Genome Analysis of The Salt-Resistant *Paludifilum Halophilum* DSM 102817\(^T\) Reveals Genes Involved In Flux-Tuning of Ectoines And Unexplored Bioactive Secondary Metabolites

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Research Article

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Abstract

*Paludilum halophilum* is the first member of the genus *Paludilum* in the *Thermoactinomycetaceae* family. The thermohalophilic bacterium was isolated from the solar saltern of Sfax, in Tunisia and was shown to be able to produce ectoines in relatively high-yield and cope with salt stress conditions. In this study, the whole genome of *P. halophilum* was sequenced and analysed. Analysis revealed 3,789,765 base pairs with average GC % content of 51.5%. A total of 3,775 genes were predicted of which 3616 were protein-coding genes and 73 were RNA genes. The genes encoding key enzymes for ectoines synthesis were identified from the bacterial genome next to a gene cluster (*ehuABCD*) encoding a binding-protein-dependent ABC transport system responsible for ectoines mobility through the cell membrane. With the aid of KEGG analysis, we found that the central catabolic network of *P. halophilum* comprises the pathways of glycolysis, tricarboxylic acid (TCA) cycle, and pentose phosphate pathway (PPP). In addition, anaplerotic pathways replenishing oxaloacetate and glutamate synthesis from central metabolism, both needed for high ectoines biosynthetic fluxes were identified through several key enzymes. Furthermore, a total of 18 antiSMASH-predicted putative biosynthetic gene clusters (BGCs) for secondary metabolites with high novelty and diversity were identified in *P. halophilum* genome, including biosynthesis of Colabomycine-A, Fusaricidin-E, Zwittermycin A, Streptomycin, Mycosubtilin and Meilingmycin. Based on these data, *P. halophilum* emerged as a promising source for ectoines and antimicrobials with the potential to be scaled up for industrial production, which could benefit the pharmaceutical and cosmetic industries.

Introduction

Among extreme niches, natural and artificial hypersaline habitats were shown to harbor several species of halotolerant and halophilic bacteria (Boujelben et al. 2015; Gibtan et al. 2017). To cope with high salinity, one of the most important strategies used by these salt-resistant and salt-loving bacteria is the transport and/or biosynthesis of organic osmolytes, the so-called compatible solutes (Kempf and Bremer 1998; Bremer and Krämer 2000; Wood et al. 2001). These highly water-soluble organic molecules can protect microorganisms against salt stress, dehydration, heat, oxydatif and UV stresses (Schröter et al. 2017; Brands et al. 2019). Among these compatible solutes, ectoines (ectoine and hydroxyectoine) are of particular interest. Besides their primary function of protecting cells against harsh conditions, the most powerful stabilizing properties on biological macromolecules (enzymes, DNA, antibodies, and even whole cells) confer ectoines attractive potentials in fields of skin caring, food processing, molecular biology, agriculture, biotechnology, and medical values in human diseases (Kanapathipillai et al. 2005; Graf et al. 2008; Pastor et al. 2010; Abdelaziz et al. 20013; Hahn et al. 2017). This led to the development of the first industrial-scale production process using the salt-tolerant bacterium *Halomonas elongata* as the production host (Schwibbert et al. 2011; Kunte et al. 2014). This strain naturally synthesizes ectoine from the precursor L-aspartate-β-semialdehyde (ASA), a central hub in microbial aspartate family amino acid production. The biosynthesis comprises a cascade of three biochemical conversions catalyzed by L-2,4-diaminobutyrate transaminase (EctB), L-2,4-diaminobutyrate acetyltransferase (EctA), and ectoine
synthase (EctC) (Schwibbert et al. 2011). This major biosynthesis pathway of ectoine has been clearly characterized to be a highly conservative cluster across halophile species (Zhao et al. 2018; Leon et al. 2018; Ma et al. 2020; Van Thuoc et al. 2020). Some microorganisms are also able to synthesize a hydroxylated derivative of ectoine, the 5-hydroxyectoine catalyzed by the ectoine hydroxylase (EctD) (Prabhu et al. 2004; Garcia-Estepa et al. 2006; Höppner et al. 2014).

According to the whole-genome organization of halophilic bacteria such as *Halomonas elongata*, *Salinivibrio proteolyticus*, and *Chromohalobacter salexigens*, the three genes (ectA, ectB, ect C) involved in ectoines biosynthesis from L-ASA are typically organized in an operon *ectABC* (Schwibbert et al. 2011; Van Thuoc et al. 2020; Czech et al. 2018). The fourth gene ectD, responsible for bioconversion of ectoine to hydroxyectoine, can be part of the *ectABC* ectoine biosynthetic gene cluster, but it is often found elsewhere in the genome (Widderich et al. 2014, 2016). ect gene clusters may sometimes contain the gene for a specialized aspartokinase (*ask_ect*) (Reshetnikov et al. 2006; Stöveken et al. 2011). Aspartokinase (Ask), and in selected cases of the afore mentioned Ask_Ect enzyme, along with L-aspartate-β-semialdehyde-dehydrogenase (Asd), provide the precursor L-ASA for ectoine biosynthesis (Reshetnikov et al. 2011; Czech et al. 2018). In addition, various types of transport systems detected in bacterial genome of halophilic strain, mediate the acquisition of these stress protectants from surrounding media. These include the TeaABC system from *H. elongata* and *Salinivibrio proteolyticus* M318, which belong to the periplasmic binding protein-dependent tripartite ATP independent periplasmic transporter family (TRAP-T) (Rosa et al. 2018). Another transport system, named Ehu (ectoine-hydroxyectoine uptake) that belongs to the binding protein-dependent ABC transporters members was also detected in the genome of *Sinorhizobium meliloti* (Ter Beek et al. 2014).

In addition to their ability for osmoprotectants production, salt resistant bacteria especially actinomycetes are among the candidates considered to have the potential to produce special or unknown bioactive metabolites. Yet, only limited attention has been given to their secondary metabolite biosynthesis (Manivasagan et al. 2014; Kim et al. 2017). Cultivability dependent methods are not very effective in searching for novel secondary metabolites because most of their biosynthesis genes cannot be expressed, or are expressed at a very low level. Meanwhile, genome sequencing methods have proven to be more effective and a panoply of hidden BGCs were revealed in halophilic bacterial genomes (Doroghazi et al. 2014; Min et al. 2018). In addition, genome mining efforts have also allowed to understand the silencing or activation of biosynthetic pathways in microbes with the development of bioinformatics software, such as antiSMASH, SMURF and PRISM (Baral et al. 2018). Traditionally, BGCs include non-ribosomal peptide synthase (NRPS), polyketide synthase (PKS), and ribosomally synthesized and post-translationally modified peptide (RiPP) family clusters. For instance, a total of 104 antiSMASH-predicted putative BGCs for secondary metabolites with high novelty and diversity were identified in nine *Ktedonobacteria* genomes (Zheng et al. 2019). In the study of Hu et al. (2018), seven of the PKS and NRPS gene clusters related to antibiotics compounds, including friulimicin, lobophorin, laspartomycin, colabomycin, borrelidin, pristinamycin and kanamycin, have been discovered in the genome of an actinomycetale streptomyces strain isolated from plant samples collected from high salt environment. The prediction of gene clusters involved in the biosynthesis of terpenoid/polyketide synthase (PKS) by
genome and transcriptome sequencing revealed a new family of diterpene cyclases in several bacteria (Yang et al. 2017).

*Paludilum halophilum* strain SMBg3 is a salt and heat-resistant bacteria from the family of *Thermoactinomycetaceae* from the phylum *Actinobacteria* (Jiang et al. 2019). It was isolated from Sfax solar salten sediment in Tunisia (Frikha-Dammak et al. 2016) and has shown be a high-yield ectoines producers under salt stress conditions (Ayadi et al. 2020). Here, we report the genome sequencing of the strain and genetic information on genes involved in ectoines and secondary metabolites synthesis. In addition, metabolic pathways for central metabolism related to ectoines accumulation were inspected.

**Materials And Methods**

**Strain culture conditions**

*P. halophilum* DSM 102817\textsuperscript{T} was routinely cultivated in an optimized mineral sea water (SW) medium (Ayadi et al. 2020) with the following composition (per liter): 5g glucose, 4.07g MgSO\textsubscript{4}.7H\textsubscript{2}O, 2.6 g MgCl\textsubscript{2}.6H\textsubscript{2}O, 0.4 g KCl, 47 mg NaBr, 13 mg NaHCO\textsubscript{3}, 67 mg CaCl\textsubscript{2}.2H\textsubscript{2}O, 278 mg FeSO\textsubscript{4}.7H\textsubscript{2}O and 1g aspartic acid. The pH of the growth medium was adjusted to 8.3-8.4 with 4 M NaOH, and the osmolarity the the set-up of the individual experiments by adding NaCl. A 100-mL medium in 250-ml Erlenmeyer flasks were inoculated with 5 mL of exponentially growing pre-cultures and incubated at 40°C in a rotary shaker incubator (New Brunswick Scientific, NJ, USA) at 200 rpm. Their growth was monitored by measuring the dry mycelial weight (DMW) from 2 mL of broth culture after incubation of the pellet at 80°C for 24 hours.

**Effect of salinity on growth and ectoines identification**

To investigate the effects of salinity on the growth of *P. halophilum*, 5 mL of seed culture was inoculated in 100 mL of glucose SW medium with NaCl concentration ranging from 5 to 25 % (w/v). The flasks were incubated as described above and samples were withdrawn to measure the increase of dry mycelial biomass and the growth was evaluated by the determination of maximal growth rate ($\mu_{\text{max}}$) in exponential phase. Ectoines extraction according to the method of Kunte et al. (1993), was done on mycelial cells harvested from a six-day culture in SW medium with 15% NaCl. Briefly, 10 mg dry cell pellets were resuspended in 570 $\mu$L of a methanol/chloroform/water mixture (10/5/4, v/v/v) and mixed for 15 min at 37°C. To precipitate proteins and extract osmolytes, 170 $\mu$L of chloroform and 170 $\mu$L of water were added. Liquid phase separation was enhanced by gentle centrifugation and the hydrophilic top layer containing compatible solutes was recovered. The identification of ectoines was determined by HPLC-UV on the basis of their retention time in comparison with standard products (sigma Aldrich) on a KNAUER-NH\textsubscript{2} analytical column, with 2 mL.min\textsuperscript{-1} of flow rate and detection at $\lambda_{\text{max}}$ of 210 nm.

**Whole genome sequencing, annotation and bioinformatic analyses**
The biomass of *P. halophilum* for genome analysis was obtained from the culture grown at 40 °C for 6 days in glucose SW-15 mineral broth with 15% salinity. A high quality genomic DNA was prepared using the DNeasy Plant Maxi Kit-Qiagen, following the manufacturer's guidelines (Thermo, USA) (www.qiagen.com). Agarose gel (1.5%) electrophoresis was used for visual assessment of DNA integrity, quantified by the nanodrop method (Garcia-Alegria et al. 2020). Genomic sequencing was carried out using a paired-end sequencing strategy at the University of Neuchatel, using next-generation sequencing technology Illumina MiSeq2000 instrument. The paired-end library had a mean insert size of 500 bp. Reads were assembled using the CLC NGS Cell v. 5.0.4 assembler (CLC bio, Waltham, MA). The genomic DNA base content (mol % GC) was directly calculated from the draft genome data. The draft genome sequence of *P. halophilum* reported in this study has been deposited at DDBJ/ENA/GenBank under the accession NOWF00000000.1 (https://www.ncbi.nlm.nih.gov/nuccore/NOWF00000000.1); BioProject: PRJNA395604 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA395604); BioSample: SAMN07411558 (https://www.ncbi.nlm.nih.gov/biosample/7411558). This WGS version of the project consists of sequences NOWF01000001-NOWF01000213.

The assembled genome sequence was annotated using the National Center for Biotechnology Information Prokaryotic Genome Annotation Pipeline version 4.2 (PGAAP, released 2013, https://www.ncbi.nlm.nih.govgenome/annotation_prok/), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al. 2012) (http://www.genome.jp/kegg/), and Clusters of Orthologous Genes (COG) database (http://www.ncbi.nlm.nih.gov/COG) (Galperin et al. 2015). Antibiotics and Secondary Metabolite Analysis Shell (Anti-SMASH) analysis of the whole genome sequence was used to identify known and novel gene clusters that may have potential for the production of secondary metabolites (Blin et al. 2019).

To ensure the exact phylogenetic position of *P. halophilum* within the *Thermoactinomycetaceae* family, multiple genome alignment was generated by Mauve software (http://asap.ahabs.wisc.edu/mauve/) (Darling et al. 2010), and a phylogenomic tree was visualized by FigTree and EvolView tools. The online OrthoVenn, available at website http://www.bioinfogenome.net/OrthoVenn/ was used for developing Venn diagram.

**Results**

**Salt tolerance and ectoine biosynthesis potential**

*P. halophilum* was selected due to its high salt resistance potential during our primary assay of the Sfax saltern strains library. This strain grows well on Bennett's and ISP2 agar, generating white spores. To determine its resistance potential against environmental salinity, it was grown in modified SW-media supplemented with different concentrations of NaCl and the results are presented in Fig. 1a. No growth was detectable up to a salinity of 5% NaCl, a property expected for a halophile. An increase in the salinity up to 20 % NaCl strongly stimulated growth, but further increases to 25% impaired it. In fact, *P. halophilum* not only depends on a considerable salt concentration for its growth but it can also cope with
a broad spectrum of salinities (from 5 to 20 % NaCl). To correlate the osmotolerance capacity of the strain with the solute compatible production, we assessed the ability of *P. halophilum* for ectoines synthesis. According to the results of the HPLC analysis, we found that at an optimum salinity growth of 15% NaCl, the most abundant osmoprotector was ectoine and hydroxyectoine that shared the same retention times of 22.609 min and 20.027 min, respectively with standard ectoines (Fig. 1b).

**Genome characteristics and phylogeny**

Upon sequencing, the chromosome of *P. halophilum* was assembled from 13 million reads resulting in a total length of 3,789,770 bp and including 213 contigs with N$_{50}$ size of 304,586 bp. The GC content of DNA for the strain is 51.5 mol% as calculated from the whole-genome sequence. The properties and statistics of the genome are summarized in Table 1. The genome annotation report by NCBI revealed that the genome constituted of a total of 3,775 genes, among which 3616 protein-coding genes and 73 RNA genes (57 tRNAs, 12 rRNAs and 4 ncRNAs). A total of 8 retroelements with a size of 2314 bp, presented by LTR elements contained seven Ty1/Copia (2002) and one Gypsy/DIRS1, two DNA transposons (158pb) were also detected (Table 1). To further reveal the evolutionary relationship of *P. halophilum* with other thermoactinomycete strains, the whole genome sequence of the strain was aligned and compared with those of the different strains selected from among the genomes submitted to the NCBI genome sequence database using Mauve software (http://asap.ahabs.wisc.edu/mauve/). It was found that *P. halophilum* clustered under a separate node with the three Thermoactinomycete, *Kroppenstedtia eburnean, Planifilum fulgidum* and *Melghirimyces thermohalophilus* as shown by the phylogenomic tree construction (Fig. 2). These results confirm a previous study based on the comparison of rRNA16S gene sequences of the strain with similar species deposed in Gene Bank (Frikha-Dammak et al. 2016). The genome-scale phylogeny constructed in this work clearly places *P. halophilum* among taxons in *Firmicute* Phylum under the family of *Thermoactinomycetaceae* and confirms that *P. halophilum* presents a new genus and a new species.
Table 1
Feature and mobile elements of *P. halophilum*

| Features                      | *P. halophilum* |
|-------------------------------|-----------------|
| Genome size                   | 3.78 Mb         |
| GC %                          | 51.5            |
| Number of contig              | 213             |
| Contig N50                    | 304,586 pb      |
| Contig N90                    | 5 Mb            |
| Secreted proteins             | 3527            |
| Gene cluster predicted        | 9               |
| retroelements                 | 8               |
| Size of retroelements         | 2314 bp         |
| Ty1/Copia(2002)               | 7               |
| Gypsy/DIRS1                   | 1               |
| DNA transposons               | 2               |
| Size of DNA transposons       | 158 pb          |

The comparison with the three closely related species shows that the genome size of *P. halophilum* is similar to that of *P. fulgidum* (3.36 Mb), *K. eburnea* (3.53 Mb) and slightly higher than *M. thermohalophilus* (3.19 Mb) (Table S1). Besides, the GC% content of *P. halophilum* is comparable to the GC% of *M. thermohalophilus* (52.9%), while it is slightly lower compared with GC % of *P. fulgidum* (58.5%) and *K. eburnea* (54.1%). In this database search, 3527 proteins were deduced from 3,702 CDSs (CDSs, CoDing Sequence), which are more important than *K. eburnea* (3360) *M. thermohalophilus* (3074) and *P. fulgidum* (3223). Also, the tRNAScan-SE predicted that a total of 57 tRNAs in the genome of strain SMBg3, that are similar to *K. eburnea* (57 tRNAs), *M. thermohalophilus* (56 tRNAs) but more important than *P. fulgidum* (54 tRNAs). The rRNAs operons present in SMBg3 (5S, 16S, 23S) are also detected in the other comparative strains *M. thermohalophilus* and *P. fulgidum* whereas the *K. eburnea* strain revealed only (5S, 16S) (Table S1).

**Distribution of gene using Gene Ontology (GO)**

Among the 3,775 annotated genes in the *P halophilum* genome, 3,702 genes with specific functions were assigned to GOCs (Gene Ontology Consortium) classified in 13 functional classes (Fig. 3). Among them, protein synthesis (23%), energy metabolism (11.8%), transcription genes (5.1%), and amino acid biosynthesis (9.4%) were abundant categories. The cell envelope (3.1%), the cofactors and prosthetic groups (14.7%) synthesis, DNA metabolism (7.4%), and purine, pyrimidines, nucleoside and nucleotides
synthesis (7.8%) are supplementary functions assigned to GOCs. The unclassified and unknown function were estimated at 4.9% and 3.6% respectively. Based on GO, genes having InterProScan hits were studied for distribution within functional categories. Allowance by GO domains, “Biological Process” and “Molecular Function”, according to generic terms at level 2 in Blast 2GO is illustrated in Fig. 4. The most “Biological process” groups were single organism process (23.23%), metabolic process (30.44%), cellular process (27.22%), biological regulation (5.35%), localization (1.5%), response to stimulus (2.78%), and cellular component organization or biogenesis (2.32%) (Fig. 4a). Distribution according to the “Molecular function” revealed the presence of those genes involved in compound binding (36.46%), catalytic activity (50.17%), transporter activity (5.69%), nucleic acid binding and transcription factor activity (2.66%), structural molecular activity (1.79%), molecular transcription factor activity (0.866%), electron carrier activity (0.835%), antioxidant activity (0.68%) and molecular function regulation (0.06%) (Fig. 4b).

To further distinguish *P. halophilum* from the three closely related thermoactinomycete species, we ran EDGAR analysis and results are shown in Fig. 5. The strain SMBg3 was shown to harbor 43 distinct genes which were not found in the other three closely associated species. Moreover, several distinctive genes were also identified in the other three species, shown in parenthesis: *K. eburnean* (21), *P. fulgidum* (26) and *M. thermohalophilus* (15), respectively (Fig. 5a). Homology searching of genes encoding known proteins involved in secondary metabolism reveals the existence of at least 1638 common gene clusters associated with the biosynthesis of secondary metabolites between the four strains. *P. halophilum* yielded 2621 metabolites, *K. eburnean* showed 2614 genes coding for secondary metabolite, *M. thermohalophilus* provided 2516 as global genes coding for secondary metabolite, and *P. fulgidum* 2264 genes coding for a global metabolic secondary production (Fig. 5b).

**Genes involved in ectoines biosynthesis and degradation**

The second objective of the study was to identify, based on the draft genome of *P. halophilum*, biosynthetic and catabolic pathways of ectoines under salt stress conditions. While the bacterial genome lacked all the genes for ectoines degradation, it contains the whole canonical *ectABCD* ectoine/hydroxyectoine biosynthetic gene cluster, a diaminobutryate-2-oxoglutarate transaminase (ectB), a L-2,4- diaminobutyric acid acetyltransferase (ectA), an ectoine synthase (ectC), and an hydroxyectoine synthase (ectD) (Fig. 6). The nucleotide blast results extracted from NCBI database showed that genes *ectA*, *ectB*, *ectC* and *ectD* of *P. halophilum* shared 100% identity with those of *Streptomyces chrysomallus*.

However, ectoine/hydroxyectoine biosynthetic gene clusters often contained other genes involved in either the transcriptional regulation (*ectR*) of the *ect* operon, the provision of the precursor L-ASA (*asD*) from aspartyl-P and the aspartyl-P from aspartate (*ask_ect*), or sometimes, even a gene for a mechanosensitive channel (*mscS*) (Reshetnikov et al. 2011; Widderich et al. 2016; Czech et al. 2018b). We found that *P. halophilum* genome lacks the mentioned *ectR*, *ask_ect*, or *mscS* genes. However, we found four genes encoding a binding-protein dependent ABC transporter located upstream of the *ectABCD* genes (Fig. 6). A closer analysis of these genes revealed that the encoded proteins are related to those of the functionally characterized ectoine/hydroxyectoine ABC-type uptake system Ehu ABCD from
S. meliloti (Jebbar et al. 2005; Hanekop et al. 2007). To compare the organization of the genomic region encoding the ectoine/hydroxyectoine synthase of P. halophilum with the industrial ectoine producer H. elongata a mauve analysis was also used (Fig. 6). Results showed that the four genes in P. halophilum were organized in a canonical ectABCD cluster, while in H. elongata, the ectD gene encoding the hydroxylase for hydroxyectoine synthesis is located apart from the ectABC cluster. In addition, Ehu genes coding for the transport of ectoine/hydroxyectoine were found only in P. halophilum.

Central carbon metabolism related to the synthesis of precursors of ectoines

As a next step, we mapped in P. halophilum and with the aid of KEGG analysis, the central carbon metabolism to investigate its potency to provide building precursors for ectoines biosynthesis (Czech et al. 2018b; Ma et al. 2020). The bacterial genome carries genes for Glycolysis, Pentose Phosphate (PPP), and Tricarboxylic Acid (TCA) pathways, but missing genes for Entner Doudoroff (ED) pathway (Fig S1). We also assessed a number of key enzymes such as, Pyruvate carboxylase, Phosphoénolpyruvate carboxylase and Oxaloacetate decarboxylase. These enzymes interconvert pyruvate, phosphoenolpyruvate, and OAA and could have a role in supporting high ectoine biosynthetic fluxes by anaplerotic pathways replenishing OAA needed for the TCA cycle. The genes encoding these enzymes, except oxaloacetate decarboxylase were identified from the genome of P. halophilum (Table 2).

| Gene        | Protein                        | ORF number |
|-------------|--------------------------------|------------|
| oad         | oxaloacetate decarboxylase     | -          |
| pcp         | pyruvate carboxylase           | 2          |
| Ppc         | phosphoenolpyruvate carboxylase| 2          |
| Alaata      | alanine aminotransferase       | -          |
| Ald         | alanine dehydrogenase          | -          |
| Glt         | glutamate synthase             | 13         |
| Gdh         | glutamate dehydrogenase        | 3          |
| asp         | Caspartate aminotransferase    | 8          |
| Ask         | aspartokinase                  | 1          |

Regarding nitogen metabolism, analysis of the ectoine biosynthesis pathways revealed the importance of glutamate and alanine in directing fluxes through ectoine synthesis pathway (Ono et al. 1999). A number of 13 copies of genes for glutamate synthase and 3 copies for glutamate dehydrogenase in the P.
halophilum genome were identified, but alanine aminotransferase and L-alanine dehydrogenase were not detected (Table 2). The enzymes specified by these genes are responsible for reductive transfer of ammonium to 2-ketoglutarate to generate glutamate, which acts as the major ammonium donor in the cell (Magasanik 1982). There were also 8 putative aspartate aminotransferases, which catalyze the reversible transfer of the amino group from glutamate to oxaloacetate, rendering aspartate and 2-ketoglutarate. This is a key enzyme as it links the TCA cycle with the first enzyme of the ectoines synthesis pathway (aspartokinase). *P. halophilum* has only one aspartokinase catalyzing the formation of aspartyl phosphate, which is a common metabolic intermediate in the biosynthesis of ectoines and aspartate family of amino acids. Together, these results support that the genome of *P. halophilum* harbors the genes for high flux of ectoines through the metabolic model shown in Fig. 7.

**Genes involved in diverse secondary metabolites biosynthesis**

Another interesting genomic trait of strain *P. halophilum*, is the presence of several new gene clusters that have low similarity with known clusters. A total of 18 gene clusters involved in secondary metabolism were predicted by antiSMASH, including 1 NRPS (non-ribosomal peptide synthetase) type, 1 PKS (polyketide synthase) type 3 and 2 hybrid clusters, namely Type 1 PKS-NRPS and NRPS- fatty acid type biosynthetic clusters (Table 3). Out of the 18 potential biosynthetic clusters, 8 exhibited some level of similarities with known BGC whereas 10 clusters represented orphan BGCs for which no known homologous gene clusters could be identified. Notably, 6 of the known clusters shared similarity with those for antibacterial compounds including colabomycine-E, Fusaricidin-A, Zwittermycin-A, Streptomycin, Meilingmycin, and Mycosubtilin, whereas the 2 others shared similarity with those S-layer-glycan or ectoine compounds. However, the levels of similarity were fairly low in most cases, which suggests the novelty of the possible metabolites from those predicted gene clusters. Several other secondary metabolites could be potentially produced by *P. halophilum*. Among them, one siderophore molecule encoded by cluster 2, one bacteriocin (cluster 16), and two other gene clusters, 8 and 9, are predicted to be responsible for terpene biosynthesis.
Table 3
List of putative secondary metabolite producing biosynthetic clusters from *P. halophilum* genome as predicted by antiSMASH

| Cluster Type From (bp) To (bp) | Most similar known MIBiG BGC-ID biosynthetic cluster* |
|--------------------------------|---------------------------------------------------|
| 1 Cf-fatty-acid 30039 54529    | Colabomycine-E (6%) BGC0000213_c1                  |
| 2 Sidophore 6532480929         | --                                                |
| 3 Cf- saccharide 122667165299  | S-layer-glycan (9%) BGC0000796-c1                  |
| 4 Cf-fatty acid-Nrps 187235255808 | Fusaricidin-A (50%) BGC00001152-c1              |
| 5NRPS 279782 325448            | --                                                |
| 6Ectoine 91720 102106          | Ectoine (100%) BGC0000853-c1                      |
| 7NRPS-T1PKS35902744171        | Zwittermycin-A (25%) BGC00001059-c1               |
| 8Terpene 8549 30450            | --                                                |
| 9Terpene 275106 295939         | --                                                |
| 10Cf -putative 95801 108118    | --                                                |
| 11Cf- saccharide152878 183803  | Streptomycin (3%) BGC0000717-c1                    |
| 12Cf -putative 290995 295475   | Mycosubtilin (20%) BGC0001103-c1                  |
| 13Cf -putative 107 5001        | --                                                |
| 14Cf -putative 81210 92644     | --                                                |
| 15T3PKS 221283 262347          | Meilingmycin (2%) BGC0000093-c1                   |
| 16Cf -putative 15440 27365     | --                                                |
| 17Cf -putative 60137 67649     | --                                                |
| 18Cf -putative 127588 142084   | --                                                |

* The percentage in parentheses indicate the number of genes showing similarity to the corresponding known biosynthetic cluster

**Discussion**

High salinity is a key determinant for the growth of *P. halophilum* in the saltern ponds from which it was originally isolated (Frikha-Dammak et al. 2016). Such a challenging habitat requires active measures by this halophile to counteract the outflow of water from the cells and to optimize the solvent properties of the cytoplasm for biochemical reactions and the functionality of cell components (Czech et al. 2018a). Analysis of the physiological response of *P. halophilum* revealed that this bacterium uses the salt-out
strategy through the accumulation of ectoines at relatively high yields (Ayadi et al. 2020). The data presented here support this conclusion and highlight the ability of \emph{P. halophilum} to cope with a broad spectrum of salinities ranging from 5 to 20%. At an optimal salinity of 15%, the synthesis and accumulation of the effective compatible solute ectoine reached its maximum yield of about 12%.

When the whole genome sequence of \emph{P. halophilum} was sequenced and taxonomically analyzed using the Mauve software pipeline, it was found to be taxonomically clustered with \emph{K. eburnea}, \emph{P. fulgidum} and \emph{M. thermohalophilus} as shown by construction of a phylogenomic tree (Fig. 2). To further infer the evolutionary relatedness of the strain relative to closely related phylogenetic species, we ran EDGAR analysis and results showed that \emph{P. halophilum} harbor 43 distinct genes which were not found in the other three closely associated species (Fig. 5). The 43 distinct genes found in \emph{P. halophilum}, made up approximately 1.7% of the total genome size of the strain. These distinct gene determinants were likely salt resistance proteins, transcriptional regulators, transporter proteins, and for sporulation proteins (Table S2). The comparative genomic analysis of the strain \emph{P. halophilum} with other three strains, confirms a strong adaptation potential possessed by the strain.

Genomics-based approaches have been developed to unveil biosynthetic pathways of ectoines from \emph{P. halophilum}. The four genes (\emph{ectA}, \emph{ectB}, \emph{ectC}, and \emph{ectD}) responsible for ectoines synthesis from L-ASA were identified from the bacterial genome (Fig. 6a). The genetic organization of the \emph{ectABC} operon from the strain is in canonical arrangement (Schwibbert et al. 2011; Widderich et al. 2014, 2016) as all genes were located in the same cluster. The presence and the arrangement of these fourth genes in an operon is also found in some other halophilic bacteria such as the industrial strain \emph{H. elongata}, \emph{C. salexigens}, and \emph{Streptomyces coelicolor} (Han et al. 2018). However, a different genetic organization of the \emph{ectABCD} genes in the genome sequence of \emph{P. halophilum} was observed. These are positioned next to a gene cluster (\emph{ehuABCD}) encoding a binding-protein-dependent ABC transport system that serves to carry ectoine and 5-hydroxyectoine to the surrounding medium (Jebbar et al. 2005). This Ehu transport system in the \emph{P. halophilum} genome seems to be primarily involved in the adjustment of the intracellular ectoines concentration by its secretion in extracellular space instead of its degradation as in \emph{H. elongata} (Schwibbert et al. 2011) or \emph{C. salexigens} (Vargas et al. 2006). Indeed, previous studies have revealed that the genetically engineered \emph{H. elongata} strain, lacked ectoine catabolic genes (Schwibbert et al. 2011; Schulz et al. 2017) and their osmotically controlled ectoine/hydroxyectoine-specific tripartite ATP-independent periplasmic (TRAP) transport system TeaABC (Grammann et al. 2002) was deleted. It excretes considerable amounts of ectoines into the growth medium and allows their recovery in a highly purified form on the scale of several tons per annum (Kunte et al. 2014). This genetic arrangement of \emph{ehu-ect} gene cluster is also found in the \emph{Nitrospina} sp. SCGCAA799_C22 isolate, a bacterium that lives in the polyextreme interfaces of Red Sea brines (Ngugi et al. 2016), and also in the thermo-halotolerant Gram-positive bacterium \emph{Paenibacilli lautus} (Richter et al. 2019).

High-level ectoines impose a biosynthetic burden on cells. For this purpose, we assessed a number of routes related to central metabolism leading to precursors of ectoines. Genome analysis revealed that the central catabolic network in \emph{P. halophilum} comprises the pathways of glycolysis, pentose phosphate
(PPP), and tricarboxylic acid (TCA) cycle that provide OAA, acetyl-coASH and NADPH2 required for ectoines production (Fig. S1). In addition, *P. halophilum* possesses specific genes encoding for pyruvate carboxylase (Pc) and phosphoenolpyruvate carboxylase (Ppc) involved in anaplerotic replenishment of the TCA cycle (Table 2). These enzymes convert respectively pyruvate and phosphoenolpyruvate to OAA and could have a role in replenishing the precursor L-ASA, the starting metabolite of the pathway for biosynthesis of ectoines and aspartate family amino acids (threonine, methionine, lysine). Theses anaplerotic replenishment pathways were also detected in *C. salexiogens* (Pastor et al. 2013) and *H. elongata* (Schwibbert et al. 2011). Since ectoines are nitrogen compounds and their biosynthesis also depends on a good supply of glutamate used as a substrate of EctB in the first step of L-2, 4-diaminobutyrate biosynthesis, we analyzed key enzymes involved in nitrogen assimilation. Interestingly, we found 3 copies of glutamate deshydrogenase, and 13 copies of glutamate synthase in *P. halophilum* genome. These enzymes are involved in anaplerotic replenishment of intracellular glutamate pool, which in turn directs the cellular metabolism toward high-levels of ectoines. It is known that glutamate dehydrogenase (GDH) catalyses a reaction between ammonium and 2-oxoglutarate to directly build glutamate in an NADPH-dependent reaction, while glutamate synthase (GS) assimilates ammonium and glutamate into glutamine; and subsequently, the glutamate synthase (GOGAT) converts glutamine and 2-oxoglutarate to form two molecules of glutamate (Magasanik 1982). These two glutamate supply cellular pathways identified in *P. halophilum* were previously identified in the model strain of actinomycete, *Streptomyces coelicolor* and showed to be activated depending on nitrogen availability (Tiffert et al. 2008; Shao et al. 2015). In addition, aspartate aminotransferase catalyzes the reversible transfer of the amino group from glutamate to oxaloacetate, and links the TCA cycle with aspartokinase, the first enzyme of the ectoines synthesis pathway. Together, these results suggested that *P. halophilum* can be considered as a new natural cell factory for high-levels production of ectoines. Indeed, our preliminary batch fermentation in lab-scale 7.5 L bioreactor showed that the conversion yield of ectoines on glucose reached the value of 37% with a yield of 0.63 g.(g dry cellular weight)\(^{-1}\), threefold higher than that of *H. elongata* and half of which was found in the fermentation supernatant (unpublished data).

Another interesting genomic trait of *P. halophilum*, the presence of several new gene clusters coding for new natural products that may be undetected under standard fermentation conditions (Winter et al. 2011). In *P. halophilum* several new gene clusters that have low similarity with known clusters have been detected (Table 3). As the typical PKSI, the biosynthetic gene cluster of Zwittermycin-A (ZmA) identified in *P. halophilum* has been first identified in *Bacillus cereus* UW85, including the condensation of five precursors (Kevany et al. 2009). Two of these precursors, L-Ser and malonyl-CoA, would be readily available for ZmA biosynthesis, being common primary metabolites. The three remaining precursors would be unique to ZmA biosynthesis and would require specific enzymes for their formation. The PKS gene cluster of ZmA from *Bacillus cereus* UW85 contains *zmaA*, *zmaB*, *zmaK*, *zmaM*, *zmaO* and *zmaQ* and encodes Transcriptional regulator, Aminotransferase, UDP-kanosamine hydrolase, UDP-glucose C3 dehydrogenase and Kanosamine transporter (Kevany et al. 2009). As the typical PKS III, Meilingmycin detected in SMBg3, a macrolide antibiotic structurally and biologically similar to avermectin was already found in *Streptomyces nanchangensis* (Yuhui et al. 2002). Meilingmycin differs from avermectin in the
fact that it has no α-L-oleandrose attached at position 13 of the macrolide ring but has an isopantenoic acid moiety at position 4, which is probably derived from valine. We also identified in *P. halophilum* genome a biosynthetic cluster showing 50% similarity with known Fusaricidin biosynthetic cluster (BGC0001152_c1). Fusaricidin is a lipopeptide antibiotics previously produced by *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*) and consists of a guanidinylated β-hydroxy fatty acid linked to a cyclic hexapeptide including four amino acid residues in the D-configuration (Kuroda et al. 2000). Besides, *P. halophilum* harbors the ectoine biosynthetic pathway that shows 100% similarity with ectoine biosynthetic cluster of *Streptomyces chrysomallus* (BGC0000853_c1). Finally, the availability of the genome sequence of *P. halophilum* SMBg3 provides a framework for biotechnological analysis and characterization of new natural products.

**Conclusion**

This work presents the first insight into the genome of the highly salt resistant bacterium *P. halophilum*. Our data revealed that the metabolism of the strain under salt stress is well adapted to support high fluxes biosynthetic toward ectoines through an *ehu-ect* gene cluster. The existence of multiple routes for the flux-tuning of ectoines precursors, the ability to release ectoines to the surrounding medium, and the inability to degrade ectoines, might allow the strain to attain high total yields of products. In addition, prediction of biosynthetic gene clusters for secondary metabolites suggested that this strain has the potential to produce novel natural products, which could be of industrial or scientific significance. Currently, our work is in progress to develop at pilot-scale, a process for efficient production of ectoines by *P. halophilum* coupling feed-batch fermentation with the downstream extraction processing.

**Declarations**

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**Conflict of interest:** The authors declare that there are no conflicts of interest.

**References**

1. Abdel-Aziz H, Wadie W, Abdallah DM, Lentzen G, Khayyal MT (2013) Novel effects of ectoine, a bacteria-derived natural tetrahydropyrimidine, in experimental colitis. Phytomed 20:585–91. [https://doi.org/10.1016/j.phymed.2013.01.009](https://doi.org/10.1016/j.phymed.2013.01.009)

2. Ayadi H, Frikha-Dammak D, Fakhfakh J, Chamkha M, Hassairi I, Allouche N, Sayadi S, Maalej S (2020) The saltern-derived *Paludilum halophilum* DSM 102817T is a new high-yield ectoines producer in minimal medium and under salt stress conditions. 3 Biotech 10: 533. [https://doi.org/10.1007/s13205-020-02512-x](https://doi.org/10.1007/s13205-020-02512-x)
3. Baral B, Akhgari A, Metsä-Ketelä M (2018) Activation of microbial secondary metabolic pathways: Avenues and challenges. Synth Syst Biotechnol 3:163–178. https://doi.org/10.1016/j.synbio.2018.09.001

4. Blin K, Shaw S, Steinke K, Villebro R, Ziemert N, Lee SY, Medema MH, Weber T (2019) antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. Nucleic Acids Res 47: W81-W87. https://doi.org/10.1093/nar/gkz310

5. Boujelben I, Martínez-García M, Pelt JV, Maalej S (2015) Diversity of cultivable halophilic archaea and bacteria from superficial hypersaline sediments of Tunisian solar salt pans. Antonie Leeuwenhoek 106:675-692. https://doi.org/10.1007/s10482-014-0238-9

6. Brands S, Schein P, Castro-Ochoa KF, Galinski EA (2019) Hydroxyl radical scavenging of the compatible solute ectoine generates two N-acetimides. Arch Biochem Biophys 674:108097. https://doi.org/10.1016/j.abb.2019.108097

7. Bremer E, Krämer R (2000) Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes, p.79-97. In: Storz G, Hengge-Aronis R (ed) Bacterial stress responses. ASM Press, Washington DC

8. Czech L, Hermann L, Stöveken N, Richter AA, Höppner A, Smits SHJ, Heider J, Bremer E (2018a) Role of the Extremolytes Ectoine and Hydroxyectoine as Stress Protectants and Nutrients: Genetics, Phylogenomics, Biochemistry, and Structural Analysis. Genes 9: 177. https://doi.org/10.3390/genes9040177

9. Czech L, Poehl S, Hub P, Stoveken N. Bremer E (2018b) Tinkering with osmotically controlled transcription allows enhanced production and excretion of ectoine and hydroxyectoine from a microbial cell factory. Appl Environ Microbiol 84, e01772-17. https://doi.org/10.1128/AEM.01772-17.

10. Darling AE, Mau B, Perna NT (2010) progressiveMauve: Multiple Genome Alignment with Gene Gain, Loss and Rearrangement. PLoS ONE 5: e11147. https://doi.org/10.1371/journal.pone.0011147

11. Doroghazi JR, Albright J, Goering AW, Ju KS, Haines RR, Tchalukov KA, Labeda DP, Kelleher NL, Metcalf WW (2014). A roadmap for natural product discovery based on large scale genomics and metabolomics. Nat Chem Biol 10:963-968. https://doi.org/10.1038/nchembio.1659.

12. Frikha-Dammak D, Fardeau ML, Cayol JL et al (2016) Paludilum halophilum gen. nov., sp. nov., a thermoactinomycete isolated from superficial sediment of a solar saltern. Int J Sys Evol Microbiol 66: 5371-5378. https://doi.org/10.1099/ijsem.0.001523

13. Galperin MY, Makarova KS, Wolf YI, Koonin E. (2015) Expanded microbial genome coverage and improved protein family annotation in the COG database. Nucleic Acids Res 43: D261–D269. https://doi.org/10.1093/nar/gku1223

14. Garcia-Estepa R, Argandona M, Reina-Bueno M, Capote N, Iglesias-Guerra F, Nieto JJ et al. (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. https://doi.org/10.1128/JB.00136-06
15. García-Alegria AM, Anduro-Corona I, Pérez-Martínez CJ, Corella-Madueño MAG, Rascon-Duran ML, Astiazaran-Garcia H (2020) Quantification of DNA through the NanoDrop Spectrophotometer: Methodological Validation Using Standard Reference Material and Sprague Dawley Rat and Human DNA. Int J Anal Chem Article ID 8896738, https://doi.org/10.1155/2020/8896738

16. Gibtan A, Park K, Woo M, Shin JK, Lee DW, Sohn JH, Song M, Roh SW, Lee SJ, Lee HS (2017) Diversity of Extremely Halophilic Archaeal and Bacterial Communities from Commercial Salts. Front Microbiol 8:799. https://doi.org/10.3389/fmicb.2017.00799

17. Graf R, Anzali S, Buenger J, Pfluecker F, Driller H (2008) The multifunctional role of ectoine as a natural cell protectant. Clin Dermatol 26:326-33. https://doi.org/10.1016/j.clindermatol.2008.01.002

18. Grammann K, Volke A, Kunte HJ (2002) New type of osmoregulated solute transporter identified in halophilic members of the bacteria domain: TRAP transporter TeaABC mediates uptake of ectoine and hydroxyectoine in *Halomonas elongata* DSM 2581T. J Bacteriol 184: 3078-3085. https://doi.org/10.1128/JB.184.11.3078-3085.2002

19. Han J, Gao QX, Zhang YG, Li L, Mohamad OAA, Narsing Rao MP, Xiao M, Hozzein WN, Alkhaliﬁah DHM, Tao Y, Li WJ (2018) Transcriptomic and Ectoine Analysis of Halotolerant *Nocardiopsis* *gilva* YIM 90087T Under Salt Stress. Front Microbiol 9:618. https://doi.org/10.3389/fmicb.2018.00618

20. Hanekop N, Höing M, Sohn-Bösser L, Jebbar M, Schmitt L, Bremer E (2007) Crystal structure of the ligand-binding protein EhuB from *Sinorhizobium meliloti* reveals substrate recognition of the compatible solutes ectoine and hydroxyectoine. J Mol Biol 374:1237-50. https://doi.org/10.1016/j.jmb.2007.09.071

21. Hahn MB, Meyer S, Schroter MA, Kunte HJ, Solomun T, Sturm H (2017) DNA protection by ectoine from ionizing radiation: molecular mechanisms. Phys Chem Chem Phys 19: 25717-25722. https://doi.org/10.1039/c7cp02860a

22. Höppner A, Widderich N, Lenders M, Bremer E, Smits SHJ (2014) Crystal structure of the ectoine hydroxylase, a snapshot of the active site. J Biol Chem 289: 29570-29583. https://doi.org/10.1074/jbc.M114.576769

23. Hu D, Chen Y, Sun C, Jin T, Fan G, Liao Q, Mok KM, Simon Lee MY (2018) Genome guided investigation of antibiotics producing actinomycetales strain isolated from a Macau mangrove ecosystem. Scientific Reports 8:14271. https://doi.org/10.1038/s41598-018-32076-z

24. Jebbar M, Sohn-Bösser L, Bremer E, Bernard T, Blanco C (2005) Ectoine-induced proteins in *Sinorhizobium meliloti* include an ectoine ABC-type transporter involved in osmoprotection and ectoine catabolism. J Bacteriol 187: 1293-1304. https://doi.org/10.1128/JB.187.4.1293-1304.2005

25. Jiang Z, Xiao M, Yang LL, Zhi XY, Li WJ (2019) Genome-based taxonomic classiﬁcation within the family Thermoactinomycetaceae. Int J Syst Evol Microbiol 69:2028-2036. https://doi.org/10.1099/ijs.0.003425

26. Kanapathipillai M, Lentzen G, Sierks M, Park CB (2005) Ectoine and hydroxyectoine inhibit aggregation and neurotoxicity of Alzheimer’s beta-amyloid. FEBS Lett 579:4775-80. https://doi.org/10.1016/j.febslet.2005.07.057
27. Kanehisa M, Goto S, Sato Y, Furumichi M, Tanabe M (2012) KEGG for integration and interpretation of large-scale molecular datasets. Nucleic Acids Res 40:109-114. https://doi.org/10.1093/nar/gkr988

28. Kempf B, Bremer E (1998) Uptake and synthesis of compatible solutes as microbial stress responses to high osmolality environments. Arch Microbiol 170: 319-330. https://doi.org/10.1007/s002030050649

29. Kevany BM, Rasko DA, Thomas MG (2009) Characterization of the Complete Zwittermicin A Biosynthesis Gene Cluster from Bacillus cereus. Appl Environ Microbio 75: 1144-1155. https://doi.org/10.1128/AEM.02518-08

30. Kim J, Shin D, Kim SH, Park W, Shin Y, Kim WK, Lee SK, Oh KB, Shin J, Oh DC (2014) Borrelidins C-E: new antibacterial macrolides from a saltern-derived halophilic Nocardiopsis sp. Marine Drugs 15: 166-177. https://doi.org/10.3390/md15060166

31. Kunte HJ, Lentzen G, Galinski E (2014) Industrial production of the cell protectant ectoine: protection, mechanisms, processes, and products. Curr Biotechnol 3: 10-25. https://doi.org/10.2174/22115501113026660037

32. Kunte HJ, Galinski EA, Trüper HG (1993) A modified FMOC-method for the detection of aminoacid type osmolytes and tetrahydropyrimidines (ectoines). J Microbiol Methods 17:129-136. https://doi.org/10.1016/0167-7012(93)90006-4

33. Kuroda J, Fukai T, Konishi M, Uno J, Kurusu K, Nomura T (2000) LI-F antibiotics, a family of antifungal cyclic depsipeptides produced by Bacillus polymyxa L-1129. Heterocycles 53, 1533–1549.

34. León MJ, Hoffmann T, Sánchez-Porro C, Heider J, Ventosa A and Bremer E (2018) Compatible Solute Synthesis and Import by the Moderate Halophile Spiribacter salinus: Physiology and Genomics. Front Microbiol 9:108. https://doi.org/10.3389/fmicb.2018.00108

35. Ma H, Zhao Y, Huang W, Zhang L, Wu F, Ye J, Chen GQ (2020). Rational flux-tuning of Halomonas bluephagenesis for co-production of bioplastic PHB and ectoine. Nature communications 11:3313. https://doi.org/10.1038/s41467-020-17223-3

36. Magasanik B (1982) Genetic control of nitrogen assimilation in bacteria. Ann Rev Genet 16: 135-168.

37. Manivasagan P, Venkatesan J, Sivakumar K, Kim SK (2014) Pharmaceutically active secondary metabolites of marine actinobacteria. Microbiol Res 169: 262-278. https://doi.org/10.1016/j.microres.2013.07.014

38. Min B, Kim S, Oh YL, Kong WS, Park H, Cho H, Jang KY, Kim JG, Choi IG (2018) Genomic discovery of the hypsin gene and biosynthetic pathways for terpenoids in Hypsizygus marmoreus. BMC Genomics 19:789. https://doi.org/10.1186/s12864-018-5159-y

39. Ngugi DK, Blom J, Stepanauskas R, Stingl U (2016) Diversification and niche adaptations of Nitrospina-like bacteria in the polyextreme interfaces of Red Sea brines. ISME J 10; 1383-1399. https://doi.org/10.1038/ismej.2015.214

40. Ono H, Sawada K, Khunajakr N, Tao T, Yamamoto M, Hiramoto M, Shinmyo U, Takano M, Murooka Y (1999) Characterization of biosynthetic enzymes for ectoine as a compatible solute in a moderately
41. Pastor JM, Bernal V, Salvador M, Argandoña M, Vargas C, Csonka L, Sevilla A, Iborra JL, Nieto JJ, Cánovas M (2013) Role of Central Metabolism in the Osmoadaptation of the Halophilic Bacterium *Chromohalobacter salexigens*. J Bio Chem 288 : 17769-17781. https://doi.org/10.1074/jbc.M113.470567

42. Pastor JM, Salvador M, Argandona M, Berna V, Reina-Bueno M, Csonka LN, et al. (2010) Ectoines in cell stress protection: uses and biotechnological production. Biotechnol Adv 28: 782–801. https://doi.org/10.1016/j.biotechadv.2010.06.005

43. 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. https://doi.org/10.1128/AEM.70.5.3130-3132.2004

44. Reshetnikov AS, Khmelenina VN, Trotsenko YA. (2006). Characterization of the ectoine biosynthesis genes of haloalkalotolerant obligate methanotroph "Methylomicrium alcaliphilum 20Z". Arch. Microbiol. 184, 286-297. https://doi.org/10.1007/s00203-005-0042-z

45. Reshetnikov AS, Khmelenina VN, Mustakhimov II, Trotsenko YA (2011). Genes and enzymes of ectoine biosynthesis in halotolerant methanotrophs. Methods Enzymol. 495 : 15-30. https://doi.org/10.1016/B978-0-12-386905-0.00002-4

46. Richter AA. et al. (2019) Biosynthesis of the stress-protectant and chemical chaperon ectoine: biochemistry of the transaminase EctB. Front Microbiol 10 : 2811. https://doi.org/10.3389/fmicb.2019.02811

47. Rosa LT, Bianconi ME, Thomas GH, Kelly DJ (2018) Tripartite ATP-independent periplasmic (TRAP) transporters and tripartite tricarboxylate transporters (TTT) : From uptake to pathogenicity. Front Cell Infect Microbiol 8 : 33. https://doi.org/10.3389/fcimb.2018.00033

48. Schröter MA, Meyer S, Hahn MB, Solomun T, Sturm H, Kunte HJ (2017) Ectoine protects DNA from damage by ionizing radiation. Sci Rep 7:15272. https://doi.org/10.1038/s41598-017-15512-4

49. Schwibbert K, Marin-Sanguino A, Bagyan I, Heidrich G, Lentzen G, Seitz H, et al. (2011) A blueprint of ectoine metabolism from the genome of the industrial producer *Halomonas elongata* DSM 2581T. Environ Microbiol 13: 1973-1994. https://doi.org/10.1111/j.1462-2920.2010.02336.x

50. 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. https://doi.org/10.1128/JB.00345-11

51. Schulz A, Stöveken N, Binzen IM, Hoffmann T, Heider J, Bremer E (2017) Feeding on compatible solutes: a substrate-induced pathway for uptake and catabolism of ectoines and its genetic control by EnuR. Environ Microbiol 19:926-946. https://doi.org/10.1111/1462-2920.13414

52. Shao Z, Deng W, Li S, He J, Ren S, Huang W, Lu Y, Zhao G, Cai Z, Wang J (2015) GlnR-mediated regulation of ectABCD transcription expands the role of the GlnR regulon to osmotic stress management. J Bacteriol 197:3041-3047. https://doi.org/10.1128/JB.00185-15
53. Sun Y, Zhou X, Liu J, Bao K, Zhang G, Tu G, Kieser T, Deng Z (2002) *Streptomyces nanchangensis*, a producer of the insecticidal polyether antibiotic nanchangmycin and the antiparasitic macrolide meilingmycin, contains multiple polyketide gene clusters. Microbiology 148: 361-371. https://doi.org/10.1099/00221287-148-2-361

54. Ter Beek J, Guskov A, Slotboom DJ (2014) Structural diversity of ABC transporters. J Gen Physiol 143: 419-435. https://doi.org/10.1085/jgp.201411164

55. Tiffert Y, Supra P, Wurm R, Wohlleben W, Wagner R, Reuther J (2008) The *Streptomyces coelicolor* GlnR regulon: identification of new GlnR targets and evidence for a central role of GlnR in nitrogen metabolism in actinomycetes. Mol Microbiol 67: 861-880. https://doi.org/10.1111/j.1365-2958.2007.06092.x

56. Van Thuoc D, Loan TT, Trung TA, Quyen NV, Tung PN, Sudesh K (2020) Genome Mining Reveals the Biosynthetic Pathways of Polyhydroxyalkanoate and Ectoines of the Halophilic Strain *Salinivibrio proteolyticus* M318 Isolated from Fermented Shrimp Paste. Marine Biotechnol 22:651-660. https://doi.org/10.1007/s10126-020-09986-z

57. Vargas C, Jebbar M, Carrasco R, Blanco C, Calderon MI, Iglesias-Guerra F, Nieto JJ (2006) Ectoines as compatible solutes and carbon and energy sources for the halophilic bacterium *Chromohalobacter salexigens*. J Appl Microbiol 100: 98-107. https://doi.org/10.1111/j.1365-2672.2005.02757.x

58. Widderich N, Höppner A, Pittelkow M, Heider J, Smits SH, Bremer E (2014) Biochemical properties of ectoine hydroxylases from extremophiles and their wider taxonomic distribution among microorganisms. PloS One 9:e93809. https://doi.org/10.1371/journal.pone.0093809

59. Widderich N, Czech L, Elling FJ, Könneke M, Stöveken N, Pittelkow M, Riclea M, Dickschat JS, Heider J, Bremer E (2016) Strangers in the archaeal world: osmostress-responsive biosynthesis of ectoine and hydroxyectoine by the marine thaumarchaeon *Nitrosopumilus maritimus*. Environ. Microbiol. 18: 1227-1248. https://doi.org/10.1111/1462-2920.13156

60. Winter JM, Behnken S, Hertweck C (2011) Genomics-inspired discovery of natural products. Curr Opin Chem Biol 15: 22–31. https://doi.org/10.1016/j.cbpa.2010.10.020

61. Wood JM, Bremer E, Csonka LN, Kraemer R, Poolman B, Van der Heide T, Smith LT (2001) Osmosensing and osmoregulatory compatible solute accumulation by bacteria. Comput Biochem Physiol A: Mol Integr Physiol 130: 437-460. https://doi.org/10.1016/s1095-6433(01)00442-1

62. Yang YL, Zhang S, Ma K, Xu Y, Tao Q, Chen Y, Chen J, Guo S, Ren J, Wang W, Tao Y, Yin WB, Liu H (2017) Discovery and characterization of a new family of diterpene cyclases in bacteria and fungi. Angew Chem Int Ed Engl 56: 4749-4752. https://doi.org/10.1002/anie.201700565

63. Zhao Q, Meng Y, Li S, Lv P, Xu P, Yang C (2018) Genome sequence of *Halomonas hydrothermalis* Y2, an efficient ectoine producer isolated from pulp mill wastewater. J Biotechnol 285:38-41. https://doi.org/10.1016/j.jbiotec.2018.08.017

64. Zheng Y, Saitou A, Wang CM, Toyoda A, Minakuchi Y, Sekiguchi Y, Ueda K, Takano H, Sakai Y, Abe K, Yokota A, Yabe S (2019) Genome Features and Secondary Metabolites Biosynthetic Potential of the Class *Ktedonobacteria*. Front Microbiol 10:893. https://doi.org/10.3389/fmicb.2019.00893
Figures

Figure 1

Growth and ectoines content of P. halophilum cells at increasing salinities (a) and HPLC-UV chromatogram (210 nm) showing the production of ectoine and hydroxyectoine by P. halophilum mycelial biomass (b). To determine the growth properties of P. halophilum in response to various
concentrations of NaCl, the cells were cultured for 6 days in optimized mineral SW medium at 40°C and their growth rates were determined by measuring the mycelial dry weight. Given are the averaged values and standard deviations derived from three independently grown cultures.

Figure 2

The phylogenomic tree showing the relationship of P. halophilum and other related taxa based on the whole genome. The numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 1000 replications. Thermoactinomyces vulgaris was chosen as an outgroup strain.
Figure 3

Pie chart of GO (gene Ontology) analysis summarized P. halophilum genes, according to molecular function.
Figure 4

GO based functional annotation of genes present in the P. halophilum genome. Biological Process (a) and Molecular function domains (b)
Figure 5

Venn diagram of the number of homologous genes between P. halophilum and K. eburnea, M. thermohalophilus and P. fulgidum with its closest functional relatives, respectively. BioVenn, a web application for the comparison and visualization of biological lists, has been used for Venn diagrams drawing.
**Figure 6**

Genetic organization of the ehu-ect cluster from *P. halophilum* and comparison with ect cluster of *H. elongata*. Genes annotation: EhuC, ectoine/hydroxyectoine ABC transporter permease; EhuB, ectoine/hydroxyectoine ABC transporter substrate-binding protein; ectA, diaminobutyrate acetyltransferase; PP_2800, diaminobutyrate-2-oxoglutarate transaminase (ectB); ectC, ectoine synthase; thpD, ectoine hydroxylase (ectD).
Figure 7

The reconstructed metabolic flux model of ectoine production in P. halophilum in relation with central catabolic pathway of glucose. pc and ppc encoding pyruvate carboxylase and phosphoenolpyruvate for enhanced accumulation of OAA. glt and gcdh encoding glutamate synthase and glutamate dehydrogenase for enhanced accumulation of glutamate. Replenishing pathways are represented by dashed lines.
Supplementary Files

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- supplementarymaterials.docx