Ribulose-1,5-bisphosphate carboxylase/oxygenase genes as a functional marker for chemolithoautotrophic halophilic sulfur-oxidizing bacteria in hypersaline habitats

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The presence and diversity of the *cbb* genes encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (a key enzyme of the Calvin–Benson cycle of autotrophic CO₂ assimilation) were investigated in pure cultures of seven genera of halophilic chemolithoautotrophic sulfur-oxidizing bacteria (SOB) and in sediments from a hypersaline lake in which such bacteria have been recently discovered. All of the halophilic SOB strains (with the exception of *Thiohalomonas nitrareducens*) possessed the *cbbL* gene encoding RuBisCO form I, while the *cbbM* gene encoding RuBisCO form II was detected only in some of the pure cultures. The general topologies of the CbbL/CbbM trees and the 16S rRNA gene tree were different, but both markers showed that the halophilic SOB genera formed independent lineages in the Gammaproteobacteria. In some cases, such as with several strains of the genus *Thiohalospira* and with *Thioalkalibacter halophilus*, the *cbbL* clustering was incongruent with the positions of these strains on the ribosomal tree. In the *cbbM* tree, the clustering of *Thiohalospira* and *Thiohalorhabdus* strains was incongruent with their branching in both *cbbL* and 16S rRNA gene trees. *cbbL* and *cbbM* genes related to those found in the analysed halophilic SOB were also detected in a sediment from a hypersaline lake in Kulunda Steppe (Russia). Most of the *cbbL* and *cbbM* genes belonged to members of the genus *Thiohalorhabdus*. In the *cbbL* clone library, sequences related to those of *Halothiobacillus* and *Thiohalospira* were detected as minor components. Some of the environmental *cbbM* sequences belonged to as yet unknown phylotypes, representing deep lineages of halophilic autotrophs.

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INTRODUCTION

The detection of key functional genes provides an excellent tool to study autotrophic microbial communities *in situ* without cultivation biases. In contrast to 16S rRNA gene-based molecular screening, protein-encoding gene analysis allows a focus on that part of the microbial community responsible for a particular function. In many cases,

Abbreviations: RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SOB, sulfur-oxidizing bacteria.

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Six supplementary figures, showing primer sets, amino acid sequence alignments, and 16S rDNA-, CbbL- and CbbM-based phylogenetic trees, are available with the online version of this paper.

functionally very important groups of the Bacteria and Archaea, such as the chemolithoautotrophs, are present in very low numbers, rendering them undetectable by 16S rRNA-based phylogenetic surveys. One such example are the chemolithoautotrophic sulfur-oxidizing bacteria (SOB), which have two sets of functional molecular markers: those encoding sulfur-oxidizing enzymes and those encoding autotrophic carbon assimilation. Since functional genes of the sulfur-oxidation pathways are not conserved and have only recently started to become the subject of molecular analysis (Friedrich *et al.*, 2001, 2005; Loy *et al.* 2009), the use of the *cbb* genes [encoding the large ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) subunit, a key enzyme of the Calvin–Benson cycle of autotrophic CO₂ fixation] as a molecular marker is an obvious choice. The *cbb* genes have been frequently used to analyse marine microbial communities, but rarely

to study autotrophic communities in extreme habitats, such as hypersaline lakes. Hitherto, the distribution and diversity of the *cbb* genes have been studied in detail only in the alkaline and saline Mono Lake, California (Giri *et al.*, 2004).

The Calvin cycle of autotrophic CO₂ fixation is the most widespread pathway among the aerobic members of the Alpha-, Beta- and Gammaproteobacteria, and is the only autotrophic cycle in Cyanobacteria and in chloroplasts of algae and plants. The key enzyme of the cycle is RuBisCO, which naturally exists in several forms. Proteobacteria contain RuBisCO forms I and II, whose large subunits are encoded by the *cbbL* and *cbbM* genes, respectively.

In our previous work, we characterized the *cbb* gene in pure cultures of haloalkaliphilic SOB belonging to the *Thiomicrospira*–*Thioalkalimicrobium* group (Tourova *et al.*, 2006) and to the family *Ectothiorhodospiraceae* (Tourova *et al.*, 2007). Recently, we discovered an unexpectedly high culturable diversity of moderately and extremely halophilic obligately chemolithoautotrophic SOB in sediments of various hypersaline habitats with neutral pH, including chloride-sulfate inland lakes in Mongolia, Russia and Ukraine, a sea saltern in Slovenia and deep-sea salt brine from the Mediterranean Sea (Sorokin *et al.*, 2006a). Moderately halophilic aerobic SOB include novel species of the known genera *Halothiobacillus* and *Thiomicrospira* (Sorokin *et al.*, 2006b), while denitrifying halophiles and extreme halophiles are represented by six novel SOB genera within the Gammaproteobacteria: *Thiohalophilus* (Sorokin *et al.*, 2007a), *Thiohalomonas* (Sorokin *et al.*, 2007b), *Thiohalospira* (Sorokin *et al.*, 2008a), *Thiohalorhabdus* (Sorokin *et al.*, 2008b), *Thioalkalibacter* (Banciu *et al.*, 2008) and *Thiohalobacter* (Sorokin *et al.*, 2010). Among these halophilic SOB, the *cbb* genes have so far been analysed only in the type species of the genus *Halothiobacillus*, *Halothiobacillus neapolitanus*, which contains both forms of RuBisCO genes.

To further advance this line of research, we tested the existing collection of pure cultures of halophilic chemolithoautotrophic SOB strains for the presence of *cbb* genes. In addition, a culture-independent study of the diversity of *cbb* genes in the sediments of a typical inland hypersaline chloride-sulfate lake was performed to broaden our view of the diversity of halophilic autotrophic populations.

METHODS

Bacterial strains and environmental samples. Two type strains of the genus *Halothiobacillus*, *Halothiobacillus hydrothermalis* and *Halothiobacillus halophilus*, were obtained from the German Culture Collection DSMZ. Other strains of halophilic SOB, represented by seven genera of the Gammaproteobacteria, have been described previously (Sorokin *et al.*, 2006a, b, 2007a, b, 2008a, b) and are maintained in our active culture collection. Sediment samples were taken from the hypersaline lake Burlinskoe (Kulunda Steppe, Altai, Russia) and kept at 4 °C before use. The lake brine had a pH of 7.45 and a total salt content of 360 g l⁻¹ with absolute domination of NaCl; the lake is a local resource for cooking salt.

DNA isolation. Genomic DNA was extracted from the pure cultures of halophilic SOB with an UltraClean Microbial DNA Isolation kit (MoBio Laboratories) according to the manufacturer's protocol. To extract DNA from salt lake sediments, the PowerSoil DNA Isolation kit (MoBio Laboratories) was used. Approximately 10 g sediment was washed twice with 1 M NaCl. Then, 1 ml of the fine clay fraction was separated from the coarse sandy fraction by three cycles of homogenization/low-speed centrifugation. Subsequently, the fine fraction was used for DNA isolation.

Amplification and cloning of the *cbbL* and *cbbM* genes from pure cultures and environmental samples. To amplify the *cbbL* genes, several primer pairs were used (see Table 1). Some of the primers were newly constructed on the basis of the alignment of *cbbL*/*cbbM* sequences derived from GenBank. Each *cbbL* and *cbbM* alignment consisted of about 100 RuBisCO sequences from chemo- and photoautotrophic bacteria. Degenerate primers were designed from conserved regions. Amplification was performed in 50 µl reaction mixtures containing 25 µl Taq PCR Master Mix (Qiagen), MgCl₂ (3 mM final concentration) and 25 pmol each of forward and reverse primers. The PCR conditions for amplification of the *cbbL* gene were as follows: an initial DNA denaturation at 94 °C for 3 min, followed by 25 cycles of denaturation at 94 °C for 30 s, a decrease of the annealing temperature from 58 to 48 °C for 30 s, and an extension at 72 °C for 45 s. In addition, another 10 cycles of 94 °C for 20 s, 48 °C for 20 s and 72 °C for 45 s were performed, followed by a final extension at 72 °C for 7 min. The amplification of the *cbbM* gene was performed under the same conditions, but the annealing temperature was lowered from 62 to 52 °C. The PCR products were subjected to 1.2% (w/v) agarose gel electrophoresis, stained with ethidium bromide and visualized by UV excitation. The PCR products of the expected size were purified and sequenced directly (for pure cultures) or used for cloning (for the products obtained from the sediment samples).

Clone libraries were constructed using the TOPO TA cloning kit and *Escherichia coli* TOP10 competent cells (Invitrogen Life Technologies) according to the manufacturer's protocol. From each clone library, 50 clones were selected randomly and screened for the presence of inserts of the expected size by PCR using the vector-specific primer pair M13Fwd/M13Rev. Purified PCR products were sequenced directly by a commercial company (Marcogen, Korea).

Comparative sequence analysis. Preliminary analysis of the new sequences was done in BLAST (<http://www.ncbi.nlm.nih.gov/blast/>), metagenomic databases at the Joint Genome Institute (Markowitz *et al.*, 2006) and CAMERA (Seshadri *et al.*, 2007). The nucleotide and inferred amino acid sequences were aligned with sequences from GenBank using CLUSTAL W (Thompson *et al.*, 1994). Phylogenetic trees were reconstructed using two different algorithms: neighbour-joining in the TREECONW program package (Van de Peer & De Wachter, 1994), and maximum-likelihood using PhyML software (Guindon & Gascuel, 2003). Calculations of rarefaction curves were performed with the Analytic Rarefaction freeware program (<http://www.uga.edu/strata/software/Software.html>). A homologous coverage was estimated according to Singleton *et al.* (2001).

RESULTS

Detection of the *cbb* genes in pure cultures of halophilic SOB

At first, the detection of *cbb* genes in pure cultures of halophilic SOB was attempted using the primer set RubIgF/RubIgR specific for the 'green-like' *cbbL* form I

Table 1. Overview of oligonucleotide primers for amplification of the different RuBisCO genes

Primer	RuBisCO form	Sequence (5'–3')	Position*	Reference or source
cbbLG1f	Form I 'green-like' <i>cbbL</i>	GGC AAC GTG TTC GGS TTC AA	343–362	Selesi <i>et al.</i> (2005)
cbbL1106r		CRT GRA TVC CRC CIG AIG CIA CVG	1106–1129	This study
RubIgF		GAY TTC ACC AAR GAY GAY GA	571–590	Spiridonova <i>et al.</i> (2004)
RubIgR		TCR AAC TTG ATY TCY TTC CA	1363–1382	Spiridonova <i>et al.</i> (2004)
gr184f		GGN ACN TGG ACC ACN GTN TGG AC	169–191	Modified after Alfreider <i>et al.</i> (2003)
gr781r		GTA RTC GWG CAT GAY GAT SGG	766–786	Modified after Alfreider <i>et al.</i> (2003)
RubII331f	Form II <i>cbbM</i>	AAC AAC CAR GGY ATG GGY GA	331–350	This study
RuIIR2		TGR CCI GCI CGR TGR TAR TGCA	851–872	Spiridonova <i>et al.</i> (2004)
RubIII113r		SGC GTT CAT GCC SSA GAT GAT CGG SGT	1090–1119	Modified after Elsaied <i>et al.</i> (2007)

*The form I primer positions correspond to the nucleotide sequence of the *Acidithiobacillus ferrooxidans cbbL* gene (AF307091). *cbbM* primer positions correspond to the nucleotide sequences of the *cbbM* gene of *Rhodospirillum rubrum* (X002860).

(Spiridonova *et al.*, 2004). Despite the good results previously obtained with these primers for the haloalkaliphilic SOB (Tourova *et al.*, 2006, 2007), they gave positive amplification among the halophilic SOB only for representatives of the genera *Halothiobacillus*, *Thiohalomonas*, *Thiohalophilus* and *Thiohalobacter*. Of the other primer pairs tested (Table 1), the one specific for the 'green-like' form IA (Alfreider *et al.*, 2003) gave the best results, but the amplified *cbbL* fragment only had a small overlap with the fragments obtained with the RubIgF/RubIgR primers and with most of the *cbbL* sequences deposited in GenBank. Therefore, we designed an additional set of primers to amplify the *cbbL* gene in representatives of the genera *Thiohalospira*, *Thiohalorhabdus* and *Thioalkalibacter*. This set consisted of primer *cbbLG1f* (Selesi *et al.*, 2005) and a newly designed reverse primer, *cbbL1106r*. The 'green-like' type 1A RubisCO was successfully detected in all tested strains of halophilic SOB except for *Thiohalomonas nitratreducens*. The common region of the *cbbL* fragments was aligned, resulting in a total of 513 nt or 171 aa that was used for phylogenetic analysis (Supplementary Figs S1 and S3).

The primer set RuIIF2/RuIIR2 (Spiridonova *et al.*, 2004) used in our previous work for the detection of the *cbbM* gene was inefficient for the investigated strains of halophilic SOB. Better results were obtained using a combination of the newly designed forward primer RubII331f and a modified version of the reverse primer (RubIII113r) described by Elsaied *et al.* (2007) (Table 1). This primer pair was successfully tested for the amplification of the *cbbM* gene from most of the pure cultures of halophilic SOB and from several photoautotrophic bacteria harbouring the form II RuBisCO. However, amplification of the *cbbM* in *Thiohalorhabdus denitrificans* HL19 and *Thiohalospira halophila* HL21 was successful only with the primer combination RuII331f/RuIIR2. In contrast to the primers for the *cbbL* gene, which were used to detect all investigated strains except for *Thiohalomonas nitratreducens*, the primer pairs for the *cbbM* gene only gave positive results for the genus *Thiohalomonas* and for some strains of the genera *Halothiobacillus*, *Thiohalospira* and *Thiohalorhabdus*. The

common region of the *cbbM* fragments was aligned, resulting in a total of 477 nt or 159 aa that was used for phylogenetic analysis (Supplementary Figs S2 and S4).

Phylogenetic analysis

According to the 16S rRNA gene-based phylogeny, the investigated halophilic SOB groups represent deep lineages within the Gammaproteobacteria not related to each other or to other groups (Fig. 1). Among them, only the genera *Halothiobacillus* and *Thioalkalibacter* (*Halothiobacillaceae*) and *Thiohalospira* (*Ectothiorhodospiraceae*) are currently classified as members of the Chromatiales, while the other groups remain unassigned. The general topology of the CbbL and CbbM trees (Fig. 2) differed from that of the 16S rRNA gene tree (Fig. 1), but the clustering pattern within most of the groups was similar. In particular, most groups of halophilic SOB branched as novel deep lineages within the Gammaproteobacteria (Supplementary Fig. S5). However, the majority of strains formed monophyletic *cbbL* clusters at the species level, with amino acid sequence identities ranging from 97 to 100%. However, there were also examples of incongruence between the 16S rRNA- and *cbbL*-based phylogenies at the species level. Two strains of *Thiohalospira halophila* (HL4 and HL21) and *Thiohalospira alkaliphila* grouped with photo- and chemotrophic Gammaproteobacteria centred around the CbbL-2 of *Allochromatium vinosum* ('*A. vinosum* group'). This is a good illustration of how ambiguous the phylogenetic assignment of an organism might be if based on either a single functional gene or a single strain. Another example was found within the genus *Halothiobacillus*. The *cbbL* sequences from this group formed a single cluster when amino acid sequences were used for phylogenetic analysis (Fig. 2), while the cluster was disintegrated when nucleotide sequences were used (data not shown). Finally, the facultatively alkaliphilic halophile *Thioalkalibacter halophilus* is a member of the *Halothiobacillus* group, according to the 16S rRNA-based phylogeny. However, the *cbbL* gene of *Thioalkalibacter halophilus* was closely related to one of the two *cbbL* genes of *Thiomicrospira halophila*.

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic affiliation of representative strains of halophilic SOB from hypersaline habitats within the Gammaproteobacteria. The strains for which RuBisCO gene sequences were determined are underlined. Tree topography and evolutionary distances are given by the neighbour-joining method with Jukes–Cantor distances. Numbers at the nodes indicate the percentage of bootstrap values for the clade in 1000 replications (the values for the maximum-likelihood method are given in parentheses). Only values above 70 % are shown.

The results of the *cbbM* gene analysis only partially correlated with the *cbbL* phylogeny. In particular, the newly isolated *Halothiobacillus* strains HL1, HL2 and HL27

and members of the genus *Thiohalomonas* formed separate clusters with high internal amino acid sequence identities between 92 and 99 % (Fig. 3 and Supplementary Fig. S6).

Fig. 2. Phylogenetic tree based on *CbbL* sequences, showing the position of representative strains of halophilic SOB in relation to environmental sequences from hypersaline lake Burlinskoe (Kulunda Steppe). The *CbbL* sequences obtained in this study are in bold type (pure cultures) or highlighted in grey (environmental clones). Tree topology and evolutionary distances are given by the neighbour-joining method with Poisson distances. Numbers at the nodes indicate the percentage of bootstrap values for the clade in 1000 replications (the values for the maximum-likelihood method are given in parentheses). Only values above 70 % are shown.

The cluster composed of strains HL1, HL2 and HL27 is separated from the type species *Halothiobacillus neopolitanus* ATCC 23641 and *CbbM* sequences of the thioautotrophic bacteria formerly included in the genus *Thiobacillus* (now reclassified as the genera *Acidithiobacillus*, *Thiomonas* and *Thiobacillus*). However, the most obvious deviation between the *cbbM*-based phylogeny and those of the 16S rRNA and *cbbL* genes was the branching position of the genera *Thiohalospira* and *Thiohalorhabdus*. Being distantly related according to the phylogenies of the 16S rRNA and *cbbL* genes, they formed a single deeply branching lineage in the *CbbM* phylogeny, with internal *CbbM* sequence identities of 96 to 100 % (Fig. 3 and Supplementary Fig. S6).

Detection of *cbb* genes in sediments of a hypersaline lake

By analysing the *cbb* genes of various halophilic SOB isolated from hypersaline habitats the existing database has been substantially increased. One of the obvious directions to continue this work was to go back to the environment from which the pure cultures of halophilic SOB were obtained and to compare the *cbb* gene diversity *in situ* with the diversity obtained with the cultivation approach. For this we chose the most typical inland hypersaline lake from the investigated set (Kulunda Steppe, Altai, Russia), which yielded all culturable forms of halophilic SOB described previously by this group, including *Thiohalophilus*, *Thiohalorhabdus*, *Thiohalomonas*, *Thiohalospira* and *Halothiobacillus*. By using the *cbbLG1f/cbbL1106r* and *RubII331f/RuIIR2* primer sets, 49 *cbbL* and 48 *cbbM* clones were recovered from the sample. Their phylogenetic analyses revealed seven phylotypes based on a nucleotide sequence identity cut-off of 99 %. The rarefaction curves (Fig. 4) did not reach clear saturation, indicating that further analysis of a larger number of clones would have revealed some additional diversity. An underestimate of the diversity was expected, as the coverages of the libraries were estimated to be 85.7 and 84.4 %, respectively.

The amplicons of the *cbbL* gene all belonged to the 'green-like' form (IA) of RuBisCO. This form is mainly found in Alpha-, Beta- and Gammaproteobacteria, although a few cyanobacterial sequences also belong to form IA (Watson & Tabita, 1997). None of the obtained *cbbL* sequences showed high similarity to sequences in GenBank (<85 % nucleotide identity), indicating the presence of previously unknown autotrophic species. However, they were clearly related to the newly obtained sequences from pure cultures of halophilic SOB (Fig. 2). The five most abundant *cbbL* phylotypes (96 % of the total) were affiliated with the

extremely halophilic deep-lineage SOB *Thiohalorhabdus*. These sequences formed a single cluster closely related to the *cbbL* sequences of *Thiohalorhabdus denitrificans*, but represented new branches in this cluster (86.6–96.7 % sequence identity to the *cbbL* gene of *Thiohalorhabdus denitrificans*). The sequence of one of the minor *cbbL* phylotypes in the clone library was almost identical to the *cbbL* gene of *Halothiobacillus halophilus*, and another minor *cbbL* phylotype was related to the atypical *cbbL* gene from *Thiohalospira halophila* (strains HL4 and HL21).

Amplicons of the *cbbM* gene were more diverse (Fig. 3). The two most abundant *cbbM* phylotypes in the clone library (77 % of the total) were affiliated to the *cbbM* gene of *Thiohalorhabdus denitrificans*, which correlated well with the results of the *cbbL*-based survey. The *cbbM* phylotype belonging to the genus *Halothiobacillus* was also present in the clone library as a minor phylotype. Some *cbbM* phylotypes, however, did not show significant similarity to any sequences in GenBank or to the sequences of halophilic SOB and formed three independent novel branches within the *cbbM* tree.

DISCUSSION

Among the chemolithoautotrophic bacteria, the SOB have one of the most efficient catabolisms, which allows them to deal with extreme conditions. This might explain the surprisingly high culturable diversity of moderate to extreme halophilic SOB recently found in hypersaline lakes and salterns (Sorokin *et al.*, 2006a; Sorokin, 2008). However, despite the relatively high viable cell counts (up to 10^6 ml⁻¹), direct detection of this important functional group in hypersaline sediments using the 16S rRNA gene as a molecular marker has proved to be inefficient (our unpublished data). Therefore, the use of genes encoding functional proteins is a better alternative. In the chemolithoautotrophic Proteobacteria, autotrophic carbon fixation via the Calvin–Benson cycle is an obvious target. Although the RuBisCO phylogeny, in general, differs from the traditional 16S rRNA gene-based phylogeny of autotrophic bacteria (Watson & Tabita, 1997), our analysis of the *cbb* genes in halophilic SOB confirms their independent deep-lineage position within the Gammaproteobacteria.

Another conclusion from the pure culture study is that the presence of multiple *cbb* genes in a single SOB species is a common phenomenon. This has been shown previously for other SOB species, such as *Acidithiobacillus ferrooxidans*, *Thiobacillus* sp. (English *et al.*, 1992), *Halothiobacillus*

Fig. 3. Phylogenetic tree based on CbbM sequences, showing the position of representative strains of halophilic SOB in relation to environmental sequences from hypersaline lake Burlinskoe (Kulunda Steppe). The sequences obtained in this study are in bold type (pure cultures) or highlighted in grey (environmental clones). Tree topology and evolutionary distances are given by the neighbour-joining method with Poisson distances. Numbers at the nodes indicate the percentage of bootstrap values for the clade of this group in 1000 replications (the values for the maximum-likelihood method are given in parentheses). Only values above 70% are shown.

Fig. 4. Rarefaction curves for the *cbbL* and *cbbM* gene sequences obtained from hypersaline lake sediments.

neapolitanus (Baker *et al.*, 1998), *Thiomonas intermedia* (Stoner & Shively, 1993) and *Thiomicrospira* sp. (Tourova *et al.*, 2006). However, most of the evidence was obtained for a single strain of a single species. Only for *Acidithiobacillus ferrooxidans* have two closely related strains been tested (Cannon *et al.*, 2003). Both strains contained two *cbbL* genes and a single *cbbM* gene. Both *cbbL* and *cbbM* showed high sequence identities between the two strains. We expected to find analogous results. However, the analysis of a large set of halophilic SOB strains closely related according to 16S rRNA gene sequence similarity demonstrated that only in the genus *Thiohalomonas* were the *cbb* and 16S rRNA gene phylogenies consistent, and all four strains of *Thiohalomonas denitrificans* contained nearly identical *cbbL* and *cbbM* genes. At the same time another species of the same genus differed significantly in its *cbb* gene set: in *Thiohalomonas nitrareducens* the *cbbL* gene was not detectable by any primer sets and so either is absent or differs significantly from those present in the type species *Thiohalomonas denitrificans*. Among the other halophilic SOB genera, the situation with *cbb* was more complicated, varying even on the level of strains of the same species. Similar ‘mosaic’ situations were found in the genera *Thiohalospira* and *Thiohalorhabdus*. In *Thiohalospira* the detected *CbbL* sequences of all tested strains divided into two distinct clusters, in contrast to *CbbM*, which formed a single cluster with the *cbbM* sequences of *Thiohalorhabdus denitrificans*, despite the obvious lack of a 16S rRNA-based phylogenetic relationship. Although this might be the result of the limitations of PCR detection, the differences in gene structure for a key functional gene among closely related strains are worth investigating further, since investigations are usually limited to the type strain.

The presence of two different *cbbL* phylotypes in strains of *Thiohalospira halophila* could be the result of an ancient gene duplication with subsequent selection of one of the duplicates, similar to other SOB, such as *Thiomicrospira*

and *Acidithiobacillus*. This might be proven by finding a *Thiohalospira* strain that bears both *cbbL* genes. On the other hand, the *cbbL* sequences of strains HLA/HL21 cluster with the group of *Allochromatium vinosum*, encompassing various unrelated chemo- and photoautotrophic bacteria, and the substantial level of sequence divergence within the group might indicate lateral *cbbL* gene transfer (Watson & Tabita, 1997; Tourova *et al.*, 2007, 2009). Lateral *cbb* gene transfer might be the reason for the isolated position of *Halothiobacillus neapolitanus* and *Thioalkalibacter halophilus* among halophilic SOB on the *cbbL* tree. The G+C content of these strains is lower than that of other halophilic SOB species; they are close to *Thiomicrospira halophila* HL5.

Lateral gene transfer is more obviously the reason for the high sequence similarity between the *cbbM* genes of two otherwise unrelated genera of extremely halophilic SOB, *Thiohalospira* and *Thiohalorhabdus*. Furthermore, variation of the *cbb* composition within a single species of these genera indicates that parallel to the *cbbM* transfer some of the donor strains have lost the gene completely. The latter might be a consequence of adaptation to a more aerobic lifestyle with a lower CO₂ concentration, which favours form I RuBisCO.

The results obtained with the different strains of halophilic SOB allowed the successful identification of the environmental *cbb* sequences retrieved from a typical inland hypersaline lake. Without the sequence information from cultured SOB, the environmental sequences would have been ‘hanging in the air’. None of the halophilic SOB phylotypes detected by cultivation or molecular techniques in the chloride-sulfate hypersaline lakes with neutral pH was related to those SOB found previously to be present in the saline alkaline Mono Lake (Giri *et al.*, 2004). All the *cbbL* sequences obtained from samples from Mono Lake were related to the alkaliphilic SOB *Thioalkalivibrio* and *Thioalkalimicrobium*, which are typical inhabitants of soda lakes.

The results of our environmental *cbbL* survey suggest the predominance of phylotypes related to the extremely halophilic *Thiohalorhabdus*–*Thiohalospira* cluster. The former genus, which is difficult to cultivate, is probably the most abundant SOB species in the hypersaline lake sediments, since it was detected by two independent PCR assays, one for *cbbL* and the other for *cbbM*. Interestingly, the *cbb* screening revealed different *Thiohalorhabdus* branches from those obtained from the isolated strains. However, the number of different *cbbM* phylotypes was less than that of *cbbL*. Other genera, such as *Thiohalospira* and *Halothiobacillus*, detected as minor *cbb* components of the clone libraries, dominated the aerobic cultures. The latter discrepancy may suggest that aerobic conditions are rare in the investigated lake sediments. This is confirmed by the presence of high sulfide concentrations (up to 1 mM) in the top 5 cm layer of the sediments. On the other hand, the complete absence of *cbb* sequences corresponding to the genus *Thiohalomonas*, consistently dominant in cultures at moderate salinity and under denitrifying conditions, might indicate that under *in situ* conditions nitrate is not freely available for this SOB type, limiting its proliferation. Indeed, it is a well-documented fact that nitrification is arrested at a salinity above 1 M NaCl (Oren, 1999).

Concluding, almost all halophilic lithoautotrophic SOB strains isolated from hypersaline lakes were shown to possess *cbbL*, while the detection of *cbbM* failed for some of the strains. Furthermore, in general, *cbb* genes are useful markers for the detection and identification of autotrophic SOB species in their habitats.

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