Different strategies of osmoadaptation in the closely related marine myxobacteria *Enhygromyxa salina* SWB007 and *Plesiocystis pacifica* SIR-1

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Only a few myxobacteria are known to date that are classified as marine, owing to their salt dependency. In this study, the salt tolerance mechanism of these bacteria was investigated. To this end, a growth medium was designed in which the mutated *Escherichia coli* strain BKA13 served as sole food source for the predatory, heterotrophic myxobacteria. This enabled measurement of the osmolytes without any background and revealed that the closely related strains *Enhygromyxa salina* SWB007 and *Plesiocystis pacifica* SIR-1 developed different strategies to handle salt stress. *Ple. pacifica* SIR-1, which was grown between 1 and 4 % NaCl, relies solely on the accumulation of amino acids, while *Enh. salina* SWB007, which was grown between 0.5 and 3 % NaCl, employs, besides betaine, hydroxyectoine as the major compatible solute. In accordance with this analysis, only in the latter strain was a locus identified that codes for genes corresponding to the biosynthesis of betaine, ectoine and hydroxyectoine.

INTRODUCTION

Micro-organisms living in a highly osmotic environment have to deal with the problem that water follows the osmotic gradient. Cells unable to cope with osmotic stress will become dehydrated. This will eventually disrupt cellular metabolism, and so is used in food conservation by pickling. One strategy to thrive in such environments involves the production of so-called osmolytes to maintain osmotic equilibrium across the cytoplasmic membrane. Osmolytes are organic compounds of low molecular mass that have no influence on cellular metabolism and are non-toxic. Therefore, these highly water-soluble molecules are also called compatible solutes (Brown, 1976; Held et al, 2010). Bacteria accumulate many different organic osmolytes in response to hypertonicity, including some amino acids, e.g. proline and glutamate, and some specialized compatible solutes, e.g. betaine and ectoine (da Costa et al., 1998; Burg & Ferraris, 2008). Ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidin-carboxylic acid) was discovered first in *Ectothiorhodospira halochloris* (Galinski, et al. 1985) and later on in many bacterial species (Severin et al., 1992; Roberts, 2005). The derivative hydroxyectoine was first discovered in *Streptomyces parvalus* (Inbar & Lapidot, 1988), before its presence was proven in a wide range of bacteria, and recently also in *Archaea* (Widderich et al., 2015). These molecules either are synthesized de novo or are taken up from the medium. The biosynthesis is well studied and the underlying genes are known (Sadeghi et al., 2014). In addition to their role in osmoregulation, compatible solutes are known to stabilize cell components under abiotic stress conditions, e.g. temperature, desiccation or oxidative stress. Their properties render them interesting for application in biotechnological research, e.g. for the stabilization of cryocultures/proteins, and as additives for PCR enhancement. Owing to the same effect, they are also applied in formulations of fragile drugs and medical products. Furthermore, compatible solutes display some biological effects. Thus, ectoine was reported to act as an anti-inflammatory upon particle-induced lung inflammation (Sydlik et al., 2009), and inhibited the aggregation of β-amyloid peptides that are

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Abbreviation: SOE, splicing-by-overlap-extension.

The GenBank/EMBL/DDBJ accession number for the *Enhygromyxa salina* SWB007 gene locus harbouring genes related to betaine, ectoine and hydroxyectoine biosynthesis identified in this work is KU237243.

Six supplementary figures and two supplementary tables are available with the online Supplementary Material.

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involved in senile diseases (Kanapathipillai et al., 2005). Ectoine is already on the market, owing to its moisturizing and protecting properties, in different skin care and cosmetic products, e.g. sun blockers (Kunte et al., 2014).

Despite the relatively moderate osmotic stress of marine habitats, which usually provide a salt concentration of about 3% NaCl, the chance of finding producers of organic osmolytes is high. Marine Vibrio species, for example, are known for their ability to produce ectoine. In recent years the first halophilic myxobacteria, which are not able to grow in the absence of NaCl, have been isolated. Their terrestrial counterparts instead grow without salt and can usually not grow at NaCl concentrations higher than 10%. However, both have the ability to lyse a variety of bacteria and fungi to obtain nutrients. The few genera of halophilic marine myxobacteria isolated to date, i.e. Halangium, Plesiocystis and Enhygromyxa (Fudou et al., 2002; Iizuka et al., 2003a, b; Schäberle et al., 2010), are of special interest, owing to their ability to produce unprecedented natural products like the anti-biotically active salimabromide (Felder et al., 2010), are of special interest, owing to their ability to produce unprecedented natural products like the anti-biotically active salimabromide (Felder et al., 2010). Despite this interest in these organisms, their salt tolerance mechanisms remain to be investigated.

This study of the closely related marine myxobacteria Enhygromyxa salina SWB007 and Plesiocystis pacifica SIR-1 is aimed at investigating their osmo-adaptation mechanisms using analytical experiments and comparative genomics.

**METHODS**

**Bacterial strains.** *Enh. salina* SWB007 (from the strain collection of the Institute for Pharmaceutical Biology, University of Bonn) was isolated from marine sediments from the German coastline near Prerow (Felder et al., 2013a, b). *Ple. pacifica* SIR-1 (DSM 14875, type strain) was isolated from a Japanese coastal sea grass (Zostera) (Iizuka et al., 2003a). *E. coli* BKA13 is a derivative of *E. coli* MH113 (Haardt et al. 1995) and cannot synthesize the compatible solute trehalose (*otsB*, *otsA*). Like its parental strain, *E. coli* BKA13 is missing all transporters for compatible solute accumulation and the genes for the conversion of choline into glycine betaine: genotype Δ*putP, proB, proA*)' .

**Generation of *otsB* gene deletion.** DNA sequences upstream and downstream from the gene *otsB* were joined by applying the splicing-by-overlap-extension (SOE) PCR technique (Horton et al., 1989) using a set of specific primers (Table S1, available in the online Supplementary Material). The resulting PCR fragments were ligated into temperature-sensitive plasmid pMAK705 (Hamilton et al., 1989) and transferred into *E. coli* by transformation. After cultivation for 24 h at 30 °C, mutants carrying chromosomally integrated pMAK705 were selected on Luria–Bertani (LB) medium containing chloramphenicol (50 µg ml⁻¹) at 43 °C. These mutants were then cultivated in LB liquid medium; chloramphenicol-sensitive *otsB* mutants, arising after double cross-over, were identified on LB solid medium by *in situ* PCR.

**Media and growth conditions.** To prepare a medium for the marine myxobacteria with no background of organic osmolytes, first an *E. coli* BKA13 culture was prepared. To this end, *E. coli* BKA13 cells were grown in the minimal medium MM63 including 0.5% NaCl (Larsen et al., 1987; Grammann et al., 2002) for 20 h, with shaking at 37 °C. The OD₆₀₀ was measured and the culture was precipitated by centrifugation for 10 min at 8873 g. The cell pellet was washed twice with tap water and subsequently resuspended in tap water and used for the preparation of *ASW*-Coli medium described below. The amount of tap water used for resuspension was calculated by the following equation: (OD₆₀₀ × initial culture volume)/50.

*Enh. salina* SWB007 and *Ple. pacifica* SIR-1 were grown in *ASW*-Coli medium containing 75% artificial seawater (ASW) and 3% *E. coli* BKA13 suspension in MilliQ water. The pH of the medium was adjusted to 7.5 with NaOH. After sterilization by autoclaving, the medium was supplemented with trace element solution (1 ml l⁻¹) and vitamin B₁₂ (1 ml l⁻¹). Standard ASW (100%) contains KBr (0.1 g l⁻¹), MgCl₂.6H₂O (10.61 g l⁻¹), CaCl₂.2H₂O (1.47 g l⁻¹), KCl (0.66 g l⁻¹), SrCl₂.6H₂O (0.04 g l⁻¹), Na₂SO₄ (3.92 g l⁻¹), NaHCO₃ (0.19 g l⁻¹) and H₂BO₃ (0.03 g l⁻¹). The trace element solution consists of 10 mg ZnCl₂, 50 mg MnCl₂.4H₂O, 5 mg H₂BO₃, 5 mg CuSO₄.5H₂O, 10 mg CoCl₂, 2.5 mg SnCl₂.2H₂O, 2.5 mg LiCl, 10 mg KBr, 10 mg KI, 5 mg Na₂MoO₄.2H₂O and 2.6 g Na₂EDTA.2H₂O dissolved in 11 distilled water; NaCl was added to reach the desired final concentration, and the solution was sterilized by filtration. Stock solution of vitamin B₁₂ was 0.5 mg ml⁻¹ cyanocobalamin in water; the solution was sterilized by filtration. To determine the salt tolerance range of *Enh. salina* SWB007, 100 ml Erlenmeyer flasks containing 30 ml *ASW*-Coli medium with different NaCl concentrations (from 0 to 5% NaCl in 0.5% steps) were prepared. The cultures were inoculated with 100 µl of dispersed fresh fruiting bodies. The dispersed fruiting body suspension was prepared beforehand by manually collecting fruiting bodies from another liquid culture using a pipette. Growth was determined by measuring the decrease in OD₆₀₀, since the *E. coli* cells served as prey and were lysed by the myxobacteria, which resulted in clearing of the medium.

For identification of compatible solutes, the myxobacteria were grown in 51 Erlenmeyer flasks containing 11 *ASW*-Coli medium with 0.5 and 3% NaCl for *Enh. salina* SWB007, and 1.0 and 3.5% NaCl for *Ple. pacifica*, respectively. Two independent experiments were performed and the mean values are given in Table 1. Incubation was performed at 30 °C at 140 r.p.m., using rotary shakers. After lysis of all *E. coli* cells (4 days, except for *Enh. salina* in 3.5% NaCl medium, which was incubated for 5 days), the fruiting bodies were collected and precipitated by centrifugation at 17 000 g for 1 min. The supernatant was discarded and the pellet was freeze-dried using a lyophilizer (Christ Beta 1-16; Martin Christ).

**Extraction of intracellular solutes.** The extraction of cytoplasmic solutes was performed following the Bligh & Dyer extraction method, as modified by Galinski & Herzog (1990). In brief, 500 µl Bligh & Dyer solution (10/5/4, by volume, methanol/chloroform/demineralized H₂O) was added to 30 mg dry material. After shaking for 5 min, 130 µl chloroform and 130 µl demineralized H₂O were added and the mixture was shaken again for 5 min. Finally, the mixture was centrifuged at 8000 g for 3 min and the hydrophilic top layer, containing the compatible solutes, was separated for subsequent analyses.

**HPLC and LCMS analysis for compatible solutes and free amino acids.** The aqueous phase was analysed by HPLC using a refractive index detector RI-71 (Shodex) and a UV detector UV1000 (Thermo Scientific) with the following HPLC conditions: column, LiChrospher 100-NH₂ 5 µm (Merck); isocratic flow of 80% acetonitrile and 20% water at 1 ml minute⁻¹. The samples were diluted 1:4 with the solvent before measurement. Additionally, reference compounds were run to identify the compatible solutes. The evaluation was performed with ChromQuest5 (ThermoQuest).
**Table 1. Content of compatible solutes and free amino acids in *Enh. salina* SWB007 and *Ple. pacifica* SIR-1**

<table>
<thead>
<tr>
<th>Osmolytes/amino acid</th>
<th><em>Enh. salina</em></th>
<th><em>Ple. pacifica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5% NaCl</td>
<td>3% NaCl</td>
</tr>
<tr>
<td>Hydroxyectoine (µmol g⁻¹)</td>
<td>ND</td>
<td>97.56</td>
</tr>
<tr>
<td>Betaine (µmol g⁻¹)</td>
<td>ND</td>
<td>48.38</td>
</tr>
<tr>
<td>Glutamine (µmol g⁻¹)</td>
<td>6.91</td>
<td>49.92</td>
</tr>
<tr>
<td>Glutamate (µmol g⁻¹)</td>
<td>76.75</td>
<td>272.63</td>
</tr>
<tr>
<td>Glycine (µmol g⁻¹)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Alanine (µmol g⁻¹)</td>
<td>ND</td>
<td>40.94</td>
</tr>
<tr>
<td>Proline (µmol g⁻¹)</td>
<td>ND</td>
<td>11.99</td>
</tr>
<tr>
<td>Hydroxyproline (mg g⁻¹)</td>
<td>ND</td>
<td>15.42</td>
</tr>
<tr>
<td>Betaine (mg g⁻¹)</td>
<td>ND</td>
<td>5.31</td>
</tr>
<tr>
<td>Glutamine (mg g⁻¹)</td>
<td>1.01</td>
<td>7.30</td>
</tr>
<tr>
<td>Glutamate (mg g⁻¹)</td>
<td>11.29</td>
<td>40.11</td>
</tr>
<tr>
<td>Glycine (mg g⁻¹)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Alanine (mg g⁻¹)</td>
<td>ND</td>
<td>3.65</td>
</tr>
<tr>
<td>Proline (mg g⁻¹)</td>
<td>ND</td>
<td>1.38</td>
</tr>
</tbody>
</table>

*Chromatograms are given in Figs S1–S6. ND, Not detected.

MS analysis was performed using an ESI-MS microTOF spectrometer (Bruker Daltonik) coupled to an HPLC. The same parameters as described above were applied, and the sample was split before transfer to ESI-MS.

Free amino acids were analysed by FMO/ADAM HPLC using a fluorescence detector (SpectraSYSTEM FL2000, 254 nm excitation, 315 nm emission; Thermo separation products); the HPLC column used was a Merck Superspher 60 RP-8, 4 µm, 250 x 4.6 mm LiChroCART system (Alltech Grom). Amino acids were derivatized with 9-fluorenylmethoxycarbonyl (FOMC) by adding 40 µl boric acid buffer (0.5 M boric acid and 25 µM norvalin) and 80 µl FOMC reagent (1 mM FOMC in acetone) to 40 µl 1:50-diluted sample material. After shaking for 45 s, 100 µl 1-aminoadamanthane (ADAM) reagent (50 % 40 mM ADAM in boric acid buffer, 50 % acetone) was added and the mixture was shaken again for 45 s. Finally, 140 µl solvent A [80 % 50 mM sodium acetate in H₂O, 20 % 0.5 % tetrahydrofuran in acetonitrile (v/v)] was added. Solvent B consisted of 20 % sodium acetate buffer and 80 % acetonitrile. The HPLC was run at 1.25 ml min⁻¹ using the following gradient: 100 % A to 91 % A in 15 min, 91 % A to 70 % A in the next 15 min, 70 % A to 40 % A in the next 10 min, 40 % A to 0 % A (i.e. 100 % B) in the next 2 min, maintained at 100 % B for the next 7 min, 100 % B to 100 % A in the next 2 min, and maintained at 100 % A for the next 2 min. The evaluation was performed with ChromQuest5 (ThermoQuest).

**Bioinformatics analyses.** The genome of *Ple. pacifica* SIR-1 (NCBI reference sequence NZ_ABCS00000000.1) and a draft genome of *Enh. salina* SWB007 (unpublished) were analysed using the bioinformatics program antisMASH3.0 (Weber et al., 2015). In the genomic data of *Enh. salina* SWB007 a gene locus was identified harbouring genes related to betaine, ectoine and hydroxyectoine biosynthesis. The gene cluster was subsequently annotated using the RAST annotation program (version 2.0) (Overbeek et al., 2014) and the UniProt Basic Local Alignment Search Tool (BLAST). The contig was submitted to GenBank (accession number KU237243).

**RESULTS**

**Generation and characterization of the otsB-deletion mutant *E. coli* BKA13**

Marine myxobacteria are usually cultivated using a medium containing autoclaved baker’s yeast. Such media are not suitable for the analysis of osmoregulated compatible solute synthesis because compatible solutes originating from yeast cells can be accumulated by the myxobacteria and falsify the results. Therefore, we established a novel medium – named ASW-Coli – which is based on *E. coli* cells as sole food source.

*E. coli* can synthesize the compatible solute trehalose *de novo* and can convert choline to glycine betaine. If provided with the medium, *E. coli* is able to transport and amass a variety of different compatible solutes, such as ectoine, proline and glycine betaine, with the help of the transporters ProP and ProU (Milner et al., 1988; May et al., 1989). In order to feed myxobacteria without contaminating compatible solutes, we designed the trehalose-free *E. coli* strain BKA-13, which is based on *E. coli* MHK-13 (Haardt et al., 1995). *E. coli* MHK13 is devoid of the compatible solute transporters ProP and ProU and the proline transporter PutP. Like its parental strain, MC4100 (Casadaban, 1976; Peters et al., 2003), MHK13 is unable to transport and convert choline into glycine betaine (betTBA). Strain MHK13 still possesses the genes otsB and otsA for trehalose synthesis. To obtain a trehalose-free *E. coli*, we deleted the gene otsB, which encodes trehalose-6-phosphate phosphatase. The DNA regions upstream and downstream of otsB were joined by applying the SOE-PCR technique. The resulting AotsB fragment was cloned into the temperature-sensitive plasmid pMAK705, which facilitated the selection for AotsB mutants. The gene otsB overlaps with ORF otsA, which is located downstream of otsB. By deleting otsB (in-frame null mutation), 26 bp of the 5′ region of otsA were removed as well (AotsB otsA) (Fig. 1).

The newly designed otsBΔ mutant BKA-13 was further characterized, and compared to MHK-13. Both strains were cultivated on minimal medium containing 2.0, 2.5, 2.7, 2.8 and 3.0 % NaCl. While strain MHK-13 grew at all salinities within 2 days, strain BKA-13 failed to grow on medium containing 2.8 and 3.0 % NaCl. On medium with 2.7 % NaCl, small colonies of strain BKA-13 were observed after 6 days of incubation. Cell extract of *E. coli* BKA-13 was further analysed by HPLC for the presence of trehalose. No trehalose was detectable in any of the BKA-13 cells grown in up to 2.5 % NaCl.

**Growth and salt tolerance of myxobacteria**

The salt tolerance ranges of the two myxobacterial strains *Enh. salina* SWB007 and *Ple. pacifica* SIR-1 were determined in ASW-Coli medium. To enable a fast and reliable method for growth determination, an indirect measurement was performed; such a method was required since the myxobacteria rapidly form fruiting bodies instead of...
disperse growth. Thus, the decrease in OD$_{600}$ was measured. OD$_{600}$ is directly related to lysis and consumption of the prey cells. Therefore, a decrease in OD$_{600}$ indicates growth of *Enh. salina* SWB007 or *Ple. pacifica* SIR-1. *Enh. salina* SWB007 was able to grow in media containing 0.5 to 3 % NaCl. The fastest lysis rate of the prey cells was in the salinity range 0.5 to 2 % (Fig. 2). After 7 days all *E. coli* cells were consumed and orange-coloured fruiting bodies appeared. Cultures supplemented with 2.5 and 3 % NaCl took 2 days longer to achieve similar results. With 0 % NaCl the culture initially seemed to grow, but after 4 days the OD$_{600}$ increased again. In cultures containing more than 3 % NaCl no growth was observed.

*Ple. pacifica* SIR-1 showed no growth in medium containing 0.5 % NaCl. The lower limit was 1 % NaCl and growth was observed up to a concentration of 4 % NaCl in ASW-Coli medium. However, the growth was decelerated and therefore a concentration of 3.5 % NaCl was used for subsequent analyses.

**Identification of the compatible solutes of *Enh. salina* SWB007 and *Ple. pacifica* SIR-1**

Both strains, *Enh. salina* SWB007 and *Ple. pacifica* SIR-1, could grow in a relatively high NaCl concentration range, i.e. 0.5–3 % NaCl for *Enh. salina* SWB007 and 1–4 % NaCl for *Ple. pacifica* SIR-1. Such a range is usually an indication that the organisms use the organic osmolyte strategy to deal with the salt stress of the environment. To prove this hypothesis, we identified the compatible solutes produced by these organisms under different salt concentrations. Thus, two 1 l cultures of each strain were grown in ASW-Coli medium: (i) 0.5 and 3 % NaCl for *Enh. salina* SWB007 and (ii) 1 and 3.5 % NaCl for *Ple. pacifica* SIR-1. These cultures were incubated until total clearing of the medium before the cells were harvested. In total, 92.9 mg (0.5 % NaCl) and 134.3 mg (3 % NaCl) dry cell mass were obtained from *Enh. salina* SWB007, and 180 mg (1 % NaCl) and 251 mg (3.5 % NaCl) from *Ple. pacifica* SIR-1 cultures. Subsequently, 30 mg dried cell masses were extracted and the aqueous phase was analysed by HPLC. Clear differences indicating accumulation of organic osmolytes were observed in *Enh. salina* SWB007 grown in 0.5 and 3 % NaCl (Fig. 3). The new peaks at high salt concentration were putatively identified as betaine and hydroxyectoine, by comparison to standard substances. To confirm these results, HPLC-MS experiments followed. These confirmed the presence of betaine and hydroxyectoine, as well as minor amounts of ectoine (Fig. 4). The most abundant compatible solute was hydroxyectoine. The peak at 20 min retention time, which also increased with salinity, however, could not be identified. The peak that eluted at 12 min was not dependent on the salt concentration, since it was present in both the 0.5 and the 3 % NaCl cultures at the same order of magnitude.

In contrast, the HPLC analysis of *Ple. pacifica* SIR-1 cultures grown in medium supplemented with either 1 or 3.5 % NaCl revealed no differences concerning the specialized osmolytes (Figs S1 and S2) when analysed under these conditions. Interestingly, this closely related organism
did not produce any of the typical organic osmolytes, e.g. betaine, ectoine or hydroxyectoine, even at a high salt concentration.

Free amino acid analysis

In addition to specialized compounds, common substances like amino acids may also serve as compatible solutes. Thus, the content of free amino acids in the cells was determined using FMOC/ADAM HPLC analysis, revealing significant changes (Table 1).

*Enh. salina* SWB007 grown under high-salt conditions accumulated the amino acids alanine, glutamate, glutamine and proline (Table 1, Figs S5 and S6). The level of glutamine increased sevenfold and the level of glutamate fourfold at elevated salinity. Furthermore, alanine and proline were accumulated whereas both amino acids were below the detection limit under low-salt conditions. Free amino acid analysis of *Ple. pacifica* SIR-1 revealed the accumulation of alanine, glutamate, glutamine, glycine and proline at high salinity (Table 1, Figs S3 and S4). The level of glutamate showed a ninefold increase compared with the level at low salinity (296 μmol g⁻¹ versus 33 μmol g⁻¹). Alanine, glutamine, glycine and proline were also significantly accumulated under high-salt conditions, while they were below the detection limit at low-salt conditions. Glycine was identified as the second major amino acid, contributing 28 % of the *Ple. pacifica* SIR-1 organic osmolyte pool. Comparing the two strains, one common feature could be detected, i.e. glutamate was the major player, making up 52 and 66 % of the total organic osmolyte pool in *Enh. salina* SWB007 and *Ple. pacifica* SIR-1, respectively. In *Enh. salina* SWB007, alanine and glutamine followed, with amounts between 40 and 50 μmol g⁻¹, comprising 19 % of the organic osmolyte pool. In *Ple. pacifica* SIR-1 in contrast these two amino acids were only present in minor amounts, comprising 2.6 % of the organic osmolyte pool. In contrast, glycine was identified as the second major amino acid used by *Ple. pacifica* SIR-1, resulting in a proportion of 28 % within the organic osmolyte pool. Proline was accumulated by both organisms only in minor amounts, contributing less than 3 % to the organic osmolytes.

Genome sequencing, annotation and bioinformatics analysis for compatible solutes biosynthesis

To elucidate the reason for the observed differences between these closely related myxobacterial strains, *in silico* analysis of the genomes was performed. The available draft genome of *Ple. pacifica* SIR-1 was screened in respect of gene clusters associated with betaine, ectoine and hydroxyectoine biosynthesis. However, no such gene cluster was identified, supporting the previous results. Instead, many solute transporters such as the Na⁺/proline...
(solute) symporters (GenBank accession numbers EDM75864.1, EDM79015.1, EDM74523.1, EDM80875.1, EDM77400.1), proton/sodium-glutamate symporter (EDM74633.1), sodium:alanine symporter (EDM78123.1), choline/glycine betaine transporter (EDM75025.1) and many ABC transporters were detected in the genome.

In *Enh. salina* SWB007, biosynthetic gene clusters for the organic osmolytes had been expected, owing to the confirmation of these compounds in the extracts. The draft genome of strain SWB007 was screened for the presence of genes corresponding to organic osmolyte synthesis. Hence, a gene locus associated with ectoine biosynthesis was identified (accession number KU237243). In addition to *ectA*, *ectB*, *ectC* and *ectD* (Fig. 5, Table 2), further ORFs putatively coding for enzymes involved in organic osmolyte biosynthesis and for osmolyte transporters are present in this gene cluster, i.e. a sensor histidine kinase (*ectR*) and an aspartokinase (*ectAsk*). These genes represent the complete biosynthetic gene cluster for the synthesis of the osmolyte hydroxyectoine. Comparison of this gene cluster to reported ectoine/hydroxyectoine gene loci revealed that *Enh. salina* SWB007 possesses a specific gene order (Fig. 6). A putative regulatory gene, i.e. *ectR*, was found in about one-quarter of the putative ectoine/hydroxyectoine producers (Widderich et al., 2016). The functional association of an *ectR* gene with ectoine biosynthesis was first demonstrated in the halotolerant alkaliphilic *Methylomicrobium alcaliphilum*. This cluster, including *ectR*, shows the highest overall identity to *Enh. salina* SWB007 (Fig. 6). However, the grade of homology varies between the different genes; *ectA*, *ectB* and *ectC* show 35–56 % identity at the protein level, while *ectR* has only about 10 % identity. This might be an indication that the regulatory mechanism differs from that of *Met. alcaliphilum*, in which it acts as a transcriptional repressor (Overbeek et al., 2014).

**Fig. 3.** Comparison of the compatible solute content of *Enh. salina* SWB007 in medium containing either 0.5 or 3 % NaCl. Black line, 0.5 % NaCl; red line, 3.0 % NaCl. Betaine and hydroxyectoine were present in cells grown in 3 % NaCl, while in 0.5 % NaCl these substances were not detectable. The peak at 20 min (**) could not clearly be assigned. The peak at 12 min (†) was present in both cultures in a similar amount, and therefore this peak was not dependent on the salt concentration. The peak at 6 min, which was only detectable in the refractive index plot, corresponds to NaCl. AU, Absorbance units.
Of special interest are the two methyltransferases (MTs), i.e. glycine/sarcosine N-MT (GS-MT) and sarcosine/dimethylglycine N-MT (SD-MT), and a betT homologue, which follow directly downstream of the ectoine-related genes, oriented in the same direction. These MTs are required for betaine synthesis from glycine, while betT codes for the transporter. Thus, the genes corresponding to the biosynthesis of betaine and hydroxyectoine are clustered together.

**DISCUSSION**

Marine myxobacteria, e.g. *Enh. salina* strains, are becoming more and more the focus of research owing to their biosynthetic potential (Felder et al., 2013a, b). To adapt these slow-growing organisms to growth under laboratory conditions, insights into their ecology, e.g. analysis of their salt tolerance mechanism, will be beneficial. To overcome the hurdle that *Enh. salina* strains are routinely grown in media containing a yeast cell suspension, which provides an unfavourable background, the medium ASW-Coli, based on *E. coli* BKA13 instead of baker’s yeast as nutrient source, was developed. This enabled the growth of marine myxobacteria to be followed by measuring OD<sub>600</sub>. Hence, the OD decrease due to the consumption of the *E. coli* cells by the predatory bacteria was measured. The values for the salt tolerance of these strains (0.5–3 % NaCl for *Enh. salina* SWB007; 1–4 % NaCl for *Ple. pacifica* SIR-1) determined with this method are in accordance with previous results (Iizuka et al., 2003a; Schäberle et al., 2010). Such a relatively high degree of flexibility to environmental salt concentrations indicates an adaptation strategy involving organic osmolyte accumulation, rather than the salt-in strategy (Sleator & Hill, 2002). At high salt concentrations, a total of 521 mmol g<sup>−1</sup> solutes was detected in *Enh. salina* SWB007. The biggest part of this consisted of glutamate (52 %), followed by hydroxyectoine and betaine, which accumulated to 19 and 10 %, respectively. Hence, glutamate, hydroxyectoine and betaine seem to be the main players in osmo-adaptation of *Enh. salina*. A comparison to marine *Vibrio fischeri* species revealed that the observed total solute pool falls within expectations (451 mmol g<sup>−1</sup> for *Vib. fischeri* DSM 7151). Here too, the major compound was glutamate (approximately 50 %), followed by ectoine (approximately 24 %) (Schmitz & Galinski, 1996). Accumulation of both glutamate and hydroxyectoine is fully congruous with metabolism and

![HPLC-MS analysis](http://mic.microbiologyresearch.org)
other cellular functions (Reuter et al., 2010). However, it seems that this mechanism cannot be regarded as a general rule for myxobacteria. The terrestrial myxobacterium Myxococcus xanthus, the model organism for myxobacteria, was shown to produce the compatible solutes betaine and trehalose under salt stress conditions (McBride & Zusman, 1989; Kimura et al., 2010). Our results indicate that in addition to betaine the marine strain acquired, or retained, the ability to use hydroxyectoine as the main compatible solute. Hydroxyectoine, a derivative of ectoine, is widespread among halophilic bacteria, and compared with ectoine it provides better protection against various stress conditions, especially desiccation and heat (Lippert & Galinski, 1992; Louis et al., 1994; Tanne et al., 2014). In the intertidal zones of the littoral environment, desiccation is at least a temporary stress factor. This might explain why the organism favours hydroxyectoine as compatible solute. The preferential accumulation of hydroxyectoine has also been observed in other salt-stressed bacteria, such as Streptomyces coelicolor A3(2) and Virgibacillus salixigenis (Sadeghi et al., 2014). Recently the ability to synthesize hydroxyectoine was also demonstrated in the acidophilic Acidiphilium cryptum, which displays only limited salt tolerance (Moritz et al., 2015). The authors concluded that hydroxyectoine, besides osmoadaptation, may serve other, hitherto unknown, functions.

In Ple. pacifica SIR-1, the total amount of solutes detected was 445 μmol g⁻¹. The major solute at high salt concentrations was also glutamate, contributing 66 % of the composition, followed by the amino acid glycine, constituting 28 %. Thus, Ple. pacifica SIR-1, the closest relative to the Enhygromyxa clade, did not produce any of the well-established compatible solutes under high-salt conditions. This marine myxobacterium instead accumulated the amino acids glutamate, glycine and proline. Glycine and proline can be enzymically synthesized or may be taken up from the environment. The accumulation of glutamate as primary response was the same as in Enh. salina SWB007. However, glycine has so far not been reported as a major bacterial osmolyte. The few reports available refer to its osmotic use in marine mussels and ciliates, in combination with alanine and taurine in the former and alanine and proline in the latter (Kaneshiro et al., 1969; Ellis et al., 1985). It is worthy of note that glycine has been applied empirically for protein stabilization during freeze–thawing of phosphate buffer systems and as a bulking agent during lyophilization of monoclonal antibodies (Pikal-Cleland et al., 2002; Meyer et al., 2009).

Fig. 5. Hypothesis for organic osmolyte biosynthesis in Enh. salina SWB007. (a) Gene locus coding for genes involved in organic osmolyte biosynthesis. Genes from left to right: ectR, sensor histidine kinase; orf2, hypothetical protein; orf3, hypothetical protein; ectAsk, aspartokinase; ectA, diaminobutyric acid (DABA) acetyltransferase; ectB, DABA aminotransferase; ectC, ectoine synthase; ectD, ectoine hydroxylase; symp, Na/solute symporter; mep, membrane protein (MarC family); gsmt, glycine/sarcosine N-methyltransferase; sdmt, sarcosine/dimethylglycine N-methyltransferase; betT, high-affinity choline uptake transporter BetT. It can be seen that the genes for ectoine/hydroxyectoine (ectAskABCD) and for betaine (gsmt and sdmt) biosynthesis are clustered in Enh. salina SWB007. (b) Hydroxyectoine biosynthetic pathway (modified from Bursy et al., 2008). (c) Glycine betaine biosynthetic pathway through methylation steps of glycine (modified from Sayyar Khan et al., 2009). SAM, S-adenosyl-L-methionine; SAH, S-adenosyl-L-homocysteine.
Therefore, the fact that glycine is one of the dominant amino acids in Ple. pacifica deserves further investigation.

In accordance with the above observations, a gene locus associated with solute biosynthesis could be identified in Enh. salina SWB007 (Fig. 5a). The biosynthesis of ectoine and hydroxyectoine has been firmly established for many micro-organisms (Sadeghi et al., 2014). Accordingly, a biosynthetic hypothesis was deduced for hydroxyectoine and betaine in Enh. salina (Fig. 5b, c).

It is striking that only hydroxyectoine is accumulated and not the direct precursor ectoine. Thus, the EctD-catalysed step has to be very efficient. In Streptomyces rimosus it was shown that the effect of salinity on ectD transcription level is threefold more prominent on ectD transcription level than on ectC (Sadeghi et al., 2014). Furthermore, it was suggested that the localization of ectD at the 3'-end of the ectABCD mRNA contributes positively to its stability. A putative regulator, encoded by ectR, is clustered with the ectABCD genes. EctR is expected to be responsible for the regulation of hydroxyectoine synthesis, dependent on natural stimuli. It was shown previously that in Gram-negative and Gram-positive bacteria the genes are induced by osmotic or temperature stress (Widderich et al., 2016). The presence of genes for several different osmolyte transporters in the genome can be regarded as a complementary mechanism for osmo-adaptation in Enh. salina SWB007. A Na⁺/proline symporter is encoded

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**Table 2. Genes encoded in the gene locus putatively corresponding to organic osmolytes biosynthesis in Enh. salina SWB007**

<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene</th>
<th>Length (bp)</th>
<th>Highest homology</th>
<th>Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ectR</td>
<td>1899</td>
<td>Histidine kinase (Marichromatium purpuratum 984)</td>
<td>43.3</td>
</tr>
<tr>
<td>2</td>
<td>orf2</td>
<td>126</td>
<td>Uncharacterized protein (Sorangium cellulosum)</td>
<td>61.3</td>
</tr>
<tr>
<td>3</td>
<td>orf3</td>
<td>153</td>
<td>Outer-membrane protein and related peptidoglycan-associated (lipo)protein (Comamonadaceae bacterium A1)</td>
<td>52.6</td>
</tr>
<tr>
<td>4</td>
<td>ectAsk</td>
<td>1458</td>
<td>Aspartate kinase (Rhodothermus marinus SG0.5JP)</td>
<td>46.6</td>
</tr>
<tr>
<td>5</td>
<td>ectA</td>
<td>537</td>
<td>Diaminobutyrate acetyltransferase (Alkalilimnicola ehrlichii)</td>
<td>53.7</td>
</tr>
<tr>
<td>6</td>
<td>ectB</td>
<td>1335</td>
<td>Diaminobutyrate aminoaminotransferase apoenzyme (Streptomyces sp. SM8)</td>
<td>63.4</td>
</tr>
<tr>
<td>7</td>
<td>ectC</td>
<td>381</td>
<td>l-ectoine synthase (Pontibacillus yanchengensis)</td>
<td>59.2</td>
</tr>
<tr>
<td>8</td>
<td>ectD</td>
<td>897</td>
<td>Ectoine hydroxylase (Castellaniella defragrans 65)</td>
<td>57.8</td>
</tr>
<tr>
<td>9</td>
<td>solute transporter</td>
<td>1446</td>
<td>SSS sodium solute transporter superfamily (Rubinisphaera brasiliensis)</td>
<td>58.5</td>
</tr>
<tr>
<td>10</td>
<td>mep</td>
<td>633</td>
<td>UPF0056 membrane protein (Lyngbya sp. strain PCC 8106)</td>
<td>53.8</td>
</tr>
<tr>
<td>11</td>
<td>GSMT</td>
<td>861</td>
<td>SAM-dependent methyltransferase (Thioploca ingrica)</td>
<td>71.4</td>
</tr>
<tr>
<td>12</td>
<td>SDMT</td>
<td>864</td>
<td>Dimethylglycine methyltransferase (Marichromatium purpuratum 984)</td>
<td>54.3</td>
</tr>
<tr>
<td>13</td>
<td>betT</td>
<td>1530</td>
<td>Choline transporter (Sphingomonas sp. Ant20)</td>
<td>56.6</td>
</tr>
</tbody>
</table>

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**Fig. 6.** Genetic organization of ectoine/hydroxyectoine biosynthesis gene clusters. The Enh. salina SWB007 gene locus is shown in comparison to closely related gene loci; related genes are shown in the same colour. The sequence homology at the protein level is given as a percentage.

http://mic.microbiologyresearch.org
directly downstream of ectD, and such transporters can mediate the accumulation of organic osmolytes, e.g. betaine, ectoine and proline, at high osmotic pressure (Wood, 2006). Furthermore, two N-methyltransferase genes are clustered with the hydroxyectoine gene locus of *Enh. salina* SWB007 (Fig. 5a). It can be assumed that these genes are linked to betaine biosynthesis (Waditee et al., 2003). Downstream of these two methyltransferase genes, there is a gene putatively coding for the high-affinity betaine/carnitine/choline transporter, BetT. In contrast, none of the established compatible solutes was detected in *Ple. pacifica* SIR-1. This is in accordance with the genome analysis, providing no gene cluster corresponding to their biosynthesis. However, the presence of many osmolyte transporters and symporters indicates that this bacterium is reliant on uptake to cope with salt stress. Such an uptake of osmolytes, e.g. amino acids, as a major strategy against the osmotic gradient represents an intelligent ploy for a predatory bacterium, which lyses prey cells and thereby creates for itself a source of these molecules. It is therefore at present unclear whether the markedly increased glycine level (28 % of total solutes) is a result of uptake or genuine osmoregulated biosynthesis.

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Different strategies of osmoadaptation


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