Carbohydrate Metabolism and Carbon Fixation in *Roseobacter denitrificans* OCh114

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Abstract

The *Roseobacter* clade of aerobic marine proteobacteria, which compose 10–25% of the total marine bacterial community, has been reported to fix CO2, although it has not been determined what pathway is involved. In this study, we report the first metabolic studies on carbohydrate utilization, CO2 assimilation, and amino acid biosynthesis in the phototrophic *Roseobacter* clade bacterium *Roseobacter denitrificans* OCh114. We develop a new minimal medium containing defined carbon source(s), in which the requirements of yeast extract were previously reported. This study provides evidence that *R. denitrificans* can be replaced by vitamin B12 (cyanocobalamin). Tracer experiments were carried out in *R. denitrificans* grown in a newly developed minimal medium containing isotopically labeled pyruvate, glucose or bicarbonate as a single carbon source or in combination. Through measurements of 13C-isotomer labeling patterns in protein-derived amino acids, gene expression profiles, and enzymatic activity assays, we report that: (1) *R. denitrificans* uses the anaplerotic pathways mainly via the malic enzyme to fix 10–15% of protein carbon from CO2; (2) *R. denitrificans* employs the Entner-Doudoroff (ED) pathway for carbohydrate metabolism and the non-oxidative pentose phosphate pathway for the biosynthesis of histidine, ATP, and coenzymes; (3) the Embden-Meyerhof-Parnas (EMP, glycolysis) pathway is not active and the enzymatic activity of 6-phosphofructokinase (PFK) cannot be detected in *R. denitrificans*; and (4) isoleucine can be synthesized from both threonine-dependent (20% total flux) and citramalate-dependent (80% total flux) pathways using pyruvate as the sole carbon source.

Introduction

Two of the most important sources of carbon sinks known in nature are the absorption of CO2 by the oceans and photosynthesis by photosynthetic organisms. The marine *Roseobacter* clade are potentially major contributors to global CO2 fixation as they make up 10–25% of the total microbial community in some surface ocean ecosystems [1–4]. Some members of the *Roseobacter* clade belong to a group known as Aerobic Anoxicogenic Phototrophs (AAPs), the only known organisms performing photosynthesis requiring oxygen but not producing oxygen, while other members are non-phototrophic. CO2 oxidation was confirmed experimentally for the non-phototrophic *Roseobacter* clade bacterium *Silicibacter pomeroyi* [5] and other *Roseobacter* clade [6], and CO2 fixation was suggested in several marine AAPs [7], while bioinformatic analysis in *Roseobacter* clade with completed genome sequence indicated that the genes encoding ribulose bisphosphate carboxylase/oxygenase (RUBISCO) and phosphoribulokinase required in the Calvin cycle for carbon fixation, as well as genes for other autotrophic CO2 fixation pathways, are missing in these bacteria [5,8–10]. Thus, it has been of great interest to determine how *Roseobacter* clade bacteria can fix CO2, if they indeed fix CO2.

Anaplerotic pathways have been proposed as an alternative mechanism for CO2 fixation in *Roseobacter* clade [5,8,11], but have not been verified experimentally. Given that organisms in the *Roseobacter* clade are known to require organic carbon sources for growth [12,13], understanding how *Roseobacter* clade bacteria utilize organic carbon and assimilate CO2 will help us understand the bio-energy metabolism, production of bioactive metabolites, and roles of global carbon cycle in these wide-spread marine bacteria.

Here, we report metabolic and biochemical studies of *Roseobacter denitrificans* OCh114 [14], which can denitrify, as its name indicates [13], and produce bacteriochlorophyll a (BChl a) aerobically. The genomic sequence of *R. denitrificans* [8] suggested that the tricarboxylic acid (TCA) cycle and anaplerotic pathways are complete, and that most of the genes for carbohydrate metabolism in the Embden-Meyerhof-Parnas (EMP, glycolysis), Entner-Doudoroff (ED), and pentose phosphate (PP) pathways are present and annotated. To understand the contributions of these enzymes and reaction pathways in the metabolism of *R. denitrificans*, we developed a minimal growth medium containing only defined carbon source(s), optimized the growth conditions of *R. denitrificans* in different defined carbon sources, and used isotopomer assisted metabolite analyses, biochemical approaches, and gene expression profiles to investigate the carbon assimilation, carbohydrate utilization, and amino acid biosynthesis pathways in *R. denitrificans*. 
Results

Growth of R. denitrificans OCh114 in different growth conditions

Similar growth curves and spectral features were obtained in the R. denitrificans OCh114 cultures grown aerobically in the light (20 W/m²), dark, and day-night cycles (Figure S1a and S1b), and slightly higher OD₆₀₀ were reached in the dark and day-light cycles compared to the continuous light growth conditions (Figure S1c). It is consistent with the reports that light has a negative effect on pigment formation in some AABs [15]. No growth was observed anaerobically regardless of the light intensity (data not shown). Multiple organic carbon sources have been tested for the growth of R. denitrificans OCh114 [13]. Here, we report studies of three carbon sources: pyruvate, D-glucose, and CO₂ (or HCO₃⁻). Spectral features of the photosynthesis system and light-harvesting complexes for the cultures grown in the minimal medium containing pyruvate or glucose are similar to the cultures grown in the rich medium (Figure 1a and 1b) as well as the results reported previously [13,16]. The image of R. denitrificans cells grown in the minimal medium with pyruvate was examined by OLYMPUS FV1000/BX61 high-resolution confocal microscope, and the morphology and average cell size is consistent with previous reports [13]. Uptake of pyruvate, 2.5×10⁻²±5×10⁻⁴ mmole per hour (Figure 1d and S1d), is approximately 2 to 3-fold faster than uptake of glucose by R. denitrificans (Figure 1c), consistent with the faster growth in pyruvate than in D-glucose (Figure 1c). Higher OD₆₀₀ and better cell growth can be obtained using 0.2% pyruvate in the minimal medium (data not shown). No differences in the ¹³C-isotopomer abundances of the protein-derived amino acids for cultures grown in the minimal medium containing pyruvate were observed under either dark or illuminated conditions (Table S1a and S1b). Similar results were also observed in the cultures grown in the minimal medium supplied with glucose.

Vitamin B₁₂ is required for the growth of R. denitrificans

The previous studies of the growth of R. denitrificans OCh114 or other AABs have been performed in either a rich medium or a medium containing either 0.02 g/liter [13] or 0.1 g/liter [17] of yeast extract (undefined carbon sources). We confirmed the necessity of yeast extract, as poor growth of the R. denitrificans cultures without yeast extract was observed (data not shown). Although it has been recognized that yeast extract contains rich vitamin mixtures, it also includes amino acids and other undefined carbon sources. Ideally, the ¹³C-isotopic labeling studies require a minimal medium containing only defined carbon sources(s), thus it is desirable to optimize the growth conditions by eliminating the yeast extract. We found that vitamin B₁₂ (cyanocobalamin) can serve as an alternative to yeast extract for growing R. denitrificans in defined carbon sources, as cultures with OD₆₀₀≥3 can be reached in our minimal growth medium with glucose as the sole carbon source (Figure 1c). Vitamin B₁₂ and different forms of cobalamin are required for methionine and protein synthesis, deoxyribonucleotide triphosphate synthesis, amino acid metabolism, and CO₂ fixation (in methanogens, although there is no such pathway identified in R. denitrificans), and are included in the growth media of many phototrophic, phototrophotrophic and chemoheterotrophic bacteria. Requirement for vitamin B₁₂ in the growth of R. denitrificans may partially explain why yeast extract was necessary to be included in the minimal medium containing either pyruvate or glucose.

The carbohydrate utilization pathways in R. denitrificans

In the cultures grown in the minimal medium containing either D-[¹³C]glucose or D-[⁶⁻¹³C]glucose, the isotopomer labeling data of serine (the precursor is 3-phosphoglycerate) and alanine (the precursor is pyruvate) were different (Table S1e, S1f, S1h, and S3). Three metabolic pathways can be employed for sugar utilization by R. denitrificans and need to be considered to account for the isotopomer abundance in these protein-derived amino acids: (1) the Embden-Meyerhof-Parnas (EMP) pathway (glycolysis), by which one [¹³C]glucose molecule is cleaved into two glyceraldehyde-3-phosphate (GAP) molecules: one is [³⁻¹³C]-labeled and the other is unlabeled using either D-[¹³C]glucose or D-[⁶⁻¹³C]glucose; (2) the Entner-Doudoroff (ED) pathway [18], by which one molecule of glucose generates one molecule of GAP, in which the third carbon is labeled using D-[⁶⁻¹³C]glucose, and one molecule of pyruvate, where the first carbon is labeled using D-[¹⁻¹³C]glucose (Figure 2a); (3) the pentose phosphate (PP) pathway, the first carbon of glucose is removed as CO₂, if the oxidative PP pathway is active, to generate ribose-5-phosphate, which can be converted into GAP through the non-oxidative PP pathway (Figure S2). As shown in Figure 2a, regardless of the pathways, GAP is converted into 3-phosphoglycerate (3-PGA), the precursor of glycine, serine, and cysteine, and then to pyruvate, the precursor of alanine, valine, leucine, and isoleucine.

If R. denitrificans predominantly uses the EMP pathway for carbohydrate metabolism, one will expect similar ¹³C-isotopomer abundance in serine and alanine (converted from pyruvate through alanineaminotransferase). However, this is not consistent with the higher M₀ value (more unlabeled carbon) in serine (0.74) than in alanine (0.56) using D-[¹⁻¹³C]glucose in our studies (Table S1e and S3). Alternatively, genes encoding two key enzymes in the ED pathway can be found in R. denitrificans: eda (RD1_2878), encoding 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14), and edd (RD1_2879), encoding phosphogluconate dehydratase (EC 4.2.1.12). If R. denitrificans uses the ED pathway as one of the alternative carbohydrate utilization pathways, [¹⁻¹³C]glucose is converted into [¹⁻¹³C]-2-keto-3-deoxy-6-phosphogluconate, which is cleaved into [¹⁻¹³C]pyruvate and unlabeled GAP, leading to unlabeled 3-PGA (Figure 2b). In this case, serine is expected to be mostly unlabeled, while half of the alanine, derived from [¹⁻¹³C] pyruvate, is labeled, consistent with our experimental data.

The QRT-PCR results indicate that both eda (RD1_2878) and edd (RD1_2879) genes are expressed and the transcript level of these genes is higher in the minimal medium containing either pyruvate or glucose than in the rich medium, similar to the gene expression profiles of other genes responsible for carbon fixation and carbon metabolism examined in this report (Figure 3). Moreover, the activity of 2-keto-3-deoxy-phosphogluconate (KDPG) aldolase and phosphogluconate dehydrase can be detected in cell-free extracts. Alternatively, the gene encoding 6-phosphofructokinase (PFK, EC 2.7.1.11), an essential enzyme for the EMP pathway, is not annotated, and no PFK activity can be detected in cell free extracts.

The proposed ED pathway was further tested using D-[⁶⁻¹³C]glucose, in which [⁶⁻¹³C]KDPG is cleaved into unlabeled pyruvate and [³⁻¹³C]GAP in the ED pathway (Figure 2b). Our data showed a smaller M₀ value in serine using D-[⁶⁻¹³C]glucose (0.30) than using D-[¹⁻¹³C]glucose (0.74), compared to slightly higher M₀ value in alanine using D-[¹⁻¹³C]glucose (0.56) versus D-[⁶⁻¹³C]glucose (0.48) (Table S1e and S1f). The M₀ value in serine using D-[¹⁻¹³C]glucose or D-[⁶⁻¹³C]glucose is not close to 1 or 0, respectively, suggesting that in addition to the ED pathway, the PP pathway is also active and generating GAP with different labeling pattern using D-[⁶⁻¹³C]glucose or D-[¹⁻¹³C]glucose (Figure 2a). Based on the isotopomer abundance of serine using D-[¹⁻¹³C]glucose and D-[⁶⁻¹³C]glucose, we estimated 25–30% of GAP is produced from the (non-oxidative) PP pathway (Table S3).
Figure 1. Spectra, image and organic carbon uptake of *R. denitrificans* OCh114. The spectra of cultures grown in the rich medium vs. in the minimal medium supplied with either 0.1% pyruvate or 0.1% glucose (a). No undefined carbon sources were included in the minimal medium reported in this paper. The normalized spectra in the 650 nm–900 nm range (b), the cell growth in the minimal medium with pyruvate, glucose, or HCO$_3^-$ (c), and the cell growth curve and uptake of pyruvate (d) or glucose (e) with or without the addition of 0.2% NaHCO$_3$ in the minimal medium containing pyruvate or glucose. More than ten biological replicates were performed in every growth conditions.

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Figure 2. Proposed carbohydrate metabolism and amino acid biosynthesis pathways in *R. denitrificans*. Predicted $^{13}$C-labeling distributions using D-$[1-^{13}C]$glucose and D-$[6-^{13}C]$glucose in the EMP and ED pathways are shown (a), and the cleavage of a hexose molecule in the EMP vs. ED pathway (b). Abbreviations: EMP, Embden-Meyerhof-Parnas (glycolysis); ED, Entner-Doudoroff; and PP, pentose phosphate. Experimentally identified $^{13}$C-labeling patterns are reported in the context.

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Historidine is synthesized from 5-phospho-ribosyl-cose is released as CO2 during the conversion of 6-phosphogluconate into D-ribulose-5-phosphate by 6-phosphogluconate dehydratase. Various pathways can lead to formate biosynthesis in R. denitrificans. THF is generated from THF and formate by transformylase, and glutamine (the nitro-10-formyl moiety of THF) is released as CO2 during the conversion of 6-phosphogluconate into D-ribulose-5-phosphate by 6-phosphogluconate dehydratase.

The oxidative PP pathway, is the precursor of PRPP. In the oxidative PP pathway (Figure S2), the first (labeled) carbon in D-[1-13C]glucose is the precursor of PRPP. The 6-phosphogluconate, an intermediate of the ED/PP pathway (Figure 4), the first (labeled) carbon in D-[1-13C]glucose. The ED and PP pathways can explain much higher M0+ value in these amino acids (0.04) using D-[1-13C]glucose than the value in these amino acids (0.04) using D-[6-13C]glucose (Table S1e) and for smaller M0+ value in histidine using D-[1-13C]glucose (0.10) (Table S1f) than using D-[1-13C]glucose (0.20). Together, our studies imply that R. denitrificans uses the non-oxidative PP pathway for histidine, ATP, coenzymes, and nucleic acids biosynthesis, as well as supplying GAP for the ED pathway (Figure 2a and 4).

**Carbon fixation in R. denitrificans**

Previous reports suggested that R. denitrificans [19] and other AAPs [11,20] can fix CO2. In our studies, no growth of R. denitrificans was observed in the minimal medium with CO2 (or bicarbonate) as the sole carbon source (Figure 1c), consistent with the finding that the key genes required for the Calvin cycle are missing in the genome of R. denitrificans OCh114 [8]. Moreover, no CO2-enhanced growth was obtained in the minimal medium containing pyruvate or glucose (Figure 1d and 1e), and 13C-isotomer distribution of protein-based amino acids suggests that the presence of bicarbonate has a rather small effect on pyruvate and glucose metabolism in R. denitrificans (Table S1e and S1f). These experimental data indicate a carbon-fixation pathway other than the Calvin cycle is utilized by R. denitrificans OCh114. Swingley et al. [8] proposed that R. denitrificans could fix CO2 by pyruvate oxidase PEP to form oxaloacetate (OAA). Using uniformly 13C-labeled D-glucose (D[U-13C6] glucose) (Table S1j and S1k), our studies show less fully labeled amino acids derived from OAA (aspartate [M+4, 0.85], methionine [M+5, 0.78] and threonine [M+4, 0.65]) than the amino acid derived from pyruvate (alanine [M+3, 0.93]) (Table S1l). Also, a smaller M0+ value can be seen in threonine (0.96) and aspartate (0.87) than alanine (0.94) using 13C-NaHCO3 and unlabeled pyruvate (Table S1d). Similar results are also obtained using 13C-NaHCO3 and unlabeled pyruvate (Table S1d). Together, our studies imply that R. denitrificans fixes approximately 10–15% of protein carbon from CO2 via the anaplerotic pathways, despite lacking the Calvin cycle. These experimental evidences support the CO2 fixation pathways by R. denitrificans proposed by Swingley et al. [8].

**The TCA cycle and anaplerotic pathways**

Our studies imply that R. denitrificans has an active TCA cycle and metabolic fluxes in the anaplerotic pathways. PEP can be converted into OAA, the precursor of aspartate, asparagine, methionine, threonine, lysine and isoleucine, OAA can be also generated from pyruvate and through the TCA cycle. Using D-[1-13C]glucose, the M0+ value is similar in aspartate (0.58), threonine (0.47), methionine (0.49) and alanine (0.36) but smaller than serine (0.74) (Table S1c), suggesting that 13C-isotomer abundance in the amino acids derived from OAA is largely from pyruvate (via the anaplerotic pathway) and/or the TCA cycle flux.

**Figure 3. QRT-PCR studies.** Gene name (encoding enzyme and gene location number): eda (2-keto-3-deoxy-6-phosphogluconate aldolase, RD1_2878), edd (6-phosphogluconate dehydratase, RD1_2879), pyc (pyruvate carboxylase, RD1_3376), pckA (phosphoenolpyruvate carboxykinase, RD1_1376), ppc (phosphoenolpyruvate carboxylase RD1_4248), pppdK (pyruvate phosphate dikinase, RD1_1948), atxA (aspire amino transferase, RD1_3802). Relative gene expression value of each gene is calculated with 2-ΔΔCt, where ΔCt = Cttarget gene - Ct16S rRNA gene and the 16S rRNA gene was used as the internal reference. Three biological replicates and eighteen technical replicates were performed for every gene.

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Four anaplerotic enzymes are known to assimilate CO₂ and replenish the intermediates of the TCA cycle, and genes encoding these anaplerotic enzymes can be all found in the genome of *R. denitrificans* OCh114: malic enzyme (*tme*, RD1_0421, EC 1.1.1.40, pyruvate = malate), PEP carboxykinase (*pckA*, RD1_1376, EC 4.1.1.49, PEP = OAA), pyruvate carboxylase (*pyc*, RD1_3376, EC 6.4.1.1, pyruvate = OAA) and PEP carboxylase (*ppc*, RD1_4248, EC 4.1.1.31, PEP = OAA). To further investigate carbon fixation by *R. denitrificans*, we used gene expression studies to confirm if the pathways are active and to identify the transcriptional level of the genes encoding enzymes for the pathways under various growth conditions, as well as 13C-isotopomer abundance to probe the anaplerotic flux. In the conditions we tested, the transcript level is higher for *pckA* (RD1_1376) and *tme* (RD1_0421) than for *pyc* (RD1_3376) and *ppc* (RD1_4248) (Figure 3). The activity of these four anaplerotic enzymes can be detected in cell-free crude extracts, consistent with the gene expression profiles. The activity is higher in PEP carboxykinase (6 umole/min·mg protein) and pyruvate carboxylase (2.5 umole/min·mg protein) than in PEP carboxylase (0.7 umole/min·mg protein) in the cell extracts from the rich dark cultures, while the activity of pyruvate carboxylase and PEP carboxylase are comparable (1–2 umole/min·mg protein) in the minimal medium supplied with pyruvate.

To investigate metabolic fluxes through the anaplerotic pathways, [3-13C]pyruvate was used and the 13C-isotopomer abundance in aspartate (from OAA), serine (from PEP, 3-PGA) and alanine (from pyruvate) was analyzed (Table S1h). Both QRT-PCR and 13C-labeling pattern suggest that the flux of pyruvate to PEP is weak (different isotopomer distribution between alanine and serine), consistent with low gene expression of *ppdK* (RD1_1948, pyruvate phosphate dikinase, EC 2.7.9.1, pyruvate→PEP) and that the pathway from OAA to PEP is likely to be active and pyruvate to OAA is not as strong. This is in agreement with the gene expression profile of *pckA* (RD1_1376) and *ppc* (RD1_3376). Hence, most of the 13C-labeling in OAA likely comes from the TCA cycle flux (from malate), in which the pyruvate to malate pathway (via malic enzyme) should be rather active, compatible with higher expression of *tme* (RD1_0421) than *ppc* (RD1_3376). The proposed anaplerotic flux is summarized in Figure 5.

The isoleucine and leucine biosynthesis pathways

In many organisms, isoleucine is converted from threonine by threonine deaminase (EC 4.3.1.19) in the threonine-dependent pathway [21], and leucine is made from valine. The 13C-isotopomer abundance in [1-13C]pyruvate cultures showed higher M+0 value in leucine (0.91) and isoleucine (0.86) compared to valine (0.25) and threonine (0.70) (Table S1a). Most of the leucine should be unlabeled because the first (labeled) carbon in 13C-(2R,3S)-3-isopropylmalate is released as CO₂ catalyzed by 3-isopropylmalate dehydrogenase (*LeuB*, EC 1.1.1.85) (Figure 6). However, highly unlabeled isoleucine is not expected if 2-oxobutanoate, a precursor...
of isoleucine biosynthesis, is derived from threonine in the threonine-dependent pathway (Figure 6). The results suggest that isoleucine is not exclusively derived from threonine. If it is, the M+0 values in threonine and isoleucine should be similar.

Alternatively, a citramalate-dependent pathway, which is a threonine-independent pathway, was recently reported in several bacteria [22–24]. These microorganisms can synthesize isoleucine from 2-oxobutanoate either exclusively from the citramalate-dependent pathway [24] or from both threonine- and citramalate-dependent pathways [23]. The higher M+0 value in isoleucine in our studies (Table S1a) can be explained if a significant portion of isoleucine is synthesized through the citramalate-dependent pathway, in which LeuB removes the first (labeled) carbon in \(^{13}\text{C}-\text{D-erythro-3-methylmalate}\) as \(\text{CO}_2\) (Figure 6). Similarly, the M+0 value is higher in isoleucine (0.90) than in threonine (0.77) using \([1-^{13}\text{C}]\text{pyruvate}\) and unlabeled \(\text{NaHCO}_3\) (Table S1c).

In the studies of the threonine-dependent pathway, QRT-PCR showed that the gene encoding threonine deaminase (thdC, RD1_0416) was active in various growth conditions (Figure 3). The activity of citramalate synthase (CimA, EC 2.3.1.182) (3±0.5 nmole/min/mg protein) and threonine deaminase (60±5 nmole/min/mg protein) can be detected in the cell-free extracts of the minimal medium containing pyruvate. Further, smaller M+0 values in isoleucine (0.83–0.85) than in leucine (0.90–0.91) using \([1-^{13}\text{C}]\text{pyruvate}\) (Table S1a and S1b) imply that some isoleucine molecules are derived from the threonine rather than pyruvate and acetyl-CoA. If \(R.\text{denitrificans}\) utilizes the citramalate-dependent pathway exclusively to generate isoleucine, the \(^{13}\text{C}\)-labeling pattern in leucine and isoleucine will be identical (and the difference is expected to be within the error of GC-MS measurements: ~0.01), since the same precursors (pyruvate and acetyl-CoA) are employed in the biosynthesis of both amino acids. Therefore, both threonine- and citramalate-dependent pathways should be contributed to synthesize isoleucine, considering that the difference in \(^{13}\text{C}\)-isotopomer abundance between leucine and isoleucine (0.06–0.07) is larger than the instrumental error (±0.01). Further, the calculated values of all \(^{13}\text{C}\)-isotopomer abundance (from M+0 to M+5) of isoleucine indicate that 80% of the in vivo metabolic flux for isoleucine biosynthesis comes from the citramalate-dependent pathway, 20% from the threonine-dependent pathway, when \([3-^{13}\text{C}]\text{pyruvate}\) is the carbon source (Table S1h and Figure S3). Together, our studies suggest that both the citramalate- and threonine-dependent pathways are active for isoleucine biosynthesis in \(R.\text{denitrificans}\), similar to the report in \(\text{Geobacter sulfurreducens}\) [23]. As the putative \(\text{cinA}\) gene is not annotated in \(R.\text{denitrificans}\), identification of the proposed citramalate synthase is in progress.

**Discussion**

**CO\(_2\)** fixation in \(R.\text{denitrificans}\) and other AAPs

In this paper, we reported that \(R.\text{denitrificans}\) uses the anaplerotic pathways to fix 10–15% of protein carbon from \(\text{CO}_2\). The amount of \(\text{CO}_2\) fixation is lower in \(R.\text{denitrificans}\) than in autotrophic bacteria, supporting previous observations that \(R.\text{denitrificans}\) cannot grow autotrophically. Other than \(R.\text{denitrificans}\), \(\text{CO}_2\) fixation in several AAPs producing BCHl \(a\) aerobically was reported previously by \(^{14}\text{CO}_2\) incorporation studies: \(\text{Erythrobacter sibiricum}\) (a very low level of \(\text{CO}_2\) (0.4%) assimilated) [7], \(\text{Erythrobacter longus}\) [7], \(\text{Acidiphilium rubrum}\) [20] and \(\text{Erythrobacter sp.}\) strains [15]. Some difference in \(\text{CO}_2\) fixation can be detected between \(R.\text{denitrificans}\) and \(A.\ rubrum\). In \(A.\ rubrum\), \(\text{CO}_2\) fixation is completely inhibited with the glucose-growth culture, and is enhanced 3–5-fold in the light (~3.0 nmole/mg dry cell weight) [20]. In contrast, our studies suggest that \(\text{CO}_2\) incorporation can be detected in \(R.\text{denitrificans}\) with either pyruvate- or glucose-growth cultures in either light or dark growth, and is not enhanced by light exposure.

Among the wide-spread marine \(\text{Roseobacter}\) clade, many of them cannot generate photosynthetic pigments. To the best of our knowledge, no \(\text{CO}_2\) assimilation data have been reported for these unpigmented strains of the \(\text{Roseobacter}\) clade. It will be very useful to compare the difference in the \(\text{CO}_2\) assimilation between \(R.\text{denitrificans}\) and un-pigmented \(\text{Roseobacter}\) clade bacteria.

**Carbohydrate metabolism in \(R.\text{denitrificans}\)**

The ED pathway has generally been viewed as an alternative to the EMP pathway. In examining the carbohydrate catabolism of both \(\text{Rhodobacter capsulatus}\) [25] and \(\text{Rhodobacter sphaeroides}\) [26] under anaerobically phototrophic and aerobically heterotrophic growth conditions, Conrad and Schlegel reported that fructose degradation was shifted to the ED pathway from the EMP pathway during the transition from anaerobically phototrophic to aerobically heterotrophic growth conditions, and that glucose catabolism was going through the ED pathway under both phototrophic and heterotrophic growth. In agreement with their experimental evidences, all of the genes in the EMP and ED pathways can be found in the genome of several \(\text{R. sphaeroides}\) strains, while the 6-phosphogluconate dehydrogenase gene (pgd) in the oxidative PP pathway cannot be annotated.
Our studies in this report indicate that the ED pathway plays a significant role in glucose metabolism of *R. denitrificans*, and that the non-oxidative PP pathway contributes to histidine, ATP, coenzyme and nucleic acid biosynthesis, and that no metabolic flux going through the EMP pathway and the oxidative PP pathway. Our experimental evidences are consistent with the genomic information of *R. denitrificans*, in which the ED pathway and the non-oxidative PP pathway are complete, but the *pfk* gene, essential for the EMP pathway, and the *pgd* gene, required for the oxidative PP pathway, are not annotated.

Searching the genomes of the *Roseobacter* clade [5,8–10], all of them have genes in the ED pathway annotated. The ED pathway is identified mainly in prokaryotes, and almost all of them are aerobes and facultative bacteria, consistent with the physiological features of AAPs. Moreover, note that the *pgd* gene is also missing in *Silicibacter pomeroyi* DSS-3, *Silicibacter* sp. TM1040 and *Dinoroseobacter shibae* DFL12, and that the *pfk* gene is not found in *Jannaschia* sp. CCS1, *Silicibacter pomeroyi* DSS-3, and *Silicibacter* sp. TM1040. Alternatively, *Dinoroseobacter shibae* DFL12 has all of the genes in the EMP pathway, and *Jannaschia* sp. CCS1 has complete
oxidative PP pathway. It will be interesting to learn carbohydrate metabolisms in these two bacteria.

Possible physiological roles of the ED pathway in \textit{R. denitrificans}

The physiological significance for \textit{R. denitrificans} and possibly some other resourceful heterotrophic marine \textit{Roseobacter} clade [12] to depend on the ED pathway, which produces 1 ATP, 1 NADH and 1 NADPH, compared to 2 ATP and 2 NADH from the more common EMP pathway, is an interesting question. The most straightforward answer could be that \textit{R. denitrificans} demands more NADPH than ATP in the cells when the oxidative PP pathway is not active. The oxidative PP pathway, not the non-oxidative PP pathway, can generate two molecules of NADPH from one molecule of glucose, and can produce approximately 60% of the NADPH required inside the cells.

NADPH is not only the reducing power for synthesizing energy-rich molecules and biofuels, but also prevents oxidative stress by reducing glutathione via glutathione peroxidase, which converts reactive \textit{H}_2\textit{O}_2 into \textit{H}_2\textit{O} by glutathione peroxidase [27]. The reactive oxygen species inside the cell can be fatal for \textit{R. denitrificans} since it is an aerobic anaerogenic bacteria that contains highly absorbing pigments that can sensitize reactive oxygen species formation. When the oxidative PP pathway is not active, the ED pathway may be the best option to generate NADPH required by the cells. It was not established whether an anaerogenic photosynthetic bacterium, like \textit{R. denitrificans}, can produce glutathione, which can be synthesized by oxygenic phototrophs, such as cyanobacteria and higher plants. Note that genes for glutathione biosynthesis (glutamate-cysteine ligase, \textit{gclA}, \textit{RD1}_1077, glutathione synthetase, \textit{gshB}, \textit{RD1}_1192), glutathione reductase (\textit{gor}, \textit{RD1}_1919), glutathione peroxidase (\textit{gpx}, \textit{RD1}_0599), and some glutathione transferases can be found in \textit{R. denitrificans} genome, suggesting possible roles of glutathione in reducing oxidative stress in \textit{R. denitrificans}.

The other possibility for \textit{R. denitrificans} adapting the ED pathway could be that many marine \textit{Roseobacter}-lineage bacteria live in coastal seawater, an environment enriched with a wider variety of organic acids, and the ED pathway is a better option for digesting the hexuronic acids (e.g. glucuronic acid). This hypothesis may be supported by the broad substrate specificity of KDPG aldolase identified biochemically [28] and structurally [29]. As gene sequencing is currently in progress for 40 different \textit{Roseobacter} strains [2], it will be interesting to learn if the patterns of carbohydrate metabolism revealed in this work may be a general theme in the \textit{Roseobacter} clade, especially the significance of the ED/non-oxidative PP pathway in these physiologically diverse, widespread and high abundance marine microorganisms.

Gene expression under different growth conditions

Throughout the QRT-PCR data analysis, we observed that the transcript level for all of the genes tested is higher in cells grown in the minimal medium containing either pyruvate or glucose than in the rich medium (Figure 3). The discrepancies may be attributed to the following possible scenarios: (1) metabolic regulation: it has been thought in some bacteria, \textit{E. coli} included, that the anaplerotic and metabolic pathways are not as much in demand in the rich medium as in the minimal medium containing defined carbon source, or some pathways may be shut down due to metabolic regulation [30,31], so genes in those metabolic pathways are not highly expressed in rich growth medium; and/or (2) the abundance of the 16S rRNA gene will be expected to remain constant with glucose (grow slowest), and highest number in the rich medium grown under dark conditions (grow fastest). Together, both possible scenarios can explain lower transcript level for all of the target genes we tested in the rich medium. Further investigations are required for addressing this important issue.

Requirement of yeast extract or exogenous vitamin B12

In this report, we demonstrate that vitamin B12 can successfully replace the undefined carbon source, yeast extract, for the growth of \textit{R. denitrificans} in our developed minimal medium containing defined organic carbon source. While it is not known why the undefined carbon source yeast extract is required for \textit{R. denitrificans} and several other photosynthetic bacteria, it is not clear why exogenous vitamin B12 is required for \textit{R. denitrificans}, because all of the genes required for vitamin B12 biosynthesis are annotated in \textit{R. denitrificans} (based on the information in the KEGG (Kyoto Encyclopedia of Genes and Genomes) database). It is also unclear how cobalamin can be utilized by \textit{R. denitrificans}, since no known cobalamin transporter genes (\textit{btkB}, \textit{btAc}, \textit{btuD} and \textit{btmA}) can be recognized in the genome. Nonetheless, the effect of exogenous vitamin B12 is clear and its inclusion permitted most of the results reported in this paper. More experimental evidences are required for understanding the roles of yeast extract and exogenous vitamin B12 reported herein for enhancing cell growth.

A common feature in Roseobacter clade?

Our studies suggest approximately 10–15% CO2 fixation in protein carbon through the anaplerotic enzymes by \textit{R. denitrificans}, supporting the proposal by Swingley et al. [8]. We also confirmed that unlike carbon fixation by autotrophs, \textit{R. denitrificans} cannot live with CO2 as the sole carbon source, consistent with lack of genes for key enzymes of the Calvin cycle. Missing genes in the Calvin cycle are also common features in the other four \textit{Roseobacter} clade bacteria with complete sequences [2,5,9], suggesting that carbon fixation mechanisms other than the Calvin cycle are needed for the AAPs.

Other than the ED pathway, our studies demonstrate that \textit{R. denitrificans} uses the non-oxidative PP pathway as the other alternative pathway for sugar degradation to make the ribose-5-phosphate for histidine, cofactors, ATP and nucleic acid biosynthesis, as well as to produce GAP for carbon metabolism. In contrast to the oxidative PP pathway, no CO2 is released in the non-oxidative PP pathway, which is an important feature for carbon fixation by autotrophs using the Calvin cycle. In these autotrophs, the non-oxidative PP pathway is part of the Calvin cycle. Although \textit{R. denitrificans} cannot use the Calvin cycle for fixing CO2, it does have a complete non-oxidative PP pathway and relies on the pathway for converting hexose phosphate into pentose phosphate.

Materials and Methods

Materials

The DNA oligomers in this report are from Integrated DNA Technology (IDT) and were used without further purification. The 13C-labeled glucose, pyruvate and sodium bicarbonate were purchased from Cambridge Isotope Laboratories (CIL), Inc (MA, USA).

The growth of bacterial strains

\textit{R. denitrificans} OCh114 (a gift from the laboratory of Dr. Beatty at University of British Columbia, Vancouver, Canada) was grown aerobically (20–30%-filled Erlenmeyer flasks) on either a nutrition-
strong medium (Difco™ Marine Broth 2216; Becton, Dickson and Company) (pH 7.5) or a reported minimal medium [13] with modifications (illustrated in next paragraph). No undefined carbon sources (yeast extract) was included in our minimal growth medium. The seawater source in our minimal medium was prepared by 3.2%/ Instant Ocean® artificial sea salts (United Pet Group, Cincinnati, OH). The minimal growth medium was adjusted to pH 7.5. All of the cultures were grown at 28°C and the growth rates were estimated by OD600. We used 2% cultures (50-fold dilution) in the late exponential growth phase grown in the rich medium to inoculate the minimal medium containing defined carbon source(s). To minimize the carryover of the rich medium, inoculated cells were centrifuged and supernatant liquid was removed. Cell pellets were washed with the minimal medium (no carbon source included) twice, resuspended in the minimal medium, and then transferred to the minimal medium containing defined carbon source(s).

In the minimal medium supplied with defined 13C-isotopically labeled carbon source, 0.1% pyruvate labeled on either the first or third carbon position, 0.1% glucose (on the first, sixth or uniform labeled), or 0.2% NaH13CO3 were added. Due to the requirement of a growth medium without including undefined carbon sources for GC/MS studies, ferric citrate [13] was replaced by ferric chloride hexahydrate, and yeast extract was substituted by 20 μg/L vitamin B12. The cultures were grown to an exponential phase, representing the pseudo steady-state, and then harvested for analyzing the isotopomer distribution in key metabolites.

RNA extraction and quantitative real-time PCR (QRT-PCR)

The methods used to extract RNA and perform QRT-PCR were described previously [35]. QRT-PCR was performed to profile the gene expression under different growth conditions of R. denitrificans OCh114. cDNA was synthesized from 1 μg RNA and 100 μM random 9mer DNA using Superscript III reverse transcriptase (Invitrogen). The QRT-PCR reactions were performed via ABI 7500 Real-Time PCR System (Applied Biosystems). The primers (shown in Table S2) for QRT-PCR reactions were designed using Primer Express 2.0 software (PE Applied Biosystems) and analyzed by OligoAnalyzer 3.0 software (IDT). The Power SYBR Green Master Mix (PE Applied Biosystem) was used for amplifying DNA [35]. The cycle threshold (Ct) was calculated as the cycle number at which a fluorescence threshold (ΔRn) crossed the baseline. Data were normalized by analyzing ΔCt = Ct of the target gene – Ct of the internal control gene (16S rRNA), and each relative gene expression value is calculated with 2–ΔΔCt. Three biological replicates, with six technical replicates for each biological sample, and total eighteen technical replicates were performed for validation, and the mean value was reported (Figure 3). The amplified DNA fragments were verified by 1% agarose gel electrophoresis, and a single fragment was obtained for all amplicons (data not shown).

Measurements of pyruvate/glucose uptake

The amount of D-glucose in solution was determined by hexokinase/glucose-6-phosphate dehydrogenase coupling assay with a D-glucose assay kit (Roche, Mannheim, Germany), and the reduction of NAD+, was followed spectrophotometrically by the increase at 340 nm in absorbance. The amount of pyruvate in the culture was estimated by lactate dehydrogenase (EC 1.1.2.3) (Sigma-Aldrich) with 25 μM NADH in 0.1 M Tris-HCl buffer at pH 8.0 [36]. The reaction was followed by the oxidation of NADH (the decrease at 340 nm in absorbance).

Enzyme assays

Enzymatic activities were performed with cell-free crude extracts prepared as follows. Cells were harvested by centrifugation at 5,000×g for 15 min at 4°C and washed with 20 mM Tris-HCl buffer at pH 8.0. Cell pellet was resuspended in the same buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF). Resuspended cells were disrupted by sonication, and cell debris was removed with centrifugation at 20,000×g for 30 min. Protein concentration in cell extracts was determined by the Bradford assay [37] using bovine serum albumin as a standard. The activity of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, phosphoenolpyruvate carboxykinase, malic enzyme, citramalate synthase, 3-phosphofructokinase, 2-keto-3-deoxy-6-phosphogluconate aldolase, 6-phosphoglucononate dehydrogenase, isopropylmalate synthase and threonine deaminase in cell extracts were assayed as described previously [22,23,38–46].

Gas chromatography (GC)/mass spectrometry (MS)

The methods for GC/MS are reported previously [44,47–49]. In brief, biomass was collected from 50 mL culture by centrifuge and then was hydrolyzed in 6 M HCl at 100°C. The resulting amino acids were dried and derivatized in tetraphospho- and N-[2,4-dimethyl]-(dimethylsilyl)-N-methyl-trifluoroacetamide (Sigma-Aldrich) at 70°C for 1 h. A gas chromatograph (GC) (GC Hewlett-Packard, model 7890A, Agilent Technologies Inc., Balwin, MO) equipped with a DB-5MS column (J&W scientific, Folsom, CA) and a mass spectrometer (5975C, Agilent Technologies Inc.) was used for analyzing metabolite labeling profiles. We report four types of MS fragment data: (1) the [M-15]+ group, which loses a methyl group; (2) the [M-57]+ group, containing un-fragmented amino acids; (3) the [M-159]+ group, which contains amino acids losing a carbonyl group; and (4) the [M-302]+ group, containing only the 1s (α-carboxyl group) and 2nd carbons in the amino acid backbone (Note that [M-302]+ and [M-159]+ cannot be detected in some amino acids). The three labeling data were used to trace the carbon metabolic route and to identify active pathways. Throughout the article, M+0, M+1, M+2… stands for the value of unlabeled (M+0) and of additional 13C-labeled protein-derived amino acids (M+1, M+2,...).

Supporting Information

Figure S1 Spectra, culture, and growth curves of R. denitrificans under different growth conditions. The culture of R. denitrificans grown in the rich medium under dark versus under light. The spectrum of the cells in the minimal medium containing defined carbon source(s).

Figure S2 The non-oxidative pentose phosphate pathway. All of the reaction steps are reversible. Possible 13C-labeling using [1-13C]glucose or [6-13C]glucose is shown in green or red dots, respectively.

Figure S3 Experimental values of the isotopomer abundance of isoleucine (red bar) using [3-13C]pyruvate as the defined carbon source, and predicted contributions of the threonine (back bar) and citramalate (blue bar) pathways for isoleucine biosynthesis in R. denitrificans.
Table S1
13C-isotopomer abundances of tert-butyl dimethylsilyl (TBDS) derivatives of protein-derived amino acids from *R. denitrificans* OCh114 grown in the defined medium containing different carbon sources. Except in (b), all of the cultures were grown in the dark. The unlabeled molecule is shown as M+0, and additional mass comes from 13C-labeled carbon source. All of the data, except leucine and isoleucine, are [M-57]+ data (tert-butyl group is removed). The [M-15]+ data (methyl group is cleaved) for leucine and isoleucine are shown, because the [M-57]+ data are overlapped in GC-MS.

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Table S2
Primers used for QRT-PCR studies reported in this paper

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