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Trimethylated homoserine functions as the major compatible solute in the globally significant oceanic cyanobacterium *Trichodesmium*

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The oceanic N_2-fixing cyanobacterium *Trichodesmium* spp. form extensive surface blooms and contribute significantly to marine carbon and nitrogen cycles in the oligotrophic subtropical and tropical oceans. *Trichodesmium* grows in salinities from 27 to 43 parts per thousand (ppt), yet its salt acclimation strategy remains enigmatic because the genome of *Trichodesmium erythraeum* strain IMS101 lacks all genes for the biosynthesis of any known compatible solute. Using NMR and liquid chromatography coupled to mass spectroscopy, we identified the main compatible solute in *T. erythraeum* strain IMS101 as the quaternary ammonium compound *N,N,N*-trimethyl homoserine (or homoserine betaine) and elucidated its biosynthetic pathway. The identification of this compatible solute explains how *Trichodesmium* spp. can thrive in the marine system at varying salinities and provides further insight into the diversity of microbial salt acclimation.

salt stress | compatible solute | cyanobacteria

The N_2-fixing (diazotrophic) filamentous cyanobacterium *Trichodesmium* spp. thrive in the present subtropical and tropical oceans, forming expansive blooms that are visible by satellite (1). *Trichodesmium* spp. are globally significant contributors of new N to the N-limited surface ocean and have thus been investigated intensely since their discovery as diazotrophs in 1960 (2, 3). *Trichodesmium*'s successful adaptive physiology includes its unique N_2 fixation strategy (4); its ability to collect and dissolve dust for micronutrients (5); its flexible P utilization and accumulation (6, 7); and its predicted expansion in the warm and acidified ocean of the future (8, 9). However, despite extensive *Trichodesmium* research, the fundamental process of osmoregulation that enables its acclimation to oceanic salinities has yet to be elucidated.

The saline environment forms one of the most fundamental properties of the marine realm, with sea-surface salinities ranging globally from ~6 parts per thousand (ppt) (Baltic) to ~41 ppt (Red Sea) and typical open-ocean salinities of ~35–36 ppt. In most microorganisms, salt acclimation is performed by using the "salt-out" strategy. In this scenario, cells export inorganic ions and accumulate low-molecular-mass organic compounds. These compatible solutes do not interfere with cell metabolism (10) and ensure osmotic equilibrium (11, 12). Currently, ~30 different structures for compatible solutes are known, and they belong to different chemical classes, such as sugars, polyols, heterosides, amino acids, and amino acid derivatives (13). Among cyanobacteria, the first compatible solute [glucosylglycerol(2-*O*-α-D-glucopyranosylglycerol (GG)] was found in the marine strain *Synechococcus* sp. RRIMP N 100 by applying ^{13}C-NMR spectroscopy (14). Subsequent searches for compatible solutes in ~200 strains revealed that GG is characteristic of marine cyanobacteria. GG provides moderate halotolerance (up to threefold seawater conditions), whereas freshwater (tolerating salinities lower than that of seawater) cyanobacteria preferentially synthesize the sugars sucrose or trehalose, and halophilic cyanobacteria, resisting saturated salt brines (up to 2.5 NaCl), accumulate a mixture of compatible solutes that is dominated by glycine betaine (15).

The >200 publicly available cyanobacterial genomes (16) represent a blueprint of cyanobacterial evolution and their adaptation to diverse environmental conditions. Although most marine strains harbor genes for GG accumulation, some exceptions were identified in our genomic analyses of potential salt tolerance in cyanobacteria (17). All strains of the ubiquitous marine *Prochlorococcus* spp. did not accumulate GG, but grew in full marine waters using sucrose and glucosylglycerate (GGA) as compatible solutes (18), whereas strains of the marine, unicellular, N_2-fixing cyanobacterium *Crocosphaera watsonii* accumulated solely trehalose (19).

The genome of *Trichodesmium erythraeum* strain IMS101 [*Trichodesmium* IMS101, National Center for Biotechnology Information (NCBI) reference sequence ID NC_008312.1] is also exceptional because it lacks genes for the biosynthesis of any known compatible solute. We therefore investigated what compound (or compounds) *Trichodesmium* uses to osmoregulate and thrive in the ocean.

Results

Identification of the Compatible Solute. To analyze the salt adaptation of *Trichodesmium* IMS101, cultures were cultivated in artificial seawater with salt supplementations ranging from 30 to 48 ppt. The maximal growth occurred at 30–37 ppt, with growth declining by 83% when salinity was increased to 43 ppt (Fig. 1*A* and Table S1). Genomic searches of *Trichodesmium* IMS101 yielded no gene for the synthesis of any known compatible solutes from cyanobacteria (GG, trehalose, sucrose, glycine betaine, and GGA; ref. 17).

Significance

Trichodesmium spp. are globally significant contributors of new nitrogen to the surface ocean. Marine organisms must accumulate compatible solutes in their cells, counteracting the high exterior osmotic pressure. However, *Trichodesmium* does not possess any known genes for the synthesis of compatible solutes, making its proliferation in the high-salinity environment enigmatic. We demonstrate that *Trichodesmium* cultures in the laboratory as well as natural populations in the ocean synthesize homoserine betaine, previously unknown as a compatible solute, and elucidated the biosynthetic pathway. The high intracellular concentrations will lead to a major injection of this organic compound into the oligotrophic ocean, when natural *Trichodesmium* blooms lyse. Such sudden releases of homoserine betaine could impact the biogeochemical cycling of carbon and nitrogen.

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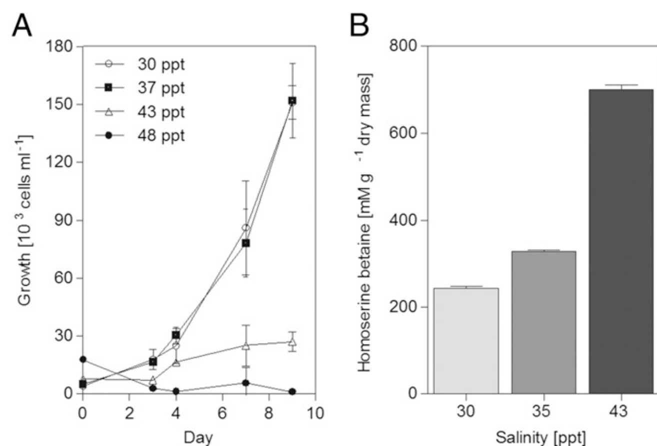

Fig. 1. Salt-dependent growth and accumulation of compatible solute in *Trichodesmium* spp. (*A*) Growth curve based on cell concentration of four *Trichodesmium* IMS101 cultures, each grown in artificial seawater medium supplemented with 30, 35, 43, or 48 ppt NaCl over the course of 9 d. (*B*) Intracellular homoserine betaine concentration in *Trichodesmium* IMS101, which was grown in artificial seawater medium supplemented with 30, 35, or 43 ppt NaCl as determined by HPLC-MS.

Moreover, corresponding peaks of these compatible solutes were absent after analyzing *Trichodesmium* IMS101 cell extracts by our established chromatographic methods.

Subsequent analyses of *Trichodesmium* IMS101 extracts by advanced liquid chromatography (LC) and HPLC coupled to mass spectroscopy (MS) yielded one prominent low-molecular-mass component with an HPLC retention time of 6.8 min and a monoisotopic mass (m/z) of 162 in cells grown at different salinities (Fig. 2*A*). This finding provided the first hint that *Trichodesmium* accumulates large amounts of an unknown, low-molecular-mass organic compound that may serve as a major compatible solute to balance the osmotic potential because its concentration increased with increasing external salinity (Fig. 1*B*). To rule out the possibility that this compound was synthesized by heterotrophic bacteria in the nonaxenic *Trichodesmium* IMS101 culture, the main contaminating bacteria were isolated and cultured at different salinities using minimal medium with glucose as carbon source. Extracts from these cultures never showed a peak at 6.8 min and a monoisotopic mass of 162. Furthermore, extracts of *Trichodesmium* spp. populations sampled from the Gulf of Eilat/Aqaba in the northern Red Sea showed the same prominent peak at 6.8 min with a mass of 162 (Fig. 2*C*), which was found before in the laboratory cultures of *Trichodesmium* IMS101. These results verified that the unknown compatible solute is synthesized by *Trichodesmium* and that it is not restricted to the laboratory strain, but is also found in naturally occurring *Trichodesmium* populations.

Because of the fragment pattern of the unknown compound in LC- and HPLC-MS systems, we assumed that the compound contains one N atom that could be directly bound to methyl groups. Accordingly, screening of all known and relevant compatible solute structures identified one candidate as carnitine, which contains one N and has the same mass (m/z) of 162 as that found in *Trichodesmium* IMS101 extracts. Carnitine is a quaternary ammonium compound that acts as a compatible solute in some heterotrophic bacteria (20) and diverse eukaryotes (21). However, carnitine showed a different retention time (6.04 min) in our HPLC-MS system (Fig. 2*C*), demonstrating that the unknown substance in *Trichodesmium* IMS101 is not carnitine.

To identify the structure of the compatible solute, we performed NMR analyses using crude ethanolic extracts of *Trichodesmium* IMS101. The ^{1}H-NMR spectrum recorded in D_2O solution comprised several defined signals (Fig. 3) and a large singlet at δ 3.23, which reflects total integration 9H and stands for three methyl groups bound to the nitrogen atom ($^{+}NMe_3$). Moreover, we observed four multiplets. The first one at δ 3.79 (2H) is assignable to the protons of the α-methine and γ-methylene groups ($CH_{(\alpha)}$ and $CH_{2(\gamma)}$), and the second one at δ 3.62 (1H) represents the proton of the γ-methylene group ($CH_{2(\gamma)}$). The last two multiplets at δ 2.24 (1H) and 2.10 (1H) were assignable to the protons of the γ-methylene groups.

The naturally abundant ^{13}C-NMR spectrum showed five distinct resonances with a chemical shift of 29.5, 52.0 (t, $^{1}J_{14N,13C} \sim 3$ Hz), 58.0, 76.2, and 171.8 ppm (Fig. S1). Distortionless enhancement by polarization transfer and 2D NMR experiments [^{1}H,^{1}H-COSY, ^{1}H,^{13}C-heteronuclear single quantum coherence (^{1}H,^{13}C-HSQC), ^{1}H,^{13}C-heteronuclear multiple bond correlation (^{1}H,^{13}C-HMBC), and ^{1}H,^{15}N-HMBC) finally confirmed the presence of one CH, one CH_3, two CH_2, and one quaternary carbon atom (Fig. S2). In addition, the compound must be chiral. Because of the existence of diastereotopic protons in both CH_2 groups, which can be seen by the two ^{13}C correlations for each CH_2 resonance in the HSQC spectrum, the structure of the compound must be dissymmetric. In addition to MS data, ^{15}N-NMR spectroscopy provided strong evidence for the existence of nitrogen in the molecule. In a 2D NMR ^{1}H,^{15}N-HMBC experiment with DMSO extracts (optimized for $J_{N,H} = 4$ Hz), correlations were found between the nitrogen resonance at −329 ppm [from δ (CH_3NO_2) = 0] and the proton signal of the methyl groups about two bonds and of one proton of the CH_2 group about three bonds. This result indicates that the nitrogen atom ought to be a quaternary substituted atom because a ^{14}N coupling splitting about one bond ($^{1}J_{14N,13C} \sim 3$ Hz; see above) in the signal of the methyl groups in the ^{13}C-NMR spectrum could be found, which is expected for tetraalkyl ammonium species. The results of the 2D NMR investigation verified that all chemical shifts corresponded to one distinct compound that accumulated in crude *Trichodesmium* IMS101 extracts. Collectively, all of our analyses revealed that *N,N,N*-trimethyl homoserine, or homoserine betaine (Fig. 3), represents the main compatible solute in *Trichodesmium* cells.

To verify the structural analysis, homoserine betaine was synthesized from L-homoserine by using methyliodide as described by Chen and Benoiton (22). The synthesized standard compound showed the same retention time and MS pattern as the native compatible solute in extracts from *Trichodesmium* after HPLC-MS analysis (Fig. 2). Furthermore, we obtained identical ^{1}H-NMR spectra for homoserine betaine extracted from *Trichodesmium* IMS101 and the synthesized compound (Fig. 3). These comparisons confirm that *Trichodesmium* accumulates high amounts of homoserine betaine in a salt-regulated fashion.

Action of Homoserine Betaine as Compatible Solute. To prove the osmoprotective function of the compatible solute, synthetic homoserine betaine was used to complement the salt-sensitive phenotype of the *Escherichia coli* mutant FF4169 that cannot synthesize its native compatible solute trehalose (23). To this end, we cultivated mutant cells in minimal medium. Without added compatible solutes, its growth rate decreased by 50% after 400 mM NaCl supplementation (Fig. 4). The addition of a 1 mM concentration of the well-known compatible solute glycine betaine that can be taken up from the medium by *E. coli* cells (12) complemented the salt-sensitive phenotype. Similar results were obtained when the same amount of homoserine betaine was added. Homoserine-supplemented mutant cultures showed similar salt tolerance to the *E. coli* wild type (Fig. 4). Subsequent HPLC-MS analyses revealed that mutant cells accumulated homoserine betaine (Fig. S3), recovering the salt-tolerant phenotype. This experiment supported the notion that homoserine betaine can act as a true compatible solute in living cells.

Identification of the Biosynthetic Pathway. Next, we aimed to identify the biosynthetic pathway of the compatible solute, homoserine betaine. The related compound glycine betaine is

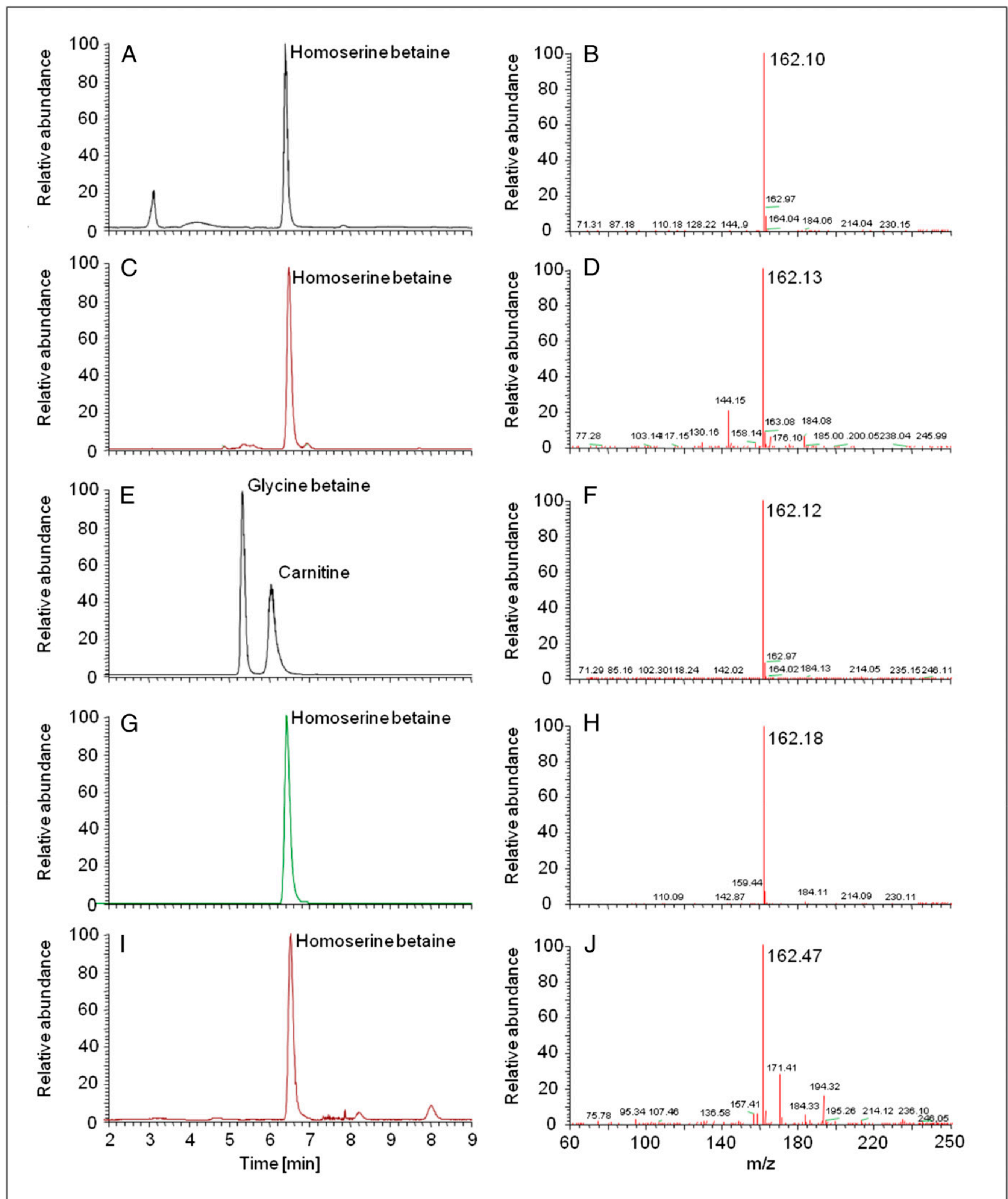

Fig. 2. Homoserine betaine detection by HPLC-MS in vivo, in vitro, and in situ. (*A* and *B*) HPLC-MS analysis of the cell extract of *Trichodesmium* IMS101 grown in YCBII medium in the presence of 35 ppt NaCl. (*C–J*) HPLC-MS analysis of cell extracts from *Trichodesmium* spp. collected from the Gulf of Eilat/Aqaba and the Red Sea (*C* and *D*), of the standard compound carnitine (*E* and *F*), of synthesized homoserine betaine (*G* and *H*), and the reaction products of the enzyme assay using heterologously expressed Tery_2447 (*I* and *J*).

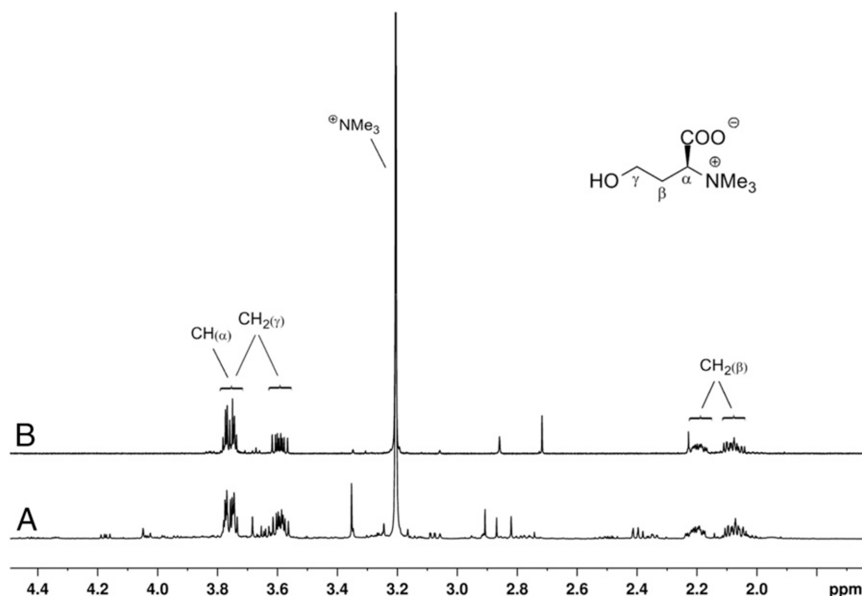

Fig. 3. Structure elucidation of homoserine betaine by NMR. ^{1}H-NMR spectra (500 MHz, 300 K) recorded in D_2O (calibrated externally against acetone 5% (vol/vol) in D_2O; 2.25 ppm): cell extract of *Trichodesmium* IMS101 grown in YCBII medium (*A*) and synthesized (L)-homoserine betaine (*B*). Approximately 5 mg homoserine betaine were dissolved per ml D_2O to obtain spectrum B. The final formula of homoserine betaine is shown in the upper right corner.

synthesized by two independent methyltransferases catalyzing the three-step methylation of the precursor glycine in the cyanobacterium *Aphanothece halophytica* (24), which made it likely that homoserine betaine is synthesized by stepwise methylation of homoserine. All genes for homoserine biosynthesis from L-aspartate are annotated in the *Trichodesmium* IMS101 genome (*tery_0087*, *tery_0587*, and *tery_4567*). Subsequent analysis of the deduced protein domains yielded 108 different genes encoding putative methyltransferases. Typically, the salt-proportional accumulation of the compatible solutes occurs concurrently with a salt-stimulated expression of the genes responsible for compatible solute biosynthesis (25). Transcriptomic analysis of *Trichodesmium* IMS101 cultivated for 9 d at different salinities (30, 37, and 43 ppt) identified one salt-regulated methyltransferase. It is encoded by the gene *tery_2447* (WP_011612023), which showed 3.5-fold more reads associated with its transcriptional start site in cultures grown at 43 ppt than at 30 ppt (average of duplicate cultures) (Fig. S4). To confirm the primary transcriptome data (26), the expression of the putative methyltransferase gene *tery_2447* was examined by quantitative PCR (qPCR) in *Trichodesmium* IMS101 cells grown at two different salinities. The *tery_2447* mRNA level increased ∼3.5-fold in cells grown at 43 ppt compared with its expression at the lower salinity (30 ppt; expression level was set to 1). Thus, both the homoserine betaine content and the *tery_2447* expression showed a salt proportional increase, making this gene product a promising candidate for a biosynthetic enzyme.

To directly verify the biochemical function, the *tery_2447* gene was expressed in *E. coli.* In vitro enzyme assays with the purified 34-kDa recombinant protein Tery_2447 (Fig. S5) were performed in the presence of different substrates. HPLC-MS analyses of the product spectrum showed that Tery_2447 is able to synthesize homoserine betaine in vitro by using the precursor L-homoserine and *S*-adenosylmethionine as the methyl group donor (Fig. 2*I*). The product was not observed with D-homoserine as the precursor, showing the stereospecificity of the homoserine methyltransferase Tery_2447. Based on these results, we propose the stepwise methylation of the precursor L-homoserine as the biosynthetic pathway (Fig. S6), which, at least in vitro, is solely catalyzed by the homoserine methyltransferase Tery_2447.

Discussion

Marine organisms depend on the accumulation of osmoprotective compounds to thrive in the saline environment of the ocean. The oceanic cyanobacterium *Trichodesmium* is well adapted to salt, as indicated by the facts that both *Trichodesmium* IMS101 (Fig. 1) and *Trichodesmium* GBRTRLI101 (27) yielded high growth rates at salinities ∼33–40 ppt and that large *Trichodesmium* blooms are found in oceanic regions with similar salinities (3, 27). By applying NMR, HPLC-, and LC-MS analyses, we discovered that *Trichodesmium* spp. accumulates high cellular amounts of the compatible solute homoserine betaine. This solute was clearly detectable in the naturally abundant ^{13}C-NMR spectra of crude extracts (Fig. S1), as found previously for the accumulation of compatible solutes such as GG (14). The structural analysis was confirmed by the identical spectra of chemically synthesized homoserine betaine.

Homoserine betaine has been previously reported in a green alga, *Monostroma nitidium*, in the ovary of shellfish *Callista brevisiphonata*, and in the viscera of turbanshell *Turbo argyrostoma* (28) and was found ether-linked to glycerolipids in many organisms (29). Its specific function in these organisms is unknown. Homoserine betaine belongs to the methylamine family, which includes efficient compatible solutes such as glycine betaine, trimethylamine *N*-oxide, sarcosine, and carnitine (30, 31). Among cyanobacteria, glycine betaine is usually accumulated by hypersaline strains that can resist almost saturated salt concentrations (15, 32). Comparable to the cyanobacterial glycine betaine synthesis (33), homoserine betaine is synthesized via the methylation of homoserine by the homoserine methyltransferase Tery_2447 (Fig. S6). Tery_2447 shows similarities to the dimethylglycine methyltransferases involved in the glycine betaine synthesis of cyanobacteria, such as *A. halophytica* PCC 7418 (45% identity), and shows similarities to other methyltransferases. The biochemical assays also confirmed the chirality of homoserine betaine, which could not be derived from the NMR studies. Because Tery_2447 only accepted L-homoserine, and not D-homoserine, as a precursor, we conclude that *Trichodesmium* accumulates L-homoserine betaine.

Our results clearly indicate that homoserine betaine accumulation is crucial for the salt tolerance of the oceanic cyanobacterium *Trichodesmium*. Its amount increased with increasing external salinity (Fig. 1*B*). Moreover, the expression of *tery_2447* encoding for homoserine betaine methyltransferase was salt-stimulated (Fig. S4).

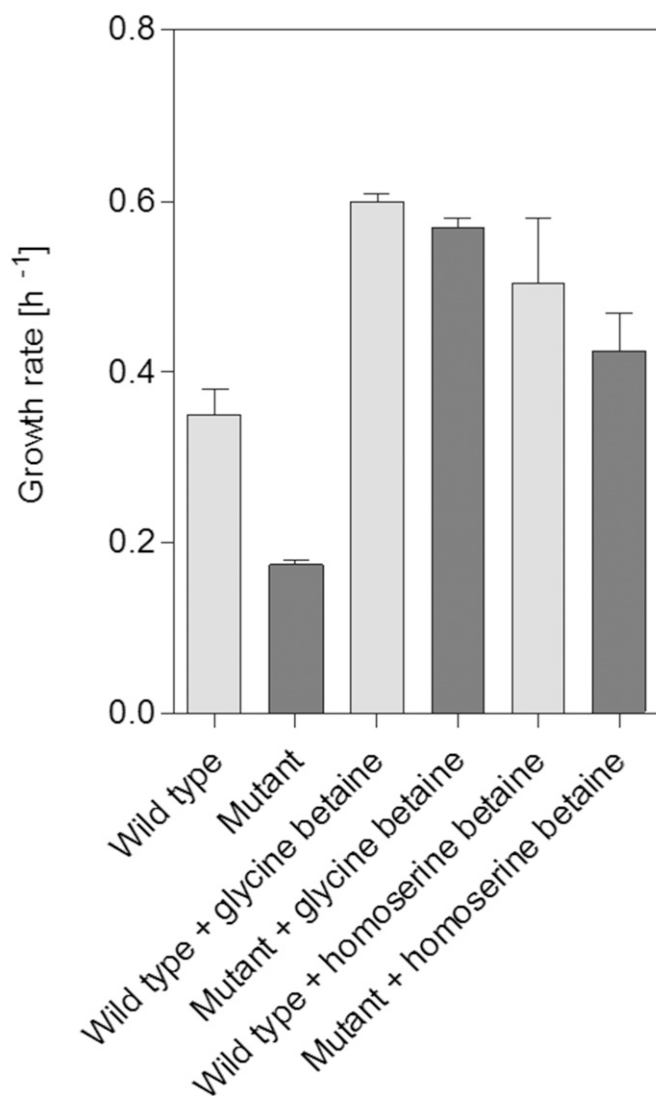

Fig. 4. Verification of the compatible nature of homoserine betaine. Growth rates of *E. coli* wild-type MC4100 and mutant FF4169 in minimal medium containing 400 mM NaCl. Growth rates were obtained in control medium and in medium supplemented with compatible solutes, 1 mM concentration of either glycine betaine or homoserine betaine.

The role of homoserine betaine as a compatible solute is supported by the similar function of related trimethyl compounds (30, 31). Because no genetic system is currently available for *Trichodesmium*, we demonstrated its compatibility with the cellular metabolism of homoserine betaine via a complementation experiment in which the salt-sensitive phenotype of an *E. coli* mutant was reverted by the addition and uptake of homoserine betaine (Fig. 4).

Our identification of homoserine betaine and its biosynthetic pathway reveals the enigmatic salt acclimation strategy of the bloom-forming cyanobacterium *Trichodesmium* spp. *Trichodesmium* belongs to the cyanobacterial subclade A, generally derived from the ancestral clade G (16). Clade A contains cyanobacteria from marine habitats that usually synthesize GG as compatible solute, but also includes strains from hypersaline habitats, such as *Arthrospira* spp. or *Lyngbya aestuarii*. The latter strain possesses a methyltransferase that is very similar in sequence and length to a methyltransferase in *Halothece* sp. PCC 7418 that is involved in glycine betaine synthesis (33). However, the most closely related methyltransferase to Tery_2447 is found in the marine cyanobacterium *Moorea producens* strain 3L (77% similarity; Table S2). *M. producens* is only distantly related to *Trichodesmium*, because it belongs to subclade B3 (16). The *Moorea* genome encodes for a second paralog, which is more similar to methyltransferases involved in glycine betaine synthesis in cyanobacteria and heterotrophic bacteria (Table S2). The evolutionary pathway of osmoregulation and the types of compatible solutes in ancestral *Trichodesmium* are as yet unknown. The early ability to synthesize GG was probably lost by *Trichodesmium's* ancestors, as was reported for *Prochlorococcus* spp. after their separation from the marine *Synechococcus* clade (18). Subsequently, the progenitor of the *Trichodesmium* lineage would have acquired a methyltransferase initially serving for glycine betaine synthesis among marine bacteria, which evolved to synthesize homoserine betaine.

The high intracellular concentration of homoserine betaine implies that, in a typical dense surface-bloom of *Trichodesmium*, a significant amount of carbon and nitrogen is diverted and stored in this compound. The fate of *Trichodesmium* in the oceans probably impacts the amount of homoserine betaine released to the ambient waters. *Trichodesmium* can be grazed by harpacticoid copepods including *Macrosetella gracilis* (34), can be lysed by phages (35), or undergo programmed cell death causing bloom termination and sinking of dead and lysing cells within 1–3 d (36). As has been reported for trimethylated amines (37), such a major injection of an organic compound may be used by other microorganisms, such as the heterotrophic bacterial consortia associated with *Trichodesmium* spp. in the oceans (38), and could therefore impact the biogeochemical cycling of C and N in the oligotrophic ocean. The residence time, fate, and biogeochemical and ecological impacts of this *Trichodesmium*-produced compatible solute within the tropical ocean remains to be further explored.

Materials and Methods

T. erythraeum strain IMS101 was grown in artificial seawater YCBII medium (39) that was supplemented with different salt concentrations: 30, 37, 43, and 48 ppt NaCl (513, 633, 736, and 821 mM NaCl, respectively). Cell concentrations were determined by using a Sedgwick–Rafter Cell (S50) and a light microscope as described by Spungin et al. (7). Homoserine betaine was analyzed by using LC-MS analysis. For NMR analyses, dried total cell extracts were recorded with a Bruker AVANCE 500 spectrometer (^{1}H: 500.13 MHz; ^{13}C: 125.8 MHz). The obtained spectra were compared with synthetic homoserine betaine, which was obtained by the protocol of Chen and Benoiton (22). The *E. coli* wild-type MC4100 and mutant FF4169 were cultivated in M63 minimal medium (40) with 400 mM NaCl and 0.4% glucose to study the osmoprotective effect of homoserine betaine. The expression of *tery_2447* was studied by using qPCR (primer sequences are in Table S3). To obtain Tery_2447 protein, the coding sequence was cloned into the expression vector pASK-IBA 43. The His-tagged Tery_2447 protein was purified by affinity chromatography, and the protein content was determined (41). The methyltransferase activity was determined in a 100-µL assay volume containing 5 mM L- or D-homoserine, 5 mM *S*-(5′-adenosyl)-L-methionine chloride hydrochloride, and ∼5 µg of purified protein in 50 mM Tris·HCl (pH 8). The detailed description of materials and methods can be found in *SI Materials and Methods*.

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