New insight into the photoheterotrophic growth of the isocitrate lyase-lacking purple bacterium *Rhodospirillum rubrum* on acetate

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Purple non-sulfur bacteria are well known for their metabolic versatility. One of these bacteria, *Rhodospirillum rubrum* S1H, has been selected by the European Space Agency to ensure the photoheterotrophic assimilation of volatile fatty acids in its regenerative life support system, MELiSSA. Here, we combined proteomic analysis with bacterial growth analysis and enzymatic activity assays in order to better understand acetate photoassimilation. In this isocitrate lyase-lacking organism, the assimilation of two-carbon compounds cannot occur through the glyoxylate shunt, and the citramalate cycle has been proposed to fill this role, while, in *Rhodobacter sphaeroides*, the ethylmalonyl-CoA pathway is used for acetate assimilation. Using proteomic analysis, we were able to identify and quantify more than 1700 unique proteins, representing almost one-half of the theoretical proteome of the strain. Our data reveal that a pyruvate:ferredoxin oxidoreductase (NifJ) could be used for the direct assimilation of acetyl-CoA through pyruvate, potentially representing a new redox-balancing reaction. We additionally propose that the ethylmalonyl-CoA pathway could also be involved in acetate assimilation by the examined strain, since specific enzymes of this pathway were all upregulated and activity of crotonyl-CoA reductase/carboxylase was increased in acetate conditions. Surprisingly, we also observed marked upregulation of glutaryl-CoA dehydrogenase, which could be a component of a new pathway for acetate photoassimilation. Finally, our data suggest that citramalate could be an intermediate of the branched-chain amino acid biosynthesis pathway, which is activated during acetate assimilation, rather than a metabolite of the so-called citramalate cycle.

INTRODUCTION

*Rhodospirillum rubrum* belongs to the purple non-sulfur bacteria, a highly metabolically versatile group of bacteria. The S1H strain of this bacterium has been selected by the European Space Agency as a major component of its regenerative life support system MELiSSA (Micro-Ecological Life Support System Alternative) (Hendrickx et al., 2006). In this artificial ecosystem, bioreactors inhabited by different organisms will recycle crew-produced waste to generate oxygen, water and food. The first compartment, which treats crude waste through thermophilic fermentation, will produce large amounts of volatile fatty acids, the most abundant of which will be acetate. *Rs. rubrum*, which is included in the second compartment of the MELiSSA loop, will be used to photoassimilate these volatile fatty acids.

The assimilation of carbon sources that enter central carbon metabolism through acetyl-CoA requires an alternative cycle to replenish the TCA cycle intermediates used for the synthesis of all cell components (Dunn et al., 2009). During photoheterotrophic metabolism, the TCA cycle is primarily employed for intermediate metabolite production, rather than acetyl-CoA oxidation and energy production. The first identified shunt allowing two-carbon compound assimilation was the glyoxylate pathway (Kornberg & Krebs, 1957), through which two acetyl-CoA molecules are converted into malate to replenish the intermediates consumed. The key enzyme involved in the glyoxylate shunt is isocitrate lyase, which converts isocitrate into succinate and glyoxylate. Glyoxylate is then combined with acetyl-CoA by malate synthase to produce malate. Through the consumption of one citrate molecule and one acetyl-CoA molecule, this cycle results in the net production of two molecules of malate.
which will be converted into oxaloacetate and 2-oxoglutarate, which are at the root of several biosynthetic pathways.

A number of bacteria, including *Rs. rubrum*, lack a functional isocitrate lyase and are thus designated L- (Kornberg & Lascelles, 1960). In these organisms, an alternative to the glyoxylate shunt is required to produce oxaloacetate and 2-oxoglutarate to feed the reactions of cell component biosynthesis (Anthony, 2011). The methylaspartate cycle was recently demonstrated to occur in halarchaea (Khomyakova et al., 2011). Moreover, a second pathway allowing acetyl-CoA assimilation, the ethylmalonyl-CoA (EMC) pathway, has been identified in *Rhodobacter sphaeroides* by Alber and colleagues (Erb et al., 2009a). All the enzymes belonging to this pathway in *Rb. sphaeroides* have been identified, purified and extensively characterized (Alber et al., 2006; Erb et al., 2007, 2008, 2009b; Meister et al., 2005). Schneider et al. (2012) recently showed that *Methyllobacterium extorquens* also employs the EMC pathway. On the other hand, *Rs. rubrum* has been proposed to assimilate acetate through the citramalate cycle (Ivanovsky et al., 1997), based on the early finding that acetate assimilation is accompanied by the production of a compound identified as citramalate (Hoare, 1963) and on the presence of enzymatic activity allowing the production of glyoxylate and propionyl-CoA from C5 compounds (Osumi & Katsuki, 1977). Berg and Ivanovsky’s group also reported the detection of enzymatic activity related to this pathway in *Rs. rubrum* cell extracts (Berg & Ivanovsky, 2009; Berg et al., 2002; Filatova et al., 2005), but the enzyme responsible for citramalate synthesis or conversion to mesaconate has not been identified/purified to date, nor has a putative gene encoding this enzyme been detected in the genome. Recently, the same group observed the expression of crotonyl-CoA carboxylase/reductase, the key enzyme of the EMC pathway, in *Rs. rubrum* grown photoheterotrophically on acetate, but the authors concluded that EMC involvement was not significant in this strain (Berg & Ivanovsky, 2009). In both the citramalate and EMC pathways, glyoxylate and propionyl-CoA are produced from mesaconyl-CoA, with 3-methylmalonyl-CoA generated as an intermediate, but the two pathways differ in the first step, leading to production of mesaconyl-CoA as well as propionyl-CoA assimilation.

Here, we used growth analysis, proteomics and enzymatic activity tests to analyse acetate photoassimilation in *Rs. rubrum* strain S1H. More than 1700 proteins, covering a significant part of the expressed proteome, were identified and quantified. Except for those shared with poly(3-hydroxybutyrate) (PHB) formation, all the proteins belonging to the EMC pathway exhibited a higher abundance under the conditions tested, suggesting that the EMC pathway could effectively be operational in our strain. In addition, activity of crotonyl-CoA carboxylase/reductase was also higher under acetate conditions. An enzyme that could be responsible for detected citramalate synthesis has also been observed in higher abundance in acetate-growing culture. Citramalate synthesis is here proposed to be a consequence of operation of part of the branched-chain amino acid biosynthetic pathway. In addition, several other proteins showed higher abundances and could be linked to the cellular redox balance of the cells and also to a potential assimilation pathway through glutaryl-CoA dehydrogenase that has not so far been identified.

**METHODS**

**Bacterial strain, culture medium and growth conditions.** *Rs. rubrum* strain S1H (ATCC 25903), hereafter named S1H, was used to study acetate assimilation under anaerobic and phototrophic conditions, in accordance with the requirements of the MELISSA project. The S1H strain differs from the S1 type strain only in its lower sensitivity to growth inhibition by L-threonine. The bacterium was cultivated on the basal salt medium of Segers & Verstraete (1983) as described by Suhaimi et al. (1987), with acetate (62 mM) or succinate (124 mM) and ammonium chloride (35 mM) as the sources of C and N, respectively, and biotin as the only vitamin provided. Carbonate was included in the medium at a concentration of 3 mM under both conditions. When acetate and propionate were used as a mixed carbon source, both components were supplied at an equivalent net carbon concentration (acetate 31 mM, propionate 20.6 mM). The cultures were grown in 50 ml serum bottles containing 40 ml medium. Pure nitrogen was used to remove oxygen from the upper gas phase and the flasks were then hermetically sealed. Unless otherwise stated, an illumination intensity of 50 μmol m⁻² s⁻¹, as determined with an underwater quantum meter (LI-193; LI-COR), was supplied with a halogen light (10 W, 100 lm; 2650 K; Senycs). The cultures were incubated at 30 °C with rotary shaking at 150 r.p.m. Growth was monitored by measuring OD₆₅₀.

**Proteomic analysis.** Bacteria were acclimated and subcultivated in acetate or succinate starting at OD₆₅₀ 0.4–0.5. On reaching OD₆₅₀ 0.9–1.2, bacteria were harvested via centrifugation at 16 000 g at 4 °C, and proteins were extracted using guanidinium chloride (6 M) and ultrasonication (3 × 10 s, amplitude 40 %, IKA U50 sonicator). The protein concentration was determined using the Bradford method (Bradford, 1976), with bovine gamma-globulin as a standard. Aliquots of protein (50 μg) were reduced, alkylated and precipitated with acetone. The protein pellets obtained were solubilized using 50 mM ammonium bicarbonate containing 1 μg trypsin, and incubated overnight at 37 °C. Digestion was stopped with 0.1 % formic acid (v/v, final concentration).

Protein identification and quantification were performed following a label-free strategy on a UHPLC-HRMS platform (Eksigent 2D UltraAB SCIEX Triple TOF 5600). Peptides (2 μg) were separated in a 25 cm C₁₈ column (Acclaim PepMap100, 3 μm; Dionex) using a linear acetonitrile (ACN) gradient [5–35 % (v/v), in 120 min] in water containing 0.1 % (v/v) formic acid, at a flow rate of 300 nl min⁻¹. To achieve the greatest possible retention time stability, which is required for label-free quantification, the column was equilibrated with 10 vols 5 % ACN prior to each injection. Mass spectra were acquired over the range 400–1300 m/z in high-resolution mode (resolution >35 000), with a 500 ms accumulation time. The instrument was operated in data-dependent acquisition mode, and MS/MS spectra were acquired over the range 100–1800 m/z. The precursor selection parameters were as follows: intensity threshold, 200 c.p.s.; maximum precursors per cycle, 50; accumulation time, 50 ms; exclusion time after one spectrum, 15 s. These parameters lead to a duty cycle of 3 s per cycle to ensure that high-quality extracted ion chromatograms (XICs) are obtained for peptide quantification.

ProteinPilot software (v4.1) was used to perform database searches against the UniProt database, restricted to *Rhodospirillum* entries (ATCC 11170 plus F11 strains). The search parameters included differential amino acid mass shifts for carbamidomethyl cysteine, oxidized methionine, all biological modifications, amino acid substitutions and missed trypsin cleavage sites.
For peptide quantification, the ‘quant’ application of PeakView was employed to calculate XICs for the five top peptides from all proteins identified at a false discovery rate below 1%. Only unmodified and unshared peptides were subjected to quantification. Peptides were also excluded if the confidence in their identification was below 0.99, as determined in ProteinPilot. A retention time window of 2 min and a mass tolerance of 0.015 m/z were applied. The calculated XICs were exported into MarkerView, which was used to normalize the chromatograms based on the summed area of the entire run. Only proteins exhibiting a fold change greater/less than 1.5/0.66 with a P-value lower than 0.05 across the three biological replicates and quantified with at least two peptides were employed for metabolic characterization.

Monitoring of volatile fatty acid consumption by HPLC analysis. Culture supernatants were obtained from culture samples after centrifugation at 16 000 g for 10 min at 4 °C. Aliquots (50 μl) of culture supernatants were analysed in isocratic mode using a Shodex Sugar SH1011 column (300 x 8 mm) with aqueous H2SO4 (5 mM) as the mobile phase. Acetate and propionate were assayed by integrating their specific peaks (retention times 11 and 13 min, respectively) and comparison with a reference curve constructed for acetate and propionate standards. Detection was performed using a refractometer.

Enzymatic activity tests. Cells were harvested by centrifugation at 16 000 g for 10 min at 4 °C in the middle of the exponential growth phase (OD680 ~1). Cells were mechanically lysed in Tris/HCl buffer (50 mM pH 7.9) using bead-beating (Sigma Aldrich); 1 : 3 beads : sample ratio; 3000 r.p.m., 5 min at 4 °C. Cell-free extract was obtained by centrifugation at 16 000 g for 10 min at 4 °C. The protein concentration was determined using the Bradford method (Bradford, 1976), with bovine gamma-globulin as standard. Acetolactate synthase activity was tested as described previously (Muhitch, 1988), using leucine and valine (10 mM each) as inhibitors of acetolactate synthase to obtain specific activity as recommended. The specific acetolactate synthase activity is defined as the part of the measured activity that can be inhibited by the presence of leucine and valine. The final activity observed in the presence of leucine and valine (the non-specific activity is attributed to pyruvate decarboxylase) was subtracted from the activity measured in their absence. Acetolactate produced after 1 h was assayed at a single end point by conversion to acetoin, which was detected by the reaction described by Westerfield (1945). The amount of acetoin produced was determined through the use of a standard curve. Values were normalized using the amount of protein determined by Bradford assay. Crotoneyl-CoA carboxylase/ reductase activity was measured as described previously (Erb et al., 2007) by following crotonyl-CoA-dependent oxidation of NADPH spectrophotometrically at 380 nm (ε=1210 l mol·1 cm·1) for 3 min. The test started when protein extract (20–40 μg) was added to the reaction mixture. Glutaryl-CoA dehydrogenase activity was measured as described previously (Blázquez et al., 2008) by following glutaryl-CoA-dependent reduction of ferricenium hexafluorophosphate spectrophotometrically at 280 nm (ε=4300 l mol·1 cm·1) for 50 min, the test starting when protein extract (20–40 μg) was added to the reaction mixture. Values obtained for each enzymatic activity were normalized using the amount of protein determined by Bradford assay. All tests were also carried out in the absence of protein extract to ensure the protein dependence of the test (data not shown).

Transmission electron microscopy. Cells were harvested by centrifugation at 16 000 g for 10 min at 4 °C in the middle of the exponential growth phase (OD680 ~1). Cells were washed with a cacodylate buffer at pH 7.8 [0.1 M cacodylate, 8 mM NaCl, 0.05% (w/v) ruthenium red] and immediately fixed for 2 h at 4 °C with 3% (v/v) glutaraldehyde in cacodylate buffer. After two additional 10 min washing steps with cacodylate buffer, samples were post-fixed for 2 h with 1% (w/v) OsO4/cacodylate buffer at room temperature, and washed again. They were subsequently dehydrated in a graded ethanol series up to 100%, and finally embedded in Spurr resin (TAAB Laboratories Equipment). Ultrathin slices (70–90 nm) obtained with a Leica Ultracut UCT ultramicrotome were stained with uranyl acetate and lead citrate. Prepared samples were observed with a Zeiss LEO 906E transmission electron microscope operated at 60 kV, and transmission electron microscopy images were acquired with analySIS software (Soft Imaging System).

RESULTS AND DISCUSSION

Rs. rubrum growth with acetate as unique carbon source – light stress sensitivity

To better characterize the growth behaviour of our strain when using acetate as the carbon source and to define culture conditions allowing almost parallel growth on acetate and succinate, which is a requirement for unbiased proteomic analysis, we first established growth curves for the acetate and succinate cultures under various conditions. In contrast with succinate, we observed a particularly long lag phase (more than 150 h) for the acetate cultures under conditions involving a small amount of succinate-obtained inoculum (starting OD680 ~0.1) and high light intensity (150 μmol m−2 s−1) (Fig 1a). Because this difference would bias the proteomic analysis, we decided to optimize culture conditions to decrease the lag phase for acetate photoassimilation. Even though such a long lag phase is unlikely to be attributable to metabolic adaptation, we tested the effect of using acetate-grown inocula without any decrease in the lag phase (data not shown). No improvement was observed when the carbonate concentration was doubled (data not shown), suggesting that the long lag phase observed did not result from the already described dependence of acetate assimilation on the presence of CO2 (Hoare, 1962; Porter & Merrett, 1972). In contrast, we were able to significantly decrease the length of the lag phase by decreasing the light intensity from 150 to 50 μmol m−2 s−1 (Fig 1b). A second major improvement was achieved by increasing the size of the inoculum so that the culture started with OD680 0.5–0.6 (Fig 1c), in place of 0.2 initially. Based on these results, it appears likely that the long lag phase could be due to a higher sensitivity to light stress when S1H is cultivated with acetate as unique carbon source. Indeed, dilution of the culture at the time of inoculation significantly increases the light intensity experienced at the cellular level. In contrast, increasing the OD at inoculation and lowering the light intensity reduce the level of ‘inoculation stress’. To confirm the higher sensitivity to light stress of S1H growing on acetate vs succinate as the carbon source, cultures reaching OD680 1.1 (the middle of the exponential growth phase) were subjected to a twofold increase in the light intensity (from 50 to 100 μmol m−2 s−1). As shown in Fig. 2, the increase in the light intensity had almost no effect on the growth rate of the bacteria on succinate but completely abolished growth on acetate. This experiment demonstrates that Rs. rubrum
S1H shows greater sensitivity to light stress when growing on acetate as the sole carbon source. We assume that a redox imbalance is responsible for this light stress and can explain the difference in sensitivity observed between these two culture conditions. A rapid increase in the light intensity would trigger an increase in the photosynthetically produced proton gradient, which would, in turn, increase the rate of NAD$^+$ photoreduction to NADH through reverse NADH dehydrogenase activity (Golomysova et al., 2010; Herter et al., 1998; McEwan, 1994). Acetate is a more reduced carbon source than succinate (McKinlay & Harwood, 2010), and the NAD$^+$/NADH ratio could be more difficult to maintain if the intensity of the light received is suddenly increased. In support of this hypothesis, the elemental composition of acetate-grown Rs. rubrum exposed to different lighting regimes clearly indicates that a more reduced biomass is produced when the bacterium is cultivated at a biomass concentration of 1 g l$^{-1}$ (high light) than at 4 g l$^{-1}$ (low light) (Favier-Teodorescu et al., 2003).

**Fig. 1.** Optimization of culture conditions for *Rs. rubrum* when using acetate as the sole carbon source. Growth was followed by measuring OD$_{680}$ (expressed as log OD$_{680}$) for cultures submitted to high light intensity (150 μmol m$^{-2}$ s$^{-1}$) and inoculated at OD$_{680}$ 0.1 (a), submitted to low light intensity (50 μmol m$^{-2}$ s$^{-1}$) and inoculated at OD$_{680}$ 0.1 (b), and submitted to low light intensity (50 μmol m$^{-2}$ s$^{-1}$) and inoculated at OD$_{680}$ 0.5–0.6 (c). All inocula were taken from precultures already acclimated to the corresponding carbon source. Means ± SD; n=3 for all conditions.

**Fig. 2.** Comparison of the effect of light stress on *Rs. rubrum* S1H grown on acetate or succinate as the sole carbon source. Acetate and succinate cultures submitted to low light intensity (50 μmol m$^{-2}$ s$^{-1}$) and inoculated at OD$_{680}$ 0.5–0.6 were subjected when they reached the middle of the exponential growth phase (OD$_{680}$ ~1.1) to a twofold increase in light intensity (t=0). Growth was followed by measuring OD$_{680}$. All inocula were taken from precultures already acclimated to the corresponding carbon source. Means ± SD; n=3 for all conditions.

**Proteomic analysis of acetate photoassimilation by *Rs. rubrum* S1H**

For proteomic analysis, cultures were grown under a low light intensity (50 μmol m$^{-2}$ s$^{-1}$), starting at OD$_{680}$ between 0.4 and 0.5, to reduce the delay in the growth of acetate cultures as much as possible. To avoid bias due to the different light intensities effectively perceived by the cells, biomass samples were collected at a constant cell density (OD$_{680}$ 1.1–1.4) from the cultures grown on acetate and succinate.

Three biological replicates, originating from different clonal populations, were analysed under acetate and succinate conditions. Altogether, more than 1700 unique proteins, representing almost 50% of the theoretical proteome (3461 and 3878 entries for ATCC 11170 and strain F11, respectively,
in UniProt), were identified and quantified. The complete dataset can be found in Table S1, available in the online Supplementary Material. Among the proteins for which a significant ($P<0.05$) fold change ($>1.5$ or $<0.66$) was detected between the acetate and succinate conditions, 352 and 225 proteins exhibited lower and higher abundances, respectively, under acetate conditions. For further interpretation, only proteins quantified with at least two peptides were taken into account.

**Ethylmalonyl-CoA pathway involvement in acetate assimilation by *Rs. rubrum* S1H**

Proteomic data revealed that all the enzymes proposed to belong to the EMC pathway (Table 1) were effectively upregulated (Fig. 3b), suggesting that this pathway could be involved in acetate assimilation in our strain. These enzymes were often badly annotated in the database but comparison to sequences of EMC enzymes studied in *Rb. sphaeroides* (Alber, 2011) allowed us to correct their annotation and clearly link them to this pathway. (Table S2 presents the best homologue candidates of all the enzymes of the EMC pathway of *Rb. sphaeroides* in the genome of *Rs. rubrum*.)

The data obtained through differential proteomic analyses are therefore largely in favour of the significant use of the EMC pathway for the assimilation of acetate in our strain. As the upregulation of an enzyme does not evidence its higher activity, crotonyl-CoA carboxylase/reductase (Ccr) activity has been measured in cell-free extracts of *Rs. rubrum* S1H grown with acetate or succinate as unique carbon source. As shown in Fig. 4b, Ccr activity was significantly higher (8.8 times higher; $P<0.01$) in acetate than in succinate conditions. This result is in contradiction with that obtained previously by Berg & Ivanovskii (2009), who observed no increase in Ccr activity in acetate conditions compared with malate conditions. This difference between the two studies could arise from the different experimental set-up (such as light intensity, which was not sufficiently detailed to be compared with our study) and also in Ccr activity test procedures. Notably, Ivanovskii's group used incorporation of radiolabelled bicarbonate while we used cofactor oxidation, and their extracts were produced using a French press while we used bead-beating. Also, the exact control used in their study is unclear.

**Citramalate involvement in acetate assimilation by *Rs. rubrum* S1H**

In the Icl$^-$ purple bacterium *Rs. rubrum*, Ivanovskii and co-workers proposed that the citramalate cycle takes place, in which acetate is assimilated via the condensation of acetyl-CoA with pyruvate through the action of citramalate synthase (Ivanovskii et al., 1997). This pathway was proposed based on the observation that citramalate is the earliest molecule to be labelled when $^{13}$C-acetate is photoassimilated (Porter & Merrett, 1972). Here, we observed slight upregulation of a protein that is currently annotated as 2-isopropylmalate synthase (LeuA) (Rru_A0695; fold change, 1.6; $P$-value, 0.01; identified peptides, 12), but for which the PRIAM method [for automated enzyme detection (Claudel-Renard et al., 2003)] predicts citramalate synthase activity with a higher probability ($E$-values, 6e$^{-54}$ for citramalate synthase versus 1e$^{-41}$ for isopropylmalate synthase). Both 2-isopropylmalate synthase and citramalate synthase are members of the LeuA dimer superfamily (Frantoul, 2012). The best-characterized citramalate synthase among proteobacteria is that of *Geobacter sulfurreducens* (Risso et al., 2008a), and this protein shares 51% identity with Rru_A0695. These data favour the annotation of this protein as a true citramalate synthase. Nevertheless, upregulation of another group of enzymes leads us to propose a new hypothesis regarding involvement of citramalate in acetate assimilation by *Rs. rubrum* S1H. Indeed, among the identified upregulated proteins, we detected a cluster of three genes encoding ketol-acid reductoisomerase (IcvC) (Rru_A0469; fold change, 4.1; $P$-value, 0.0005; identified peptides, 54) and the acetolactate synthase small (Rru_A0468; fold change, 3.1; $P$-value, 0.003; identified peptides, 22) and large subunits (Rru_A0467; fold change, 3.1; $P$-value, 0.003; identified peptides, 33). These proteins are involved in the first step of branched-chain amino acid (isoleucine, leucine and valine, ILV) synthesis (Fig. 3a). Dihydroxy-acid dehydratase (IlvD), which is also involved in this pathway, was also more abundant under acetate conditions (Rru_A1786; fold change, 1.6; $P$-value, 0.04; identified peptides, 32). Citramalate is an intermediate in the atypical isoleucine synthesis pathway, which is independent of threonine supply (Wu et al., 2010). In this alternative pathway, citramalate is converted to 2-oxobutanoate by the action of 3-isopropylmalate/(R)-2-methylmalate dehydratase large and small subunits (LeuC and LeuD, Rru_A1189 and Rru_A1190) and 3-isopropylmalate dehydrogenase (LeuB, Rru_A1191). These three proteins were identified in this study but were not significantly differentially regulated under acetate condition (Fig. 3a). Acetolactate synthase can equivalently transfer an acetaldehyde from pyruvate to 2-oxobutanoate, forming 2-ethyl-2-hydroxy-3-oxobutanoate, a reaction involved in the ILV biosynthesis pathway. As the upregulation of an enzyme does not evidence its higher activity, we measured the acetolactate synthase activity in cell-free extracts of *Rs. rubrum*. As shown in Fig. 4a, acetolactate synthase activity was slightly but significantly higher in acetate conditions than in the succinate control ($P<0.05$), suggesting this enzyme could be involved in acetate photoassimilation.

However, we observed a decrease in abundance of some branched-chain amino acid transaminases (Ruu_A2223; fold change 0.4, $P$-value 0.01, identified peptides 16; Rru_A1040, fold change 0.03, $P$-value 0.001, identified peptides 16), suggesting that the outcome of acetate assimilation through reactions belonging to the ILV pathway is not the production of branched-chain amino acids. 3-Methyl-2-oxopentanoate and/or 2-oxoisovalerate could, for example, be directed to another metabolic pathway, such as the valine degradation.
Table 1. Differentially expressed proteins

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The complete dataset can be found in the supplementary data.

*The fold change is the ratio of the abundance of a protein under acetate conditions to the abundance under succinate conditions.

†No. identified peptides denotes the number of identified peptides with a confidence higher than 95%.

‡Due to interference in MS signal, fold change for this protein was manually corrected and results from quantification of four peptides rather than five.

§Fix, Electron transfer flavoprotein.
(a) Acetate photoassimilation in *Rhodospirillum rubrum*

1. Acetate $\rightarrow$ Pyruvate
2. Pyruvate $\rightarrow$ Acetyl-CoA
3. Acetyl-CoA $\rightarrow$ Acetoacetyl-CoA
4. Acetoacetyl-CoA $\rightarrow$ Malyl-CoA
5. Malyl-CoA $\rightarrow$ Succinyl-CoA

(b) Alternative pathways:

1. Acetoacetyl-CoA $\rightarrow$ (R)-3-Hydroxybutyryl-CoA
2. (R)-3-Hydroxybutyryl-CoA $\rightarrow$ Crotonyl-CoA
3. Crotonyl-CoA $\rightarrow$ Butyryl-CoA
4. Butyryl-CoA $\rightarrow$ Propionyl-CoA
5. Propionyl-CoA $\rightarrow$ Methylmalonyl-CoA
6. Methylmalonyl-CoA $\rightarrow$ Malyl-CoA

Fold change:

- $<0.2$
- $<0.5$
- $<0.66$
- $<1.5$
- $<2$
- $<5$
- $>5$

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pathway (Sykes et al., 1987), which is conducive to propionyl-CoA production. However, despite intensive researches for an enzyme similar to the already described keto-acid dehydrogenase in the genome of *Rs. rubrum*, we could not find any candidate fulfilling this function. Further investigation, notably including metabolite analyses, will thus be needed in order to clarify the involvement of reactions belonging to the ILV synthesis pathway in acetate photo-assimilation in *Rs. rubrum*.

Acetolactate synthase combines two molecules of pyruvate to produce one molecule of acetolactate and releases one molecule of CO₂. Substantial activation of this pathway would result in enhanced consumption of pyruvate, which would need to be replenished continuously. We detected the presence of a pyruvate : ferredoxin oxidoreductase, also known as NifH (Rru_A2398; fold change, 1.8; *P*-value, 0.01; identified peptides, 80), which is commonly reported to catalyse oxidative decarboxylation of pyruvate to generate acetyl-CoA. It has been shown that this enzyme is able to catalyse the reverse reaction as well, thus producing pyruvate via the reductive carboxylation of acetyl-CoA (Furdui & Ragsdale, 2000; St Maurice et al., 2007). At physiological concentrations, Furdui & Ragsdale (2000) measured a significant carboxylating activity and showed that the rate of the reaction depends only on the concentration of acetyl-CoA and pyruvate. As the intracellular concentration of acetyl-CoA is likely to be high in cells grown on acetate and as pyruvate level should be kept low through consumption by acetolactate synthase, the net flux through this enzyme is expected to favour pyruvate synthesis when the bacteria are grown on acetate. Noticeably, the high level of acetyl-CoA can be maintained in the cell through the upregulation of acetyl-CoA transferase (Rru_A1927; fold change, 1.8; *P*-value, 0.004; identified peptides, 34), which could be responsible for acetyl-CoA activation, as mentioned by Alber et al. (2006). The preference for reductive carboxylation activity is further supported by the observation of major downregulation of all subunits of the pyruvate dehydrogenase (lipoamide) complex (Rru_A1878, Rru_A1879, Rru_A1881). Indeed, pyruvate dehydrogenase (lipoamide), dihydrolipoamide acetyltransferase and dihydrolipoamide dehydrogenase were all clearly downregulated under acetate conditions, suggesting that oxidative decarboxylation of pyruvate activity is lowered in cells grown on acetate. Importantly, reductive carboxylation activity relies on the presence of an electron supplier, which has been proposed to be ferredoxin (Furdui & Ragsdale, 2000). In our dataset, we identified 4Fe–4S ferredoxin as differentially regulated under acetate conditions, but its abundance was greatly decreased (Rru_A0077; fold change, 0.25; *P*-value, 0.003; identified peptides, 8). Nevertheless, a second ferredoxin encoded in the fix gene cluster was clearly upregulated under acetate conditions (Rru_A2264; fold change, 6.2; *P*-value, 0.0003; identified peptides, 6) and could therefore serve as the electron supplier for reductive carboxylation of acetate. Altogether, these data are in favour of a major involvement of carboxylation of acetyl-CoA to pyruvate, which in turn feeds the branched-chain amino acid synthesis pathway. This interpretation of our result is also in agreement with the data of Porter & Merrett (1972), who...
reported that citramalate was one of the earliest and most abundant radiolabelled compounds in short-term exposure experiments with NaH\textsuperscript{14}CO\textsubscript{3} and acetate-assimilating Rs. rubrum. Altogether, these data lead us to propose that the extensively reported contribution of citramalate synthase in acetate assimilation by Rs. rubrum is linked to the use of part of the ILV biosynthesis pathway (Fig. 3a) rather than to the so-called citramalate cycle, and that the ILV biosynthesis pathway plays a significant but not yet fully understood role in acetate assimilation. Further experiments such as flux analyses will be needed to definitely demonstrate and quantify the involvement of this proposed pathway in acetate photoassimilation in Rs. rubrum.

Glutaryl-CoA dehydrogenase involvement in acetate assimilation by Rs. rubrum S1H

Differential proteomic analysis also revealed the intense upregulation of the clustered glutaryl-CoA dehydrogenase (Rru_A2005; fold change, 176; P-value, $7 \times 10^{-6}$; identified peptides, 26) and carnitine hydratase (best annotated as a CoA transferase) (Rru_A2006; fold change, 8; P-value, $9 \times 10^{-6}$; identified peptides, 26). Glutaryl-CoA dehydrogenase is generally observed to be involved in the anaerobic degradation of benzoate in bacteria (Härtel et al., 1993; Wischgoll et al., 2009), in which it catalyses the decarboxylation of glutaryl-CoA to produce crotonyl-CoA. Under our acetate conditions, this compound could be easily assimilated through the EMC pathway (Fig. 3b), but the origin of glutaryl-CoA and the role of the linked CoA transferase remain enigmatic. Glutaryl-CoA dehydrogenase has been shown to use electron transfer flavoproteins as electron acceptors (Ramsay et al., 1987). Under acetate conditions, we observed a large increase of the abundance of the four proteins encoded by the fix gene cluster (FixABCX; Rru_A2267, Rru_A2266, Rru_A2265 and Rru_A2264, respectively). FixA and FixB are electron transfer flavoproteins that are usually responsible for electron transfer to nitrogenase in diazotrophically growing Rs. rubrum (Edgren & Nordlund, 2004). However, nitrogenase was clearly downregulated in response to the use of acetate as the carbon source for growth (Rru_A0793, fold change 0.06, P-value 0.01, identified peptides 3; and nitrogenase iron protein Rru_A0795, fold change 0.02, P-value 0.0017, identified peptides 12). We thus analysed the glutaryl-CoA dehydrogenase activity in cell-free extracts of Rs. rubrum S1H grown in acetate and succinate conditions. As shown in Fig. 4(c), glutaryl-CoA dehydrogenase activity was not detected in the succinate conditions but was readily detected in the acetate conditions [17 nmol min$^{-1}$ (mg protein)$^{-1}$, $P<0.01$]. The detection of glutaryl-CoA dehydrogenase activity only in acetate-grown cells is in total agreement with the very high fold change observed in proteomic data which reveal absence/presence of the enzyme. We also tested (data not shown) glutaryl-CoA dehydrogenase activity in cultures assimilating butyrate and propionate as sole carbon source in the same conditions but neither showed glutaryl-CoA dehydrogenase activity. Non-decarboxylating glutaryl-CoA dehydrogenase exists, which releases the intermediate glutaconyl-CoA in place of crotonyl-CoA. While the released compound was not identified in our enzymatic test, we consider Rru_A2005 most probably belongs to the decarboxylating group of glutaryl-CoA dehydrogenases, based on conservation of key amino acids highlighted by the Boll group (Schaarschmidt et al., 2011; Wischgoll et al., 2009), namely E91, S99 and Y373. Our data thus suggest that glutaryl-CoA dehydrogenase could play a previously unsuspected role in acetate photoassimilation. It is clear that only a detailed flux analysis could definitely identify the relative importance of the multiple pathways proposed here. Nevertheless, comparison of specific activities obtained for crotonyl-CoA carboxylase/reductase [47 nmol min$^{-1}$ (mg protein)$^{-1}$] and glutaryl-CoA dehydrogenase [17 nmol min$^{-1}$ (mg protein)$^{-1}$] suggests that the latter potentially contributes significantly to acetate assimilation.

Acetate transport in Rs. rubrum S1H

Among the observed downregulated proteins, we identified a Na\textsuperscript{+}/solute symporter (Rru_A0635; fold change, 0.2; P-value, 0.0006; identified peptides, 5) that is highly similar (70% ID) to the acetate permease (ActP) of Escherichia coli. Downregulation of the acetate transporter in response to acetate in the medium could appear to be counterproductive, but Gimenez et al. (2003) clearly demonstrated that, in E. coli, ActP was only needed for growth at low acetate concentration. In addition, Risso et al. (2008b) have shown that acetate transporter genes in Geobacter species were only expressed at low concentration of acetate. Moreover, we observed that propionate, which has been shown to inhibit acetate transport through ActP in E. coli (Gimenez et al., 2003), had no effect on acetate assimilation in Rs. rubrum when the two carbon sources were present simultaneously and at the same concentration in the medium (Fig. 5). Altogether, these observations lead us to
suggest that ActP is not involved in acetate transport in our strain under our conditions.

Respiratory chain modification during acetate assimilation by *Rs. rubrum* S1H

Five of the ten members of the NADH-quinone oxidoreductase complex identified in the present study were detected at higher abundances under acetate conditions. The activation of respiratory chain electron transport makes sense only if a final diffusible/excretable electron acceptor is present, such as O₂ or nitrate. In this study, cells were grown under anaerobic conditions with ammonium as the nitrogen source, and neither O₂ nor nitrate was present as a final electron acceptor. The absence of O₂ was confirmed based on pigment expression, which is immediately repressed at a very low O₂ partial pressure (Sistrom, 1965). Upregulation of members of NADH-quinone oxidoreductase in acetate conditions will need further investigation.

Redox balance during acetate assimilation by *Rs. rubrum* S1H

Photoheterotrophic growth of purple bacteria has been extensively reported to depend on the Calvin–Benson–Bassham (CBB) cycle playing the role of an 'electron sink' (Falcone & Tabita, 1991; McKinlay & Harwood, 2010; Wang et al., 2010). In our dataset, we also observed a decreased abundance of RuBisCO when acetate is used as sole carbon source (Rru_A2400; fold change, 0.52; P-value, 0.04; identified peptides, 54) as compared with succinate-containing cultures. This finding seems to be in agreement with the conclusion of Laguna et al. (2011) that, in *Rb. sphaeroides*, CO₂ fixation through the EMC pathway is sufficient to equilibrate redox balance even in the absence of a functional CBB cycle. Nevertheless, we observed that other redox-balancing mechanisms could be playing a role during acetate assimilation. Notably, we observed that, as already mentioned by Kim et al. (2012) for *Rb. sphaeroides*, our strain produced larger amounts of PHB during acetate assimilation (Fig. 6). Hauf et al. (2013) have shown that the reduction state of the cofactor pool can influence PHB production. PHB production observed here could be the result of an increased level of NAD(P)H and indirectly be a way to deal with NAD⁺ photoreduction (Hertez et al., 1998), notably occurring at the beginning of the growth curve. Further experiments are needed to confirm this hypothesis, notably by following PHB accumulation along the complete growth curve in acetate conditions. NifJ catalyses the reductive carboxylation of acetyl-CoA, which could also play the role of redox-balancing reaction. Interestingly, in a kinetic experiment in which we compared the proteome of a culture in lag phase with the proteome of the same culture just after the initiation of growth on acetate as a carbon source (data not shown), NifJ was the most highly overexpressed protein when growth was initiated (fold change, 11.2; P-value, 0.003; identified peptides, 86). This result suggests that NifJ plays a major role at the beginning of the growth curve, corresponding to the period during which light stress due to culture dilution likely triggered the previously mentioned redox imbalance (Favier-Teodosescu et al., 2003). CO₂ fixation through acetyl-CoA carboxylation could therefore represent a new redox-balancing reaction developed by bacteria to combine acetate assimilation and maintenance of the redox balance.

CONCLUSIONS

In conclusion, our proteomic data suggest that *Rs. rubrum* S1H assimilates acetate through multiple pathways. Combination of proteomic analysis and enzymatic activity testing reveal that the EMC pathway could be operational in *Rs. rubrum* when growing photoheterotrophically on acetate. Indeed, all the enzymes specific to the EMC pathway were detected in higher abundances and crotonyl-CoA carboxylase/reductase activity was increased under acetate culture conditions. Our data also lead us to propose a new outcome for citramalate. Indeed, citramalate could be an intermediate of an alternate route of acetate assimilation through pyruvate and part of the ILV biosynthesis pathway. NifJ, which has been shown to produce pyruvate through acetyl-CoA reductive carboxylation, was upregulated in
acetate conditions. This could represent a new redox-balancing reaction, and the pyruvate generated could be directed to acetolactate synthase and the IlvC and IlvD enzymes, which were also detected in higher abundances in acetate conditions. The outcome of the activation of part of the ILV biosynthesis pathway during acetate assimilation is still not understood and requires further investigation. Finally, glutaryl-CoA dehydrogenase could play a previously unsuspected but important role in phototroph acetate assimilation since this protein was the mostly upregulated in this study.

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REFERENCES


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