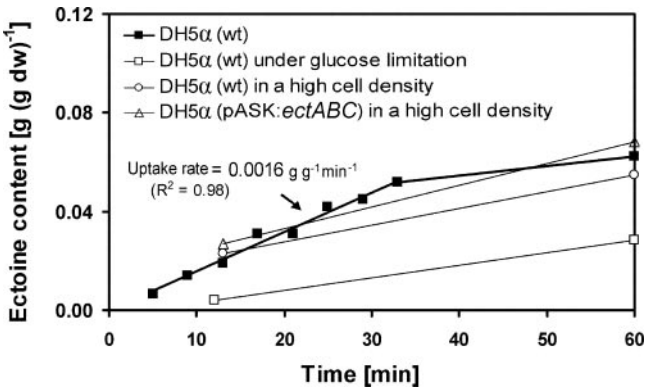

FIG. 5. Uptake of exogenous ectoine (1 mM) from the medium into the cells of *E. coli* DH5α or noninduced *E. coli*(pASK-*ectABC*) under salt stress (final concentration, 4% NaCl). Intracellular ectoine was not found in the absence of exogenous ectoine source under salt stress. Without salt stress, only small intracellular amounts (ca. 0.1%) were detectable. The uptake rate of exogenous ectoine was 1.6 mg g^{-1} (dry weight [dw]) min^{-1}. wt, wild type.

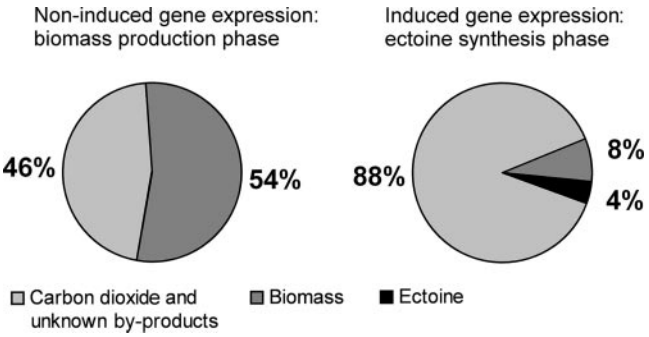

FIG. 6. Carbon balance of the conversion of glucose in moles of C converted into biomass (growth), ectoine, and known by-products (e.g., CO_2 [not measured]) under induced and noninduced conditions.

little protein was released while ectoine was excreted. The excretion of ectoine into the medium could have occurred via the well-investigated unspecific mechanosensitive channels (35) or specific transporters. In the first case, other amino acids should have been extruded as well, since *E. coli*, for instance, contains roughly 100 nmol glutamate per mg cell (dry weight) (35). This means that the glutamate concentration after activation of the mechanosensitive channels could have increased to 2,000 μM. This is far above the sensitivity level of our HPLC method (5 μM). Since we found no significant amounts of amino acids in the medium, we suppose that ectoine had been released by a specific efflux system. Osmoregulated secondary transporters for uptake of the compatible proline, glycine, betaine, and ectoine from the surrounding media are found in *E. coli* (14). Our own experiments showed that *E. coli* DH5α is able to take up ectoine when the salinity of the medium increases. This transport is independent from the genetic modifications as well as from the cultivation conditions (Fig. 5). Furthermore, Poolman and Glaasker (30) have described that such an osmotransporter can also function unidirectionally when activated by a slight increase in turgor pressure. It is therefore conceivable that the recombinant *E. coli* discharged ectoine by a specific transporter.

Furthermore, the DNA sequence between the *tet* promoter and the *ectA* start codon was analyzed as a 50-bp fragment with the sequence 5′- TTT GTA GCA CAA AGC TGA AAT GAA TAG TTC GAC AAA CAT CTA GCA TGC AT-3′. No additional functional genes that could be involved in the transport of ectoine were found.

Potential for further improvement of the biocatalyst. Figure 4 shows that the growth and production phases can be well controlled by using an anhydrotetracycline promoter. During the growth phase, 54% of the substrate carbon (C-mol) flowed into the growth and multiplication phases (Fig. 6). Although the ectoine synthesis rate and the ectoine/biomass ratio are promising indicators for the biotechnological application of our recombinant strain, the channeling of 88% of the carbon substrate into carbon dioxide and unknown by-products opens up opportunities for further strain optimizations. The main goal should be the search for metabolic reasons for the high catabolic rate and the low ectoine yield coefficient. Furthermore, other promoters should be tested as substitutes for the antibiotic anhydrotetracycline, since other inducers could be cheaper and less risky in terms of the induction of resistances or the contamination of the product. Another target for strain improvement is the integration of the heterologous genes into the chromosome to improve their stability in the host organism.

We have described here for the first time an engineered *E. coli* strain carrying the ectoine genes of the halophilic bacterium *Chromohalobacter salexigens.* The recombinant *E. coli* strain is promising for industrial application because it produces ectoine at high rates and excretes the product into the medium so that it can be easily separated from the biocatalyst. The observed low biomass formation during ectoine synthesis is rather advantageous for a potential bioprocess with immobilized cells. The established bioproduction of ectoine relies on cell recovery by cross-flow filtration that bears the risk of blockage. However, the main weaknesses of the conventional bioprocess, i.e., the complex downstream processing with extreme fluctuations in salinity and the resulting corrosiveness of the medium, can be overcome with our strain.

ACKNOWLEDGMENTS

The AiF (German Federation of Industrial Research Associations Otto von Guericke) (KF 0218803 KMD3) is gratefully acknowledged for its financial support.

We thank Elke Häusler for her skilled technical assistance.

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