The Calvin--Benson--Bassham (CBB) cycle has been extensively studied in proteobacteria, cyanobacteria, algae and plants, but hardly at all in Gram-positive bacteria. Some characteristics of ribulose bisphosphate carboxylase/oxygenase (RuBisCO) and a cluster of potential CBB cycle genes in a Gram-positive bacterium are described in this study with two species of *Sulfobacillus* (Gram-positive, facultatively autotrophic, mineral sulfide-oxidizing acidophiles). In contrast to the Gram-negative, iron-oxidizing acidophile *Acidithiobacillus ferrooxidans*, *Sulfobacillus thermosulfidooxidans* grew poorly autotrophically unless the CO₂ concentration was enhanced over that in air. However, the RuBisCO of each organism showed similar affinities for CO₂ and for ribulose 1,5-bisphosphate, and similar apparent derepression of activity under CO₂ limitation. The red-type, form I RuBisCO of *Sulfobacillus acidophilus* was confirmed as closely related to that of the anoxygenic phototroph *Oscillochloris trichoides*. Eight genes potentially involved in the CBB cycle in *S. acidophilus* were clustered in the order *cbbA, cbbP, cbbE, cbbL, cbbS, cbbX, cbbG* and *cbbT*.

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INTRODUCTION

The utilization of CO₂ by Gram-positive, mineral sulfide-oxidizing *Sulfobacillus* species is of interest with regard to the environmental biogeochemical activity of the organisms and to their industrial utilization in biomining. These bacteria have been used industrially in bioreactors at 50 °C for liberation of gold from arsenopyrite (Miller, 1997) and in the development of base metal extraction processes with various mineral sulfides (Dew et al., 1999). Their ribulose bisphosphate carboxylase/oxygenase (RuBisCO) activity has indicated that the Calvin--Benson--Bassham (CBB) cycle is likely to be the primary route of CO₂ fixation when these nutritionally versatile acidophiles grow autotrophically (Wood & Kelly, 1984). However, there has been no characterization of the CBB cycle’s enzymes, encoding genes or regulation in these organisms. Form I RuBisCOs have been divided into red or green types with reference to their presence in red algae or chlorophytes respectively (Delwiche & Palmer, 1996). Both types, and the two major subgroups of each, contain examples from prokaryotes (Watson & Tabita, 1997). The partial sequences of RuBisCO large subunits predicted from *cbbL* gene fragments of *Sulfobacillus* strain NW-6 (Holden & Brown, 1993) and *Sulfobacillus acidophilus* (Clark, 1995) were reported as most similar to sequences of those proteobacteria, such as *Ralstonia eutropha*, that have red-type RuBisCOs. Analysis of a larger fragment of the *S. acidophilus* RuBisCO inferred from a partial *cbbL* gene PCR product confirmed this affiliation but showed a close relationship only to RuBisCO of the anoxygenic phototroph *Oscillochloris trichoides* (Tourouva et al., 2006).

*Sulfobacillus thermosulfidooxidans* grows strongly on mineral sulfides and *S. acidophilus* grows well on sulfur (Norris et al., 1996), but both grow poorly autotrophically unless the CO₂ concentration in culture aeration is enhanced (Clark & Norris, 1996). *S. thermosulfidooxidans* has been used here to investigate some aspects of its CO₂ assimilation, including some relevant enzyme activities in comparison to those of the well-studied Gram-negative, mineral sulfide-oxidizing acidophile, *Acidithiobacillus ferrooxidans*. The genes encoding RuBisCO and other enzymes potentially involved in the CBB cycle in *S. acidophilus* are described.

METHODS

**Bacterial strains and culture conditions.** *Sulfobacillus thermosulfidooxidans* strain BC1 and *Sulfobacillus acidophilus* (DSMZ 10332) were grown autotrophically at 45 °C on ferrous iron (50 mM) at
pH 1.6 as described previously (Norris et al., 1996). Acidithiobacillus ferrooxidans (DSMZ 583) was grown in the same medium but at 30 °C. Cultures (0.5 l) were grown in stirred reactors (1 l capacity) gassed with air or with 1 % (v/v) CO2 in air (0.25 l min−1). S. thermosulfidooxidans was also grown mixotrophically with ferrous iron in the presence of glucose (1 mM) and 1 % (v/v) CO2 in air. S. acidophilus was grown lithotrohetrotrophically with ferrous iron and yeast extract (0.02 %, w/v) to provide biomass for DNA extraction. Rhodobacter sphaeroides (NCIMB 8253) was kindly provided by N. H. Mann (University of Warwick). It was harvested from an illuminated, continuous culture operated with a dilution rate of 0.05 h−1 under CO2 limitation in butyrate/bicarbonate medium (Gibson & Tabita, 1977). The culture was grown at 30 °C and degassed with O2-free N2.

Preparation of cell suspensions and cell-free extracts. Autotrophic cultures of S. thermosulfidooxidans (grown with CO2 supplementation) and A. ferrooxidans were harvested from the exponential growth phase by centrifugation for 10 min at 15 000 g. Cell pellets were washed with 50 mM Tris/HCl (pH 8) and 10 mM EDTA. Permeabilized cells were prepared by incubation with Triton X-100 or cetyl trimethylammonium bromide (CTAB) at selected concentrations (see Results) for 15 min at 45 °C (S. thermosulfidooxidans) or 30 °C (A. ferrooxidans). Cell-free extracts were prepared at 4 °C by four passes through a French pressure cell at 138 MPa (S. thermosulfidooxidans) or 276 MPa (A. ferrooxidans) followed by centrifugation (15 000 g for 15 min) to remove cell debris. A stromal extract of pea (Pisum sativum) chloroplasts was prepared by hypertonic lysis.

Partial purification of bacterial RuBisCO. The procedure was based on those of Bowien (1977) and Cook et al. (1991). Membrane-free fractions of S. thermosulfidooxidans and A. ferrooxidans were prepared from cell-free extracts by centrifugation at 40 000 g for 2 h. Fractions precipitated with 30–55 % (w/v) ammonium sulfate were the most active fractions after resuspension and dialysis. These were concentrated by ultrafiltration (10 kDa cut-off) and fractionated on 0.2–0.8 M sucrose gradients. Active fractions were again pooled and subjected to FPLC with a Green-A dye-ligand column (Amicon). Proteins were eluted with a 0–2 M KCl gradient.

Enzyme assays. RuBisCO, phosphoribulokinase (PRK) and phosphoenolpyruvate (PEP) carboxylase were assayed with minor modifications to procedures described by Smith et al. (1980). An assay buffer (0.9 ml) was added to cell-free extracts or permeabilized cells (0.3 ml aliquots containing approximately 0.5 mg cell protein). The buffer contained Tris/HCl, pH 8 (78 mM), MgCl2 (25 mM), reduced glutathione (2.1 mM) and NaH14CO3 (44 mM; 6.9 × 105 Bq mmol−1). PEP carboxylase activity was assayed with addition of sodium glutamate (8 mM) and acetyl-CoA (0.32 mM) and replacement of Tris/HCl and MgCl2 by Tris/H2SO4 and MgSO4 as described by Smith et al. (1980). PRK was assayed in the presence of ATP (4.6 mM), NADH (0.85 mM) and RuBisCO (4.5 mg spinach enzyme, Sigma). Enzymes were activated by 10 min incubation of assay mixtures at 45 °C (S. thermosulfidooxidans) or 30 °C (A. ferrooxidans) before addition (0.3 ml) of the appropriate substrates. These were ribulose 1,5-bisphosphate (RuBP) (10 mM), PEP (60 mM) or ribose 5-phosphate (10 mM). Samples (200 μl) were taken and added to 6 M phosphoric acid (200 μl) in glass scintillation vials that were heated for 1 h at 40 °C to release unfixed CO2 before addition of 10 ml Optiphase Safe scintillation fluid (LKBI) for counting. The substrate affinities of RubisCOs were determined essentially as described by Pierce et al. (1982) and Cook et al. (1991). Partially purified enzymes from S. thermosulfidooxidans and A. ferrooxidans and pea enzyme in a stromal extract were used. A range of RuBP concentrations (0.025–0.4 mM) in the presence of 30 mM NaHCO3 and a range of NaH14CO3 concentrations (0.4–6.6 mM) in the presence of 1 mM RuBP were used in a buffer of Bicine (100 mM), dithiothreitol (0.5 mM), EDTA (0.2 mM) and MgCl2 (20 mM). Protein concentrations were approximately 0.1 mg ml−1. Reactions were terminated with HCl before release of unfixed CO2 and counting as above. Reported enzyme activities are the means of five independent experiments with permeabilized cells and three independent experiments with cell-free extracts.

Electrophoresis and Western blotting. Whole-cell lysates were prepared by incubation with lysozyme (1 mg ml−1) for 15 min at 37 °C and subjected to standard slab gel SDS-PAGE using 10 % (w/v) acrylamide gels. Electrophoresed proteins were stained with Coomassie blue R250 or transferred to Hybond C nitrocellulose paper (Amersham) and probed with anti-R. sphaeroides form I RuBisCO antibodies kindly provided by G. A. Codd (University of Dundee) and J. C. Murrell (University of Warwick). Native PAGE used 3–40 % (w/v) acrylamide gels and 5 % (w/v) acrylamide tube gels. RuBisCO activity was assayed in tube gels after electrophoresis of sucrose gradient-fractionated cell-free extracts. These fractions were prepared as described above in buffer containing Tris/HCl (20 mM), MgCl2, H2O (10 mM), NaHCO3 (50 mM), EDTA (1 mM) and β-mercaptoethanol (5 mM). Following native PAGE of fractions that showed RuBisCO activity, tube gels were frozen at −20 °C and sliced into 1 mm discs. These were incubated in RuBisCO assay mix (200 μl) with shaking at 4 °C for 3 h to facilitate enzyme activation. After pre-incubation for 10 min at 30 °C (A. ferrooxidans and R. sphaeroides) or 45 °C (S. thermosulfidooxidans), RuBisCO activity was determined during 30 min further incubation after addition of 20 μl RuBP (10 mM).

Identification of Calvin–Benson–Bassham cycle genes. Routine DNA manipulations were performed by standard methods. N-Hybrid membrane (Amersham), digoxigenin (DIG) nucleic acid labelling and chemiluminescence detection systems (Boehringer Mannheim) were used in Southern blotting. Genomic DNA from S. acidophilus was digested with several endonucleases, electrophoresed and blotted. Two probes, each of 80 oligonucleotides, were based on sequence positions 1–80 and 221–300 of a 387 nucleotide fragment of the gene (cbbL) predicted to encode the RuBisCO large subunit of S. acidophilus (Clark, 1995). The DNA fragments labelled most strongly with each probe were the same size for any single enzyme digest, with the exception of EcoRI-digested DNA because of an EcoRI site between the regions specific for each probe. A BamHI-digested fragment of approximately 9 kb was eluted and cloned in pUC18 with an Escherichia coli host (Invitrogen). Inverse PCR (Ochman et al., 1993) was used to allow sequencing upstream of the cloned region after initial sequencing indicated that the gene encoding the RuBisCO large subunit (cbbL) was close to the 5' end of the cloned fragment. Genomic DNA was digested with restriction endonucleases (Sall, SacI and BamHI) and recircularized at a concentration of 5 ng ml−1. Inverse PCR used the religated DNA as template to amplify approximately 6 kb upstream of the cbbL gene. The GenBank accession number of the S. acidophilus genomic DNA sequence containing putative cbb genes and flanking sequences is U75301.

Sequence alignments and phylogenetic analysis. Alignments of translated gene sequences produced with CLUSTAL_X (Chenna et al., 2003) were manually adjusted as required and gaps were removed before analysis with PHYLIP programs (Phylogeny Inference Package version 3.6a3; Felsenstein, 2002). Preliminary trees were constructed using distance and maximum-likelihood programs generally with 25–35 appropriate sequences. Maximum-likelihood trees with a limited number of sequences that were representative of the larger trees are presented.
RESULTS

Influence of CO₂ concentration on growth and enzyme activities

Poor autotrophic growth of *S. thermosulfidooxidans* on ferrous iron under air was reproducible indefinitely through serial subcultures. In the time taken for oxidation of 40 mM ferrous iron in a CO₂-supplemented culture, only 6 mM ferrous iron was oxidized and about sevenfold less biomass was produced during growth under air (data not shown). In contrast, growth of *A. ferrooxidans* was similar with or without CO₂ supplementation. The maximum enzyme activities obtained with permeabilized *S. thermosulfidooxidans* and *A. ferrooxidans* were not influenced by the permeabilization agent (Triton X-100 or CTAB) but there were sharp peaks in RuBisCO activity when cells were permeabilized with 0.03–0.05 % (w/v) CTAB. This contrasted with similar maximum activities over a wide range of Triton X-100 concentrations. Greatest RuBisCO activity was found in *A. ferrooxidans* permeabilized with between 2 and 5 % (v/v) Triton, the highest concentration tested. Activity was greatest in *S. thermosulfidooxidans* permeabilized with 0.5–2 % (v/v) Triton and was reduced by 15 % with 5 % (v/v) Triton. Enzyme activities were determined with *S. thermosulfidooxidans* and *A. ferrooxidans* permeabilized with 1 % and 3 % (v/v) Triton X-100 respectively and in cell-free extracts (Table 1). The organisms showed similar RuBisCO activity and an increase of between 2.2- and 2.7-fold in this activity when growth under air, PRK and PEP carboxylase activities were not clearly influenced by the CO₂ concentration during growth.

Presence and form of RuBisCO in cell-free extracts

The major polypeptides of *S. thermosulfidooxidans* and *A. ferrooxidans* revealed by Western blots with anti-*R. sphaeroides* form I RuBisCO antibodies had apparent molecular masses of 54 kDa and 53 kDa respectively. These probable RuBisCO large-subunit polypeptides were more prominent in extracts from CO₂-limited cells of both species (Fig. 1). Further SDS-PAGE coupled to densitometry of Coomassie-stained bands with correction for protein loading differences indicated they were about threefold more abundant in CO₂-limited cells of *S. thermosulfidooxidans* compared to cells grown with 1 % (v/v) CO₂ in air. The increase was between twofold and threefold for the putative RuBisCO in similarly treated *A. ferrooxidans*. Whole-cell extract fractions of *S. thermosulfidooxidans*, *A. ferrooxidans* and *R. sphaeroides* that showed RuBisCO activity after sucrose gradient centrifugation were pooled and analysed by native PAGE. The activity in native gel slices resided in two peaks with *R. sphaeroides*, indicating form I and form II enzyme activities, and in single peaks with *S. thermosulfidooxidans* and *A. ferrooxidans* which corresponded to the activity of the *R. sphaeroides* form I enzyme (data not shown).

Substrate affinities of partially purified RuBisCOs

The specific activities of RuBisCOs in cell-free extracts of *S. thermosulfidooxidans* and *A. ferrooxidans* were enhanced by partial purification procedures prior to estimation of substrate affinities. The specific activity of the *S. thermosulfidooxidans* RuBisCO was increased from 5 to 302 nmol CO₂ fixed (mg protein)⁻¹ min⁻¹ after ammonium sulfate precipitation of a membrane-free extract and Green-A dye ligand-binding steps. The specific activity of the *A. ferrooxidans* RuBisCO was increased from 12 to 63 nmol CO₂ fixed (mg protein)⁻¹ min⁻¹ after ammonium sulfate precipitation of a membrane-free extract and a sucrose-gradient step. Almost all activity with *A. ferrooxidans* was lost with the subsequent Green-A FPLC step that was useful with *S. thermosulfidooxidans*. Fractions that had most RuBisCO activity showed bands that co-migrated with spinach RuBisCO on native gels (not shown). Elution of the *S. thermosulfidooxidans* putative

Table 1. Enzyme activities of *S. thermosulfidooxidans* and *A. ferrooxidans*

All activities are given as nmol CO₂ fixed (mg protein)⁻¹ min⁻¹. ND, Not determined.

<table>
<thead>
<tr>
<th>Culture gassing:</th>
<th>1 % (v/v) CO₂ in air</th>
<th>Air</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Permeabilized cells</td>
<td>Cell-free extracts</td>
</tr>
<tr>
<td><em>S. thermosulfidooxidans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RuBisCO</td>
<td>5.3 (± 2.4)</td>
<td>5.2 (± 2.1)</td>
</tr>
<tr>
<td>PRK</td>
<td>24.0 (± 10.8)</td>
<td>ND</td>
</tr>
<tr>
<td>PEP carboxylase</td>
<td>0.8 (± 0.4)</td>
<td>4.1 (± 1.7)</td>
</tr>
<tr>
<td><em>A. ferrooxidans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RuBisCO</td>
<td>5.6 (± 2.5)</td>
<td>4.8 (± 1.9)</td>
</tr>
<tr>
<td>PRK</td>
<td>25.0 (± 11.2)</td>
<td>ND</td>
</tr>
<tr>
<td>PEP carboxylase</td>
<td>8.0 (± 3.6)</td>
<td>6.4 (± 2.6)</td>
</tr>
</tbody>
</table>
RuBisCO protein band and subsequent SDS-PAGE indicated that the principal component was a polypeptide of approximately 54 kDa, in accordance with the apparent molecular mass of the RuBisCO large subunit detected by Western blotting. The apparent affinities of the RuBisCOs were estimated with a membrane-free stromal extract of pea assayed as a control enzyme for which substrate affinities were known (Table 2).

**cbb genes in S. acidophilus**

A cluster of eight putative CBB cycle genes (cbb genes) were found in the genomic DNA fragment that hybridized with a cbbL-directed oligonucleotide probe. The cluster was not preceded by a cbbR gene, which is upstream of cbb gene clusters in many autotrophic proteobacteria and encodes a LysR-type transcriptional regulator of the cbb genes (Gibson & Tabita, 1996; Kusian & Bowien, 1997). The upstream genes encoded a putative RNA polymerase factor sigma70 (orf1) and PEP carboxylase (ppc) and those downstream of the cluster encoded a putative acetyltransferase (orf2), a sensor histidine kinase (orf3) and response regulator receiver (orf4), and a transcription terminator factor Rho (orf5). The gene arrangement is shown in Fig. 2 and the proteins inferred from translations of the putative cbb genes are listed in Table 3. The translational start sites were predicted from similarity searches in protein databases and putative Shine–Dalgarno (SD) regions. The mol% G+C contents of the eight putative cbb genes were between 54.6 and 59.5, with most close to the 55–56 mol% G+C value for *S. acidophilus* genomic DNA.

**Table 2. Affinities (apparent $K_m$) of partially purified RuBisCOs for CO$_2$ and RuBP estimated from Lineweaver–Burk plots**

<table>
<thead>
<tr>
<th></th>
<th>CO$_2$ (µM)</th>
<th>RuBP (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. thermosulfidoxidans</em></td>
<td>41, 46</td>
<td>63, 72</td>
</tr>
<tr>
<td><em>A. ferrooxidans</em></td>
<td>54, 40</td>
<td>83, 88</td>
</tr>
<tr>
<td>Pea</td>
<td>24</td>
<td>30</td>
</tr>
</tbody>
</table>

Separate values indicate work with different batches of cells.

The cbbL, cbbS and cbbX, cbbL and cbbS (genes encoding large and small subunits of form I RuBisCO) were adjacent to cbbX, as in many proteobacteria that have red-type RuBisCOs. The residues of the large subunit that are conserved and involved in catalysis and RuBP binding (Hanson & Tabita, 2001) are present in the inferred *S. acidophilus* protein sequence. This was placed on a phylogenetic tree close to red-type enzyme sequences, but before these subdivided into groups C and D (Fig. 3a). Ten amino acids were conserved in 95 % of the examples in a comparison of green- and red-type RuBisCO small-subunit sequences (Spreitzer, 2003) and these were all present in the *S. acidophilus* sequence. The *S. acidophilus* small subunit was phylogenetically most closely related to those from organisms with red-type algal and bacterial small subunits (Fig. 3b) and, like those, it has an extended C-terminal region that could augment interaction with the large subunit (Hansen *et al.*, 1999; Sugawara *et al.*, 1999). The cbbX-encoded protein, which might be required for synthesis of active red-type RuBisCOs (Gibson & Tabita, 1997; Bowien & Kusian, 2002), differed in only one sequence position of those described as invariant when similar sequences were first compared (Gibson & Tabita, 1997). Its phylogenetic relationship appears closer to proteobacterial than red algal proteins (Fig. 3c) in an analysis with an outgroup that reflects the relationship of
these sequences to those of stage-specific *Bacillus* sporulation factors and a group of mycobacterial hypothetical proteins (Maier *et al.*, 2000). Two similar sequences from cyanobacteria with green-type RuBisCOs were grouped separately from those of the proteobacteria: the *cbbX* genes in these cyanobacteria are among a cluster of RuBisCO and carboxysome genes rather than adjacent to *cbbS*.

**cbbP and PRK.** *cbbP*-encoded PRK catalyses the regeneration of the CO$_2$-acceptor RuBP. The C-terminal region of bacterial PRKs, principally involved in protein–protein contacts in the multimeric enzyme, has little sequence conservation with the region in eukaryotic enzymes. Therefore, the inferred putative *Sulfobacillus* PRK sequence was compared to other sequences over the region of clear sequence similarities, from its N terminus (residue 5) to residue 209 (in *R. sphaeroides* numbering, the end of $\beta$-strand 5 in the protein). Over this region, it appeared most closely related, although with low bootstrap values, to those of cyanobacterial PRK sequences affiliated with those of red algae and plants (Fig. 3d). The partial sequence was 49–53 % identical to these cyanobacterial PRK sequences and 40–46 % to a second group of related sequences in the cyanobacteria (Fig. 3d).

**Other cbb genes.** The putative *cbb* genes in the *S. acidophilus* cluster that could encode carbohydrate-transforming enzymes that are not unique to the CBB pathway have inferred products that appear most closely related to similar proteins of other Gram-positive bacteria, particularly species of *Carboxydothetermus, Geobacillus* and *Bacillus* (Table 3). Amino acid sequence analysis has shown no homology between class I and class II fructose-1,6-bisphosphate (FDP) aldolases, and a division of class II types into A and B groups (Plaumann *et al.*, 1997). The *S. acidophilus* *cbbA* sequence encodes a putative class IIB enzyme that appears most closely affiliated with those of Gram-positive bacteria (Fig. 3e). Ribulose-5-phosphate-3-epimerase (RPE) catalyses the interconversion of ribulose 5-phosphate and xylulose 5-phosphate in reductive and oxidative pentose phosphate pathways. RPE is encoded by genes termed *cbbE* if they are associated with the CBB pathway or *rpe* if they are not. In *E. coli*, the *rpe* gene is one of three ORFs that share conserved motifs (Sprenger, 1995): the sequence of the inferred product of one of the two genes of unknown function that are less closely related to confirmed *rpe/cbbE* genes was used as an outgroup in analysis of the *S. acidophilus* sequence. Its placement closest to those of low G+C Gram-positive bacteria was only supported by low bootstrap values (Fig. 3f) with identities of about 60 % to sequences from species of *Bacillus* and *Synechococcus* and 50 % to the *cbbE*-encoded sequences from the proteobacteria *Bradyrhizobium japonicum* and *Rhodobacter capsulatus*. The inferred transketolase of *S. acidophilus* encoded by the putative *cbbT* contains the conserved thiamine diphosphate-binding motif and all of the residues described as invariant when similar sequences were previously compared (Schenk *et al.*, 1997). Using a yeast enzyme sequence outgroup, there was poor bootstrap support for the branching order close to the *S. acidophilus* sequence, which was among those from a varied collection of mostly Gram-positive bacteria, and separate from most sequences previously described as *cbbT*-encoded (data not shown). In relation to three groups of divergent, class I glyceraldehyde-3-phosphate dehydrogenases (GAP) (Figge *et al.*, 1999), the *S. acidophilus* *cbbG* gene product sequence was placed with those of the GAP II subgroup (Fig. 3g).

**DISCUSSION**

The presence and activity of a form I RuBisCO was demonstrated in *S. thermosulfidooxidans*. No other RuBisCO activity was found after mixotrophic growth with glucose and excess CO$_2$ in order to minimize possible discrimination against a form II enzyme, which could have had a lower affinity for CO$_2$ than a form I enzyme as reported for *R. sphaeroides* (Jordan & Ogren, 1981). Partially purified RuBisCOs from *S. thermosulfidooxidans*...
and *A. ferrooxidans* showed similar affinities for CO₂ and for RuBP (Table 2). The apparent affinities of the *A. ferrooxidans* enzyme for CO₂ and RuBP were slightly lower and similar respectively to the 28 μM CO₂ and 80 μM RuBP reported for *A. ferrooxidans* by Holuigue *et al.* (1987). Values in control assays with pea were close to published values (Yeoh *et al.*, 1981). The apparent affinity of the *S. thermosulfidooxidans* enzyme for CO₂ was in the range (25–100 μM) reported (Horken & Tabita, 1999) for bacteria (e.g. *Bradyrhizobium japonicum* and *Xanthobacter*).
Fig. 3. Unrooted, maximum-likelihood trees of putative cbb-encoded protein sequences, with the number of aligned residues given in parentheses, as follows: (a) form I RuBisCO large subunits (463), including examples of green-type subgroups A and B and red-type subgroups C and D, and a *Rhodospirillum rubrum* form II RuBisCO outgroup; (b) RuBisCO small subunits (100), with the sequence of *Allochromatium vinosum* as outgroup (A–D refer to the RuBisCO subgroups defined by large-subunit sequences); (c) *cbbX* gene translated products (282), with *B. subtilis* stage V sporulation protein K outgroup; (d) PRKs (181 N-terminal residues), with *B. subtilis* uridine kinase outgroup; (e) class IIB aldolases (281), with a yeast class IIA aldolase as outgroup; (f) RPEs (201), with RPE-like *E. coli* sgcE gene product as outgroup; (g) GAPs (295), with an outgroup sequence from GAP subgroup III (*Synechococcus elongatus*). Scale bars indicate 0.1 substitutions per site. Bootstrap values of 70 or greater out of 100 replicates are indicated.

RuBisCO and cbb genes in *Sulfobacillus*
flavus) with enzymes that are phylogenetically close to that of *S. acidophilus* (Fig. 3a) and its affinity for RuBP was slightly lower than those found in the *Bradyrhizobium–Xanthobacter* group (17–55 μM RuBP). The apparent derepression of RuBisCO with CO₂ limitation of *A. ferrooxidans* and *S. thermosulfidooxidans* was similar to that with CO₂ limitation of other sulfur-oxidizing bacteria such as *Thiobacillus* (now *Halothiobacillus*) neapolitanus (Beudeker et al., 1980). In summary, the different growth responses of the *Sulfobacillus* and *Acidithiobacillus* species to CO₂ limitation appeared unrelated to characteristics of the key enzymes directly involved in CO₂ fixation. The responses were more likely related to differences in CO₂ uptake capacities and the presence of carboxysomes in *A. ferrooxidans* but not in *S. thermosulfidooxidans* (data not shown).

The number and order of genes in CBB cycle operons in bacteria vary considerably ( Gibson & Tabita, 1996; Kusano & Bowien, 1997). The inferred products of eight potential *cbb* genes found in a cluster in *S. acidophilus* have the key residues required for activity. Further work is required to link directly the observed RuBisCO, PRK and PEP carboxylase activities to expression of potential *cbbL*, *cbbS* and *cbbP* genes, and so far the activities and genes have been described for different, although closely related, species. The relationship of the *S. acidophilus* RuBisCO to red-type enzymes was indicated by analysis of the predicted large- and small-subunit sequences and by the presence of a *cbbX* gene immediately following *cbbL* and *cbbS*. The identity of the inferred sequence of the *S. acidophilus* CbbL to the most closely related full sequences available for comparison is high (Table 3) but a closer relationship exists to the *RuBisCO* of *Oscillochloris trichoides*. Over the 224 amino acid sequence positions described for *O. trichoides* (GenBank accession no. DQ139403), *S. acidophilus* shared 89% identity compared to 76–78% with the D-subgroup enzyme sequences used in the phylogenetic analysis (Fig. 3). In contrast to its RuBisCO, the predicted *S. acidophilus* PRK sequence did not show a close evolutionary relationship to the respective protein of proteobacteria with red-type RuBisCO (Fig. 3c). While PRKs from prokaryotes and eukaryotes share key features of the active site for RuBP, there are major differences in primary sequence, quaternary structure and regulation of the enzymes (Miziorko, 1998). Over the region of sequence comparison between spinach and *R. sphaeroides*, one relative insertion (of 15 residues) in the plant sequence and three (of 10, 11 and 3 residues) in the proteobacterial sequence were all absent in cyanobacteria and *S. acidophilus*. The inferred protein of *S. acidophilus* has the key residues that have been implicated in catalysis in *R. sphaeroides* (R49, H45 and K165 involved in Ru5P binding, key catalytic sites D42, E131, R168, D169 and R173, and R187, which may influence cooperativity of substrate binding). Those involved in Ru5P binding and R187 are changed in the uridine kinase of *B. subtilis* while the others are conserved. The closeness of the conserved Y98 and H100 to the active site in *R. sphaeroides* was noted (Harrison et al., 1998) and H100 significantly influences catalytic efficiency (Runquist & Miziorko, 2006). Y98, but not H100, is present in the *B. subtilis* kinase while both are in the *S. acidophilus* putative PRK sequence. A uridine kinase-like enzyme in a *Sulfobacillus* ancestor could have evolved to RuBP binding and enzymes of carbohydrate central metabolism could have adapted to fulfils CBB cycle functions, in keeping with the consideration that it is the reactions of the cycle that are conserved rather than the origins of the enzymes that carry them out (Martin & Schnarrenberger, 1997). The potential CBB cycle enzymes CbbA, CbbE, CbbG and CbbT of *S. acidophilus*, in contrast to those described in other bacteria, appear affiliated with proteins of low mol% G + C Gram-positive bacteria, although with some low bootstrap values (Fig. 3). A suggestion of a Gram-positive bacterium as a possible origin of a CBB-active enzyme of some proteobacteria arose previously from the apparent relatedness of their CbbA to the class II FBP aldolase of *B. subtilis* (van den Bergh et al., 1996). However, the RuBisCO of *S. acidophilus* shows no closer relationship than other red-type RuBisCOs to the distantly related RuBisCO-like proteins (including examples in several *Bacillus* species) which could be closer to a common ancestor of CBB cycle-active RuBisCOs (Ashida et al., 2005), giving no support for particularly early evolution of a bacterial CBB cycle in *Sulfobacillus*-like species. Acquisition of CBB cycle genes by *Sulfobacillus* (or an ancestor) from an unknown organism could also have led to their autotrophy. It is not yet known if the close relationship of the *Sulfobacillus* and *Oscillochloris* CbbL sequences might extend to those of other CBB cycle proteins in these bacteria. Neither genus has other closely related genera with organisms that use the CBB cycle.

Any significance to the location of the *ppc* gene in the opposite orientation adjacent to the genes presumably encoding the major CO₂ assimilation pathway is unknown but PEP has been discussed as a signal metabolite with regard to the carbon status of cells and therefore to *cbb* gene expression (Shively et al., 1998). The redox state of the cell, and therefore the availability of reduced inorganic substrates in chemoaustrophes, is also related to expression of the CBB cycle, which could act as an electron sink (Shively et al., 1998; Dubbs & Tabita, 2004). A ferrous iron oxidation-minus mutant of *S. acidophilus*, grown in the presence of iron and yeast extract, did not produce RuBisCO until reversion to iron oxidation after several serial cultures with the same substrates (N. P. Burton & P. R. Norris, unpublished work). This first description of a *cbb* gene cluster in the nutritionally versatile *Sulfobacillus* species could facilitate investigation of possible interaction between the CBB cycle and iron oxidation.

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