The phototrophic Chloroflexus aurantiacus (32) and related Chloroflexi are facultative autotrophic green nonsulfur bacteria. They grow phototrophically in thermal springs, forming microbial mats with cyanobacteria (9, 35). During the day cyanobacteria produce glycolate as a product of photosynthesis and excrete fermentation products at night. C. aurantiacus uses the 3-hydroxypropionate bi-cycle for autotrophic carbon fixation. This pathway was thought to be also suited for the coassimilation of various organic substrates such as glycolate, acetate, propionate, 3-hydroxypropionate, lactate, butyrate, or succinate. To test this possibility, we added these compounds at a 5 mM concentration to autotrophically pregrown cells. Although the provided amounts of H₂ and CO₂ allowed continuing photoautotrophic growth, cells immediately consumed most substrates at rates equaling the rate of autotrophic carbon fixation. Using [14C]acetate, half of the labeled organic carbon was incorporated into cell mass. Our data suggest that C. aurantiacus uses the 3-hydroxypropionate bi-cycle, together with the glyoxylate cycle, to channel organic substrates into the central carbon metabolism. Enzyme activities of the 3-hydroxypropionate bi-cycle were marginally affected when cells were grown heterotrophically with such organic substrates. The 3-hydroxypropionate bi-cycle in Chloroflexi is unique and was likely fostered in an environment in which traces of organic compounds can be coassimilated. Other bacteria living under oligotrophic conditions acquired genes of a rudimentary 3-hydroxypropionate bi-cycle, possibly for the same purpose. Examples are Chloroherpeton thalassium, Erythrobacter sp. strain NAP-1, Nitrococcus mobilis, and marine gammaproteobacteria of the OM60/NOR5 clade such as Congregibacter litoralis.

Chloroflexus aurantiacus uses the 3-hydroxypropionate bi-cycle for autotrophic carbon fixation. This pathway was thought to be also suited for the coassimilation of various organic substrates such as glycolate, acetate, propionate, 3-hydroxypropionate, lactate, butyrate, or succinate. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. They grow phototrophically in thermal springs, forming microbial mats with cyanobacteria (9, 35). During the day C. aurantiacus uses the 3-hydroxypropionate bi-cycle for autotrophic carbon fixation. This pathway was thought to be also suited for the coassimilation of various organic substrates such as glycolate, acetate, propionate, 3-hydroxypropionate, lactate, butyrate, or succinate. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration. At night they may excrete fermentation products due to oxygen limitation, as do anaerobes underneath photorespiration.
propionyl-CoA) formation. Finally, beta-alanine and the abundant osmoprotectant dimethylsulfoniopropionate can be metabolized via 3-hydroxypropionate (2, 3, 28, 39, 43). Interestingly, various widespread bacteria appear to have acquired genes of the first part of the 3-hydroxypropionate bi-cycle, possibly for the same purpose of coassimilation. Genes operating in the second part of the bi-cycle are found predominantly in proteobacteria. The purposes of the elements of the rudimentary 3-hydroxypropionate bi-cycle and where they were obtained are open questions.

This work aimed to study the response of \textit{C. aurantiacus} to different organic substrates provided in addition to CO$_2$. We show that the enzymes of the 3-hydroxypropionate bi-cycle are not significantly downregulated in this situation. Moreover, the bacterium readily assimilates many different organic acids by using the enzymes of the autotrophic 3-hydroxypropionate bi-cycle. Rudimentary elements of this bi-cycle may be used by various heterotrophic bacteria for the same purpose.

**MATERIALS AND METHODS**

Materials. Chemicals were obtained from Fluka (Neu-Ulm, Germany), Sigma-Aldrich (Deisenhofen, Germany), Merck (Darmstadt, Germany), Serva (Heidelberg, Germany), or Roth (Karlsruhe, Germany). Biochemicals were from Roche Diagnostics (Mannheim, Germany), Applichem (Darmstadt, Germany), or Gerbu (Craiberg, Germany). [U-14C]acetate was obtained from Hartmann Analytic (Braunschweig, Germany).

Syntheses. 3-Hydroxypropionate was synthesized chemically from \textit{H$_2$}-propiolactone. \textit{H$_2$}-Propiolactone (1.25 ml, 25 mmol) was added dropwise with stirring to 6 ml of 5 M NaOH. The solution was lyophilized, and the dry powder was stored at room temperature. Acetyl-CoA and propionyl-CoA were synthesized from the anhydrides of the respective carbonic acids by the method of Stadtman (37). (S)-Methyl-CoA was synthesized enzymatically. The reaction mixture (1 ml) contained 200 mM 3-(N-morpholino)propanesulfonic acid (MOPS)-K$^+$ buffer (pH 7.8), 5 mM MgCl$_2$, 30 mM glyoxylate, 10 mM acetyl-CoA, and 1.5 U of recombinant (S)-methyl-CoA$\beta$-methylmalyl-CoA lyase/(S)-citramalyl-CoA (MMC) lyase. The reaction was carried out at 55°C for 15 min and stopped on ice by addition of 50 \mu{l} of formic acid, and precipitated protein was removed by centrifugation. Maly-CoA was purified via preparative high-pressure liquid chromatography (HPLC) using a reversed-phase C$_{18}$ column (end capped, 5 \mu{m}, 125 by 4 mm; LiChrospher 100 [Merckl]). CoA-thioesters were detected by UV

**FIG. 1.** The autotrophic 3-hydroxypropionate bi-cycle in \textit{Chloroflexus aurantiacus} and potential entry sites for various organic cosubstrates. 1, acetyl-CoA carboxylase; 2, malonyl-CoA reductase; 3, propionyl-CoA synthase; 4, propionyl-CoA carboxylase; 5, methylmalonyl-CoA epimerase; 6, methylmalonyl-CoA mutase; 7, succinyl-CoA:(S)-malate-CoA transferase; 8, succinate dehydrogenase; 9, fumarate hydratase; 10a/b/c, (S)-malyl-CoA/\textit{H$_2$}-methylmalyl-CoA/(S)-citramalyl-CoA lyase; 11, mesaconyl-C1-CoA hydrolase (\textit{H$_2$}-methylmalyl-CoA dehydratase); 12, mesaconyl-CoA C1:C4 CoA transferase; 13, mesaconyl-C4-CoA hydrolase.
absorbance at 260 nm with a Waters 996 photodiode array detector (Waters, Eschborn, Germany). A 15-mL gradient from 2 to 6% acetonitrile in 40 mM ammonium formate (pH 4.0) with a flow rate of 1 mL min\(^{-1}\) was applied, the corresponding peak was collected on ice and lyophilized, and the dry thioester was stored at \(-20^\circ C\). (S)-Malyl-CoA was dissolved in water or buffer directly before use and kept on ice.

**Organisms and cultivation.** *C. aurantiacus* strain OK-70-B (DSMZ 363) was grown anaerobically and phototrophically under autotrophic conditions on minimal medium with \(H_2\) and \(CO_2\) (80:20, vol/vol) gas through a sterile cell-free supernatant using 600-mL bottles with a rubber stopper. The 500-mL contaminations, centrifuge tubes and bottles were autoclaved before use. Cloning of *Chloroflexus* was also grown aerobically in the dark on minimal medium supplemented with sodium acetate (2 g liter\(^{-1}\)) as the sole carbon source at 55°C; the fermentor was aerated under stirring. Growth was determined by measuring the optical density at 600 nm (OD\(_{600}\)) (1-cm light path) of the cultures. Cells were harvested during exponential growth at an OD\(_{600}\) of \(\sim 2\) by centrifugation (10 min, 6,000 \(\times\) g, 25°C). For enzyme assays, cells were stored at \(-20^\circ C\).

**Preparation of cell extracts.** For preparation of cell extracts with a mixer mill, cells (0.2 to 0.3 g [wet mass]) were suspended in a 3-fold volume of 20 mM MOPS-K\(^+\) buffer (pH 7.5) in microtubes (1.5 mL). After addition of 1.2 g of glass beads (0.1 to 0.25 mm), the cooled cell suspension was treated in a mixer mill (type MMZ; Retsch, Haare, Germany) for 10 min at 30 Hz. The supernatant obtained after centrifugation (15 min, 16,000 \(\times\) g, 4°C) was used for enzyme assays. The membranes prepared from cell extracts were washed with a French press, cells were suspended in a 2-fold volume of 20 mM MOPS-K\(^+\) buffer (pH 7.5) with 0.1 M DNaSe 1 mL\(^{-1}\). The cell suspensions were passed twice through a chilled French press cell at 137 MPa. The cell lysate was ultracentrifuged (1 h, 100,000 \(\times\) g, 4°C), and the supernatant was used immediately or stored at \(-20^\circ C\) in the presence of 20% (vol/vol) glycerol. Extracts were freshly prepared prior to enzyme activity measurements.

**Experiments with suspensions of autotrophically grown cells.** To exclude contaminations, centrifuge tubes and bottles were autoclaved before use. Centrifuged fresh cells (2.6 g to 3.2 g [wet mass]) were suspended in 100 mL of the cell-free supernatant using 600-mL bottles with a rubber stopper. The 500-mL headspace was exchanged with \(H_2\)-\(CO_2\) (80:20, vol/vol) gas through a sterile cell-free supernatant with either sodium acetate (2 g liter\(^{-1}\)) and sodium bicarbonate (3 g liter\(^{-1}\)), or sodium bicarbonate (3 g liter\(^{-1}\)), or sodium succinate (2 g liter\(^{-1}\)). Under these conditions the fermentors were not gassed during growth; thus, no hydrogen was present. *Chloroflexus* was also grown aerobically in the dark on minimal medium supplemented with sodium acetate (2 g liter\(^{-1}\)) as the sole carbon source at 55°C; the fermentor was aerated under stirring. Growth was determined by measuring the optical density at 600 nm (OD\(_{600}\)) (1-cm light path) of the cultures. Cells were harvested during exponential growth at an OD\(_{600}\) of \(\sim 2\) by centrifugation (10 min, 6,000 \(\times\) g, 25°C). For enzyme assays, cells were stored at \(-20^\circ C\).

**Materials and methods.** All reagents were purchased at 99% purity from Sigma-Aldrich (St. Louis, MO) or from Roche Diagnostics (Mannheim, Germany). Cell extracts were freshly prepared and mixed right before use. Liquid scintillation counting was performed using a liquid scintillation counter (TriCarb 2100TR; Packard, Meriden, CT). The counting efficiency (75 to 85%) was determined by the channel ratio, and measured values were corrected accordingly.

**Other methods.** Protein was determined using the Bradford method (7). DNA and amino acid sequences were analyzed with the BLAST network service at the National Center for Biotechnology Information (Bethesda, MD). BLAST searches were performed using the genome sequence of *Chloroflexus aurantiacus* strain 3-10-fl. Phylogenetic trees were constructed using the neighbor-joining method (36) and the program MEGA 4.0.2 (24).

**RESULTS**

Regulation of enzymes of the central carbon metabolism in response to organic carbon sources. The activities of characteristic enzymes of the 3-hydroxypropionate bi-cycle and the two key enzymes of the glyoxylate bypass, isocitrate lyase and malate synthase, were measured in extracts of cells that grew exponentially in the presence of different carbon sources. These analyses aimed at determining whether the bi-cycle or
TABLE 1. Activities of characteristic enzymes of the 3-hydroxypropionate bi-cycle and of the glyoxylate bypass in extracts of C. aurantiacus cells that were grown photoautotrophically under different conditions

<table>
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<tbody>
<tr>
<td>Acetyl-CoA carboxylase</td>
<td>7</td>
<td>6</td>
<td>3</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Propionyl-CoA carboxylase</td>
<td>20</td>
<td>20</td>
<td>15</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Malonyl-CoA reductase</td>
<td>60</td>
<td>60</td>
<td>50</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Propionyl-CoA synthase</td>
<td>60</td>
<td>50</td>
<td>70</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>(S)-Malyl-CoA lyase</td>
<td>280</td>
<td>70</td>
<td>140</td>
<td>160</td>
<td>20</td>
</tr>
<tr>
<td>Isocitrate lyase</td>
<td>30</td>
<td>80</td>
<td>50</td>
<td>60</td>
<td>70</td>
</tr>
<tr>
<td>Malate synthase</td>
<td>400</td>
<td>310</td>
<td>370</td>
<td>400</td>
<td>1,800</td>
</tr>
</tbody>
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\(^a\) Mean values (deviations were <20 %) were obtained from at least two determinations at 55°C. Key genes of the glyoxylate pathway are indicated by a gray background.

All substrates tested except succinate were readily consumed by these autotrophically pregrown cells. Succinate, however, is a dicarboxylic acid, and therefore its import may be regulated differently than the import of monocarboxylic acids. The consumption of acetate, glycolate, 3-hydroxypropionate, propionate, lactate, and butyrate started without a lag phase. The consumption of C\(_2\) and C\(_3\) compounds was generally faster.

![FIG. 2. Assimilation of organic substrates by thick suspensions of autotrophically grown cells. The cells were pregrown autotrophically and harvested by centrifugation. Cells were suspended in a small aliquot of the culture supernatant and concentrated cell suspension was then incubated at 55°C (pH 8) in the light with stirring in the presence of 80% H\(_2\) and 20% CO\(_2\). Simultaneously the following organic compounds were added at a 5 mM final concentration each: a mixture of acetate, succinate, and 3-hydroxypropionate (a); a mixture of lactate, propionate, and butyrate (b); glycolate (c); or [U-\(^{14}\)C]acetate (d). In panel d, the acetate concentration and the amount of label in the supernatant were determined, as well as the amount of label incorporated into cell mass. Volatile \(^{14}\)CO\(_2\) was removed by acidifying and shaking the samples. Decreasing concentrations in panels a to c were taken as indication of assimilation of the respective compounds.](http://aem.asm.org/)
TABLE 2. Consumption of organic carbon from the medium by thick suspensions of autotrophically grown cells of *C. aurantiacus* 

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Avg consumption of compound (nmol min⁻¹ mg⁻¹ protein)</th>
<th>Carbon consumption per compound (nmol min⁻¹ mg⁻¹ protein)</th>
<th>Total organic carbon consumption (nmol min⁻¹ mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>10</td>
<td>2</td>
<td>48</td>
</tr>
<tr>
<td>3-Hydroxypropionate</td>
<td>8</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Succinate</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Propionate</td>
<td>11</td>
<td>33</td>
<td>66</td>
</tr>
<tr>
<td>Lactate</td>
<td>7</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Butyrate</td>
<td>3</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Glycolate</td>
<td>6</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>[¹⁴C]acetate</td>
<td>12</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

*In vitro* consumption per compound and total organic carbon consumption were calculated as described in Table 1. Values are averages of two or three independent experiments. 

*Vol. 77, 2011 COASSIMULATION OF ORGANIC SUBSTRATES IN CHLOROFLEXUS* 6185

than that of the C₄ substrate butyrate, which is consistent with its higher carbon content. Only glycolate (6 nmol min⁻¹ mg⁻¹ cell protein) was assimilated more slowly than acetate (10 to 12 nmol min⁻¹ mg⁻¹ cell protein). The consumption rates of the substrate mixtures were 48 and 66 nmol organic carbon min⁻¹ mg⁻¹ cell protein, respectively (Table 2). These values are even higher than the previous CO₂ fixation rate of the autotrophically grown cells of 32 to 38 nmol min⁻¹ mg⁻¹ protein.

**Test of whether the coassimilated substrates were partly oxidized.** To test whether these compounds were partly oxidized anaerobically in the light in the presence of H₂ and CO₂, we exemplarily used uniformly [¹⁴C]-labeled acetate (Fig. 2d). Disappearance of acetate and [¹⁴C] from the supernatant proceeded almost in parallel; only a small fraction (20%) of non-volatile, [¹⁴C]CO₂ free label remained after 3 h of incubation, when all of the acetate was used up. Half of the [¹⁴C] that was originally added was incorporated into cell mass. Hence, approximately 30% of the added acetate carbon was converted to volatile products, most likely [¹⁴C]CO₂, and 20% was covered in soluble products (which may be partly due to cell lysis). The majority of acetate served as a carbon source, and the rate of carbon assimilation from acetate was consistent with the rate of autotrophic carbon fixation. This shows that, despite the presence of CO₂ and H₂, the cells preferentially used acetate as a carbon source. Only a minor part of the acetate was oxidized to CO₂, probably in the course of the assimilation process. Complete oxidation of acetate is unlikely since no external electron acceptor was added.

**Biotin stain for carboxylases.** The measured activities of the biotin-dependent carboxylases in cell extracts were generally quite low. These enzyme complexes are notorious for disassembling in *vitro*, resulting in inactivation. The genome of *C. aurantiacus* harbors genes for three different biotin-dependent carboxylases and for two membrane-associated sodium ion-translocating biotin-dependent decarboxylases (presumably methylmalonyl-CoA decarboxylase) (see Table S1 in the supplemental material). The genes encoding different subunits of the carboxylases are not clustered in the *C. aurantiacus* genome. The question was whether all of these carboxylases are expressed and whether acetyl-CoA and propionyl-CoA are carboxylated by the same enzyme. Furthermore, there may be different isoforms of acetyl-CoA carboxylase, one for CO₂ fixation and another one for fatty acid biosynthesis. Thus, we used a highly specific biotin stain (avidin coupled to peroxidase) to detect biotin-containing proteins in the soluble protein fraction in cell extracts after SDS-PAGE and blotting onto a nitrocellulose membrane.

As shown in Fig. 3, there are at least three different biotinylated proteins present, which corresponds to what was found in the *C. aurantiacus* genome. These bands correlate with genes encoding biotin carboxyl carrier proteins/protein domains (Caur_1378, 64.3 kDa; Caur_2832, 69.4 kDa; and Caur_3739, 19.3 kDa) of acetyl-CoA/propionyl-CoA carboxylases. However, all the biotin carboxyl carrier proteins migrated as if their molecular mass was increased, presumably due to some kind of modification. The smaller, faint bands may correspond to the biotin/lipoyl attachment proteins (Caur_3053, 13.6 kDa; and Caur_3433, 16.2 kDa) of sodium ion-translocating decarboxylases (see Table S1 in the supplemental material). Although a slightly varying pattern of expression was observed, this variation does not correlate with the measured activities (Table 1). Note that only the carboxyl transferase subunits dictate substrate specificity. Also, the genes encoding biotin carboxyl carrier proteins are not clustered together with genes of other subunits of the carboxylases. Hence, the regulation of transcription may differ for all subunits. This approach did not discriminate genes encoding acetyl-CoA carboxylase, propionyl-CoA carboxylase, or a less specific carboxylase acting on both CoA thioesters.

**Occurrence of characteristic genes of the 3-hydroxypropionate bi-cycle in other bacteria.** BLAST searches in the NCBI database (http://blast.ncbi.nlm.nih.gov/) were used for a survey

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**FIG. 3. Biotin staining of different extracts of *C. aurantiacus* cells grown under different conditions.** Streptavidin-coupled peroxidase was used for the detection of biotin carrier subunits. The two upper bands correspond to fusion proteins Caur_1378 (64.3 kDa) and Caur_2832 (69.4 kDa), each comprising a biotin carboxyl carrier protein and a biotin carboxylase. The lower band corresponds to a protein with only the biotin carboxyl carrier domain, Caur_3739 (19.3 kDa). The Page-Ruler unstained protein ladder (Fermentas, St. Leon-Rot, Germany) was used as a molecular mass standard.
of the distribution among prokaryotes of those genes/enzymes that are related to the 3-hydroxypropionate bi-cycle. Some of these enzymes also belong to other common metabolic pathways. Acetyl-CoA carboxylase catalyzes the first committed step of fatty acid biosynthesis. Propionyl-CoA carboxylase, methylmalonyl-CoA epimerase, and methylmalonyl-CoA mutase take part in propionate and odd-chain fatty acid metabolism. Other enzymes, such as succinate dehydrogenase and fumarase, belong to the citric acid cycle. Hence, only a limited set of genes was chosen for this search, i.e., in the first partial cycle the key genes for malonyl-CoA reductase and propionyl-CoA synthase and in the second glyoxylate-assimilating partial cycle the genes for the characteristic C5-transforming enzymes. The occurrence of these genes in representative other bacteria is summarized in Table 3. The organization of the genes is shown in Fig. 4.

Only members of the family of Chloroflexaceae (Chloroflexus aggregans and Roseiflexus spp.) harbor all the genes required to establish the complete 3-hydroxypropionate bi-cycle (22, 40, 44). Interestingly, Oscillochloris trichoides (also Chloroflexi) possesses only genes encoding malonyl-CoA reductase and propionyl-CoA synthase but lacks genes of the second glyoxylate assimilation cycle (21, 25). Note that this genome is still in draft form, and it is not certain that these genes are missing. However, Oscillochloris uses the Calvin-Benson cycle for autotrophic growth (5, 20), and malonyl-CoA reductase and propionyl-CoA synthase were not found to be active in cell extracts (5). Genes for malonyl-CoA reductase and propionyl-CoA synthase are shown in Fig. 4. The occurrence of genes in representative other bacteria is summarized in Table 3. The organization of the genes is shown in Fig. 4.

![Fig. 4](http://aem.asm.org/)

**Fig. 4.** Organization of characteristic genes of the 3-hydroxypropionate bi-cycle in different bacteria. The enzymes are mesaconyl-C4-CoA hydratase (meh), succinyl-CoA-(S)-malate-CoA transferase (smtAB), mesaconyl-CoA C1-C4 CoA transferase (mct), (S)-malyl-CoA methylmalonyl-CoA lyase/S-citramalyl-CoA lyase (mcl), mesaconyl-CoA hydratase (meh), propionyl-CoA synthase (pcs), malonyl-CoA reductase (mcr), acetyl-CoA carboxylase (accACD), beta-ketothiolase (bkt) (not required for the bi-cycle), and acyl-CoA dehydrogenase (acd) (not required for the bi-cycle). White arrows show open reading frames (ORFs) of unknown function. For catalyzed reactions, see Fig. 1.
CoA synthase were also found in various ubiquitous marine proteobacteria (gammaproteobacteria, unnamed strains NORS-3, NORS1-B, and HTCC2080; alphaproteobacteria, *Erythrobacter* sp. strain NAPI). The propionyl-CoA synthase gene alone is present in the marine gammaproteobacteria *Congo* *gigabacter* *litoralis* and *Nitroco* *occus* *mobilis* as well as in *Chlo* *roherpent* *on thalass* *ium* (*Chlorobiaceae*), whereas malonyl-CoA reductase is lacking. Note that the *Nitroco* *occus* propionyl-CoA synthase lacks the CoA ligase domain, but this function may be taken over by another CoA ligase. These bacteria may use a rudimentary cycle for the mixotrophic assimilation of acetate, 3-hydroxypropionate, and/or propionate.

**DISCUSSION**

**Functioning of 3-hydroxypropionate bi-cycle enzymes under mixotrophic and heterotrophic growth conditions.** Autotrophically grown cells of *C. aurantiacus* readily took up organic carbon compounds without any lag phase. This observation corroborates the idea that the 3-hydroxypropionate bi-cycle is a means of co-assimilating organic carbon (Fig. 1). The rates of organic carbon uptake were consistent with the assumption that these compounds are completely assimilated. Not unexpectedly, the rate of carbon acquisition from organic substrates was even higher than the autotrophic carbon assimilation rate. Mixotrophy thus enables *Chloroflexus* to increase growth rate and yield compared to those in purely autotrophic growth. The 3-hydroxypropionate bi-cycle makes a balanced redox state of the cell possible, since CO₂ fixation consumes electrons (up to 10 electrons in one complete turn). Even an incomplete bi-cycle may be a necessity for anaerobic growth on substrates that are more reduced than the average cell carbon. A similar mechanism has been described for the Calvin-Benson cycle functioning in redox homeostasis under anaerobic photoheterotrophic conditions (27, 29, 34).

The characteristic enzymes of the 3-hydroxypropionate bi-cycle were active in cells grown under mixotrophic (in the presence HCO₃⁻) or purely heterotrophic conditions. In addition, the key enzymes of the glyoxylate cycle were active and malate synthase was induced under aerobic-dark conditions with acetate, indicating that the glyoxylate cycle may be responsible mainly for aerobic acetate assimilation. In fact, the incomplete 3-hydroxypropionate bi-cycle may represent another strategy of acetyl-CoA assimilation, as an alternative to the glyoxylate cycle and the ethylmalonyl-CoA pathway. Other products such as 3-hydroxypropionate and propionate can be assimilated simultaneously along with CO₂ and acetate. C₅ compounds need to generate some acetyl-CoA, which requires malyl-CoA lyase and glyoxylate assimilation. Otherwise, formation of acetyl-CoA as well as pyruvate would involve the unfavorable decarboxylation of C₅ compounds. Coassimilation of glycocolett/glyoxylate via the second half of the 3-hydroxypropionate bi-cycle would result in additional CO₂ fixation, in contrast to the case for other glycolate salvage pathways, which are associated with a loss of CO₂. This feature may hold some potential for bioengineering.

The general anabolic use of the bi-cycle is reflected by the minor regulation of the key enzymes. The original purpose of the 3-hydroxypropionate bi-cycle may have been the assimilation of reduced organic compounds under conditions where oxygen is only sometimes available. Autotrophic CO₂ fixation via this pathway might have been just a secondary develop-

ment, which was beneficial for *Chloroflexus* because it could exploit CO₂ as a carbon source as long as an electron donor was available. Thus, the complete bi-cycle may be a late and singular invention in the *Chloroflexi*.

**Acquisition of genes of the 3-hydroxypropionate bi-cycle.** The organization of genes encoding characteristic enzymes of the pathway may be disadvantageous for gene transfer. Although the genes necessary for the glyoxylate assimilation part are clustered in the *C. aurantiacus* genome, the genes encoding malonyl-CoA reductase and propionyl-CoA synthase are located (each separately) far away from that gene cluster (Fig. 4). Therefore, a number of gene transfer events would be required to establish the whole bi-cycle in other organisms. However, in two closely related *Roseiflexus* species these two genes form another cluster together with genes probably encoding acetyl-CoA carboxylase (22).

Interestingly, the gammaproteobacterium HTCC2080 (38) seems to possess the genes for a chimeric 3-hydroxypropionate/4-hydroxybutyrate cycle that may allow even for autotrophic growth: genes required for the conversion of acetyl-CoA plus two bicarbonate molecules to succinyl-CoA are of the *Chloroflexus* type. In contrast, the regeneration of acetyl-CoA from succinyl-CoA may proceed as in the pathways found in autotrophic *Sul* *folobales*, *Thermoproteales*, and *Desulfiurococales* (6, 18, 33). There, succinyl-CoA is reduced to 4-hydroxybutyrate, which is activated and further converted to two molecules of acetyl-CoA.

Besides *Chloroflexus*, only a few bacteria possess all genes required for the glyoxylate-assimilating partial cycle. One outstanding example is "*Candidatus Accumulibacter phosphatis*" (17), a betaproteobacterium that appears to harbor the whole *Chloroflexus*-like glyoxylate assimilation gene cluster. Under anoxic conditions "*Candidatus Accumulibacter phosphatis*" uses its glycolgen and polysaccharide storages, while taking up acetate and propionate from the surroundings and producing polyhydroxybutyrate/polyhydroxyvalerate (8, 15, 16, 42). Remobilization of polyhydroxyvalerate yields acetyl-CoA and propionyl-CoA. The *Chloroflexus*-type enzymes of the glyoxylate assimilation cycle may be associated with the assimilation of propionyl-CoA.

**Phylogenetic trees for characteristic enzymes.** To address the origin of key genes of the 3-hydroxypropionate bi-cycle, we constructed phylogenetic trees for bifunctional malonyl-CoA reductase, trifunctional propionyl-CoA synthase, promiscuous (S)-malyl-CoA/beta-methylmalyl-CoA/(S)-citramalyl-CoA lyase, and enzymes of the glyoxylate assimilation cycle (see Fig. S1 to S5 in the supplemental material). The neighboring-joining trees for malonyl-CoA reductase and propionyl-CoA synthase look very similar, suggesting that both genes were transferred or gained together. The (S)-malyl-CoA/beta-methylmalyl-CoA/(S)-citramalyl-CoA lyase family forms three clusters: the *Chloroflexus*-type lyase cluster of a 360-amino-acid enzyme; a *Rhodobacter*-like cluster of a 320-amino-acid enzyme, Mel1, that plays a role in the ethylmalonyl-CoA pathway for acetyl-CoA assimilation in many bacteria (11–14); and a distinct Mel2 cluster representing a specific (S)-malyl-CoA hydrolase (thioesterase), also associated with the ethylmalonyl-CoA pathway (12). Interestingly, *Methylobacterium* spp. harbor all three types of enzymes (31).

All enzymes required for the glyoxylate assimilation part of the 3-hydroxypropionate bi-cycle form clades with similar topologies. Each of these clades comprises the respective enzymes of *Chloroflexaceae* and "*Candidatus Accumulibacter*..."
phosphatis.” This strongly indicates that the whole Chloro-flexus-like cluster of genes for glyoxylate assimilation was transferred in a singular event to “Candidatus Accumulibacter.” Considering each enzyme separately, one will find that they are all widely spread among bacteria, whereas only a few species possess more than two or three of the genes together. This may be an example of the modularity of metabolic pathways, where enzymes are gained through lateral gene transfer, sometimes mutated in order to achieve new substrate specificities, and combined to pathways serving completely different purposes.

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REFERENCES