# Analysis of the photoheterotrophic assimilation of valerate and associated PHA production by *Rs. rubrum*

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**Running title**

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## Abstract

Purple non-sulfur bacteria are increasingly regarded for industrial applications in bioplastics, pigment and biomass production. In order to optimize the yield of future biotechnological processes, the assimilation of different carbon sources by *Rhodospirillum rubrum* (*Rs. rubrum*) has to be understood. As they are released from several fermentation processes, volatile fatty acids (VFAs) represent a promising carbon source in the development of circular industrial applications. To obtain an exhaustive characterization of the photoheterotrophic metabolism of *Rs. rubrum* in the presence of valerate, we combined phenotypic, proteomic and genomic approaches. We obtained evidence that valerate is

cleaved into acetyl-CoA and propionyl-CoA and depends on the presence of bicarbonate ions. Genomic and enzyme inhibition data showed that a functional methylmalonyl-CoA pathway is essential. Our proteomic data showed that the photoheterotrophic assimilation of valerate induces an intracellular redox stress which is accompanied by an increased abundance of phasins (the main proteins present in PHA granules). Finally, we observed a significant increase in the production of the copolymer P(HB-co-HV) presenting an outstanding (more than 80%) percentage of HV monomer. Moreover, an increase in the PHA content was obtained when bicarbonate ions were progressively added to the medium. The experimental conditions used in this study suggest that the redox imbalance is responsible for PHA production. These findings also reinforce the idea that PNSB are suitable for PHA production through another strategy than the well-known feast and famine process.

## Importance

The use and the littering of plastics represent major issues that humanity has to face. Polyhydroxyalkanoates (PHAs) are good candidates for the replacement of oil-based plastics as they exhibit comparable physicochemical properties but are biobased and biodegradable. However, the current industrial production of PHAs is curbed by the production costs, which are mainly linked to the carbon source. Volatile fatty acids issued from the fermentation processes constitute interesting carbon sources as they are cheap and easily available. Among them, valerate is gaining interest regarding the ability of many bacteria to produce a copolymer of PHAs. Here, we describe the photoheterotrophic assimilation of valerate by *Rhodospirillum rubrum*, a purple non-sulfur bacterium mainly known for its metabolic versatility. Using a knowledge based optimization process, we display a new strategy for the improvement of PHA production, paving the way for the use of *Rhodospirillum rubrum* in industrial processes.

## 1 Introduction

*Rhodospirillum rubrum* is a purple non-sulfur bacterium (PNSB) belonging to the α-proteobacteria group. For decades *Rs. rubrum* has been studied in order to understand the photoheterotrophic metabolism. Indeed, this bacterium is able to assimilate a broad range of carbon sources during anoxygenic photosynthesis (1,2). Among these carbon sources, volatile fatty acids (VFAs) are studied in the biotechnology field, as they are cheap and easily available. The use of the by-products issued from the fermentation bioprocesses could also increase the circularity of the bioindustry (3–5). As acetic acid is the most abundant VFA resulting from the fermentation processes, its assimilation has been broadly studied (6–12). It is now well accepted that, in isocitrate lyase gene lacking organisms (*icl*$^-$)(13), such as *Rs rubrum*, an alternative pathway, the ethylmalonyl-CoA pathway (EMC) is used(11) for acetate photoassimilation. The role of this pathway was first characterized by Alber's group(9,10). Beside the EMC pathway, the use of additional metabolic routes, such as the isoleucine biosynthesis and degradation pathways (MBC pathway) or the pyruvate ferredoxin oxydoreductase (PFOR), remains obscure and further studies are still needed to attest to their involvement in acetate assimilation (11,12,14,15) .

Valerate constitutes the fourth most abundant VFA resulting from the majority of fermentation processes (16,17). However, some studies have revealed that the fermentation of pig feces resulted in equal proportions of each VFA, or an even higher proportion of valerate than the other VFAs (18). Moreover, some processes deliberately enriched their fermentation effluent with valerate in order to induce the production of the polymer (poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)) P(HB-*co*-HV) (19) which possesses more interesting physicochemical properties than PHB (*e.g.* higher elasticity, flexibility,…). This further demonstrates the importance of understanding the photoheterophic assimilation of this VFA

by *Rs. rubrum*. Despite this interesting feature, few studies related to the photoheterotrophic assimilation of valerate are available (20). In this study by Janssen and Harfoot, it was stated that all *Rhodospirillaceae* were able to photoassimilate valerate, but no information was given about the underlying metabolic pathway. In *Escherichia coli*, the assimilation of valerate has been reported to rely on the ATO system responsible for short chain fatty acid degradation (21).

The use and littering of petroleum-based plastics is one of the major issues that humanity faces. Polyhydroxyalkanoates (PHAs) represent good candidates to replace conventional plastics as they exhibit physicochemical properties close to oil-based plastics (22–24) with the advantage of being biobased and biodegradable (25,26). However, the production costs today still limit the economic sustainability of the PHA industry. In some processes, the carbon source may account for more than 50% of the total production costs (27). The use of cheap substrates, such as VFAs, may thus represent a way to significantly decrease these costs. Moreover, the nature of the carbon source influences the type and composition of the PHAs produced. In this context, the use of carbon sources with an odd number of carbons, such as propionate or valerate, is known to induce the production of the co-polyester poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate (P(3HB-*co*-3HV)) (28,29) which exhibits enhanced physicochemical properties with a higher elasticity and lower melting point and crystallinity(26).

In this paper, we investigate valerate assimilation by *Rs. rubrum* S1H using global analyses of the metabolism (proteomic and functional genomic approaches) and targeted analyses using knock out mutant and chemical inhibition of key enzymes. Our results demonstrate that the presence of bicarbonate ions is mandatory for the assimilation of valerate. We also show that valerate is first cleaved into propionyl-CoA and acetyl-CoA. Our whole metabolism analyses reveal that a fully functional methylmalonyl-CoA pathway is

mandatory for the photoheterotrophic assimilation of valerate and that the ethylmalonyl-CoA pathway is not necessary for the assimilation of acetyl-CoA issued from valerate cleavage. We suggest that other pathways, such as pyruvate ferredoxin oxydoreductase (PFOR) or isoleucine biosynthesis may take part in this assimilation process as has already been suggested by our previous studies (11,12,14,15). We also investigat PHA production by *Rs. rubrum*, highlighting the production of copolymers with an outstanding HV monomer percentage, even in conditions of full nutrient availability. We finally highlight the capacity of controlling the composition of polymers by the sequential addition of VFAs, which would represent a major advance in controlling the production of biopolymers.

## 2. Materials and methods

### 2.1 Bacterial strain, medium composition and cultivation conditions

The strain *Rhodospirillum rubrum* S1H (ATCC25903) as well as the Δ*ccr*::*km*$^R$ strain were cultivated in medium as previously described (11,12). The medium was supplemented with valerate (24.9 mM) as a carbon source, $NH_4Cl$ (35 mM) as a nitrogen source, biotin (0.06 mM) and a defined amount of added bicarbonate (3 or 50 mM). Stock cultures were cultivated in a supplemented malate ammonium (SMN) rich medium. The precultures used here were all cultivated in the presence of succinate as a carbon source. Cultures were submitted to 50 µmol photons/m² s (Sencys; 10 W; 100 lumens; 2,650 K). The upper gaseous phase was flushed using pure $N_2$ and flasks were hermetically sealed.

Growth was followed through optical density ($OD_{680nm}$) measurement. *Rhodospirillum rubrum* was cultivated under anaerobic phototrophic conditions. Cultures were inoculated at a starting $OD_{680nm}$ between 0.450 and 0.550 and incubated at 30°C with rotary shaking at 185 rpm. Five clonal biological replicates were used for each culture

129 condition. Cell dry weight was approximated using a standard (conversion factor: CDW=
130 0.6521*$OD_{680nm}$).

131 ## 2.2 Mutant strain control
132

133 The presence of *ccr* and *kmR* cassette genes in both the WT and mutant strain was
134 verified through PCR and electrophoresis gel. Genomic DNA (RNA-free) was isolated
135 from 500 µl of bacterial culture using the QIAamp DNA Mini Kit (QIAGEN) according
136 to the manufacturer's instructions. The concentration and quality of the isolated DNA
137 samples were measured using a BioSpec-Nano (Shimadzu) micro-volume
138 spectrophotometer based on UV spectra. The samples were adjusted to a concentration of
139 1 ng/µl. Extracted DNA was then submitted to PCR using a specific primer for Crotonyl-
140 CoA reductase/carboxylase (Ccr-Rru_A3063) and Kanamycin Resistance gene (KmR)
141 (12). DNA was then submitted to electrophoresis and the presence of the targeted genes
142 was revealed using Gel Red.

143 ## 2.3 Monitoring the carbon source concentration in the medium
144

145 Culture supernatants were obtained from culture samples after centrifugation at 16,000
146 g for 10 min at 4°C using a Shodex Sugar SH1011 column (300 mm x 8 mm) with aqueous
147 $H_2SO_4$ (0.01N) as the mobile phase, as previously described (11). Valerate and succinate
148 concentrations were determined by the integration of their specific peak and comparison with
149 a reference curve prepared with the corresponding standard. Detection was performed using a
150 refractometer.

151 ## 2.4 Polyhydroxyalkanoate extraction and quantitation
152

153 PHAs were isolated as previously described (30) with some modifications. Briefly 500
154 µl of culture were centrifuged (8000 rpm, 15 minutes) and stored at -20°C until analysis.

PHAs were extracted and methanolysed by resuspending pellets in 500 µl of chloroform and 2 mL of methanolysis solution consisting of UHPLC methanol: concentrated HCl (90:10). The methanolysis solution also included 0.1 mg/ml of 3-methylbenzoic acid as an internal standard. The mixture was then incubated at 100°C for 2 hours before being cooled on ice. 2 mL of distilled water was then added and the bottom chloroformic part was recovered and analyzed by GC-MS (Shimadzu GC-MS QP2010S).

### 2.5 Mutant fitness assay

The mutant library was produced following the protocol described by Wetmore *et al.*(31) and described in De Meur *et al.* (12).

The mutant fitness assays were performed in five replicates starting with five different glycerol stocks of the mutant library. Each aliquot of the mutant library was independently recovered in SMN with 50 µg/ml kanamycin under dark aerobic conditions. The recovered library was rinsed and resuspended in defined medium to reach a starting $OD_{680nm}$ of 0.1. Samples of biomass were collected at "time zero" (before growth selection) and after five generations of growth in the selected condition, and the genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen). The concentration and purity of the extracted DNA were assessed with a BioSpec-Nano (Shimadzu Biotech) before storage at -80°C. We performed DNA barcode sequencing (BarSeq) as described by Wetmore *et al.*(31) to quantify the barcodes and, consequently, the abundance of each mutant in each experimental condition. The strain fitness was determined as the normalized log2 ratio of bar code counts between samples after five generations and the "time-zero" reference samples. Gene fitness was calculated as the weighted average of the individual strain fitness values for a given gene, as described. Only genes exhibiting fitness value below -0.5 were considered for further analysis.

## 2.6 Proteomic analysis

Bacteria were harvested via centrifugation (16,000 g, 4°C) at the beginning of the exponential phase ($OD_{680nm}$:0.9-1). Proteins were extracted using guanidinium chloride (6 M) and ultrasonication (3*10 sec, IKA U50 sonicator (Staufen, Germany), amplitude 40%). Protein concentration was determined using the Bradford method(32) with Bovine Gamma Globulin as standard. 50 µg of proteins were reduced (DTE), alkylated (iodoacetamide) and finally precipitated overnight using acetone. The obtained protein pellets were then solubilized using 50 mM ammonium bicarbonate containing 1 µg of trypsin and incubated overnight at 37°C. Digestion was stopped by adding 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 ultra-AB SCIEX TripleTOF™ 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. Data were acquired in a data-dependent acquisition mode (DDA). Search parameters included differential amino acid mass shifts for carbamidomethyl cysteine, oxidized methionine, all biological modifications, amino acid substitutions and missed trypsin cleavage sites. ProteinPilot Software (v4.1) was used to perform database searches against the UniProt database, restricted to *Rhodospirillum* entries (ATCC11170). The areas under the XIC curves of peptides were computed using PeakView™ 2.1 (ABSciex, USA) and individually normalized based on a summed area of all peptides for each sample. Five biological replicates were considered for each condition. Markerview™ 1.2.1 (ABSciex, USA) was used for the statistical treatment of the data.

All computed data, as well as raw data, were uploaded to the MassIVE repository and are freely accessible (dataset identifier: MSV000085300) https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=b5561aeb53f940248f6032205e12e014

## 3. Results and discussion

### 3.1 Bicarbonate ions are necessary for the photoheterotrophic assimilation of valerate

In order to first characterize the photoheterotrophic assimilation of valerate, *Rhodospirillum rubrum* S1H (*Rs. rubrum*) was cultivated with valerate as the sole source of carbon. As bicarbonate ions are already known to act as a limiting factor in VFA assimilation such as acetate, propionate or butyrate (12,14,33–35), we tested the effect of an addition of a low and high concentration of bicarbonate ions (3 and 50 mM $HCO_3^-$) in the medium and used succinate as the control condition. Indeed, previous studies have demonstrated that bicarbonate ions are necessary during VFA assimilation to handle redox stress through the activation of a redox sink, namely the Calvin-Benson-Bassham cycle (34,36,37). Moreover, we have previously shown that bicarbonate ions are necessary for *Rs. rubrum* growth under high light intensity (15). As shown in Fig. 1A, the phototrophic growth of *Rs. rubrum* with valerate as the sole carbon source, and supplemented with a low concentration of bicarbonate ions, stopped at an $OD_{680nm}$ of 1.5 ± 0.2, whereas growth with a high concentration of bicarbonate ions reached an $OD_{680nm}$ of 5.9 ± 0.1 (Fig. 1A). Interestingly, an identical carbon supply (124 mM of C equivalent and 50 mM $HCO_3^-$) led to higher biomass production in the presence of valerate than in the presence of succinate (calculated biomass concentration: Val = 3.73 mg/mL vs Succ = 2.40 mg/mL). Indeed, 1.79±0.08 mg of biomass were accumulated per mg of available carbon in the presence of valerate, whereas 1.48±0.03 mg of biomass were accumulated per mg of available carbon in the presence of succinate (p-value < 0.001),

suggesting a significant assimilation of bicarbonate ions under valerate condition. In order to attest to the essentiality of bicarbonate ions during photoheterotrophic growth with valerate as the sole source of carbon, *Rhodospirillum rubrum* was cultivated in the presence of valerate with only 3 mM of added bicarbonate ions. As expected, the growth stopped around $OD_{680nm}$ ~ 1.5. Once growth stopped, 10 mM of bicarbonate ions (final concentration) were added to the culture and growth was monitored. The growth immediately restarted after this addition of bicarbonate ions, reaching a final $OD_{680nm}$ comparable to those observed with high bicarbonate supplementation (Fig. 1B). The essentiality of bicarbonate ions for the assimilation of acetate, propionate or butyrate has already been documented(14,15,34,38). As the valerate constitutes the most reduced VFA compared to the biomass (redox state valerate:-2.5; biomass: -0.5)(39,40), the requirement of bicarbonates ions could be linked to the redox stress induced by the photoheterotrophic assimilation of valerate.

Consistently, proteomic analysis revealed that the Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase (RuBisCO), the main enzyme of the Calvin-Benson-Bassham (CBB) cycle, was more abundant (Rru_A2400; fold change = 1.71, *p*-value = 0.01, # peptides = 89) during the photoheterotrophic assimilation of valerate compared to succinate. This observation further corroborates the strict necessity of bicarbonate ions for the assimilation of valerate and the higher biomass productivity. Moreover, the mutant fitness assay showed that a functional CBB cycle was essential in both conditions, as already demonstrated for succinate (37). This observation corroborates the results obtained during the phenotypic analyses. Considering this observation, it seems that $HCO_3^-$ is, at least partly, consumed through the Calvin-Benson-Bassham cycle, suggesting a role of this pathway in the dissipation of the reduced equivalents generated during the photoheterotrophic assimilation of valerate.

### 3.2 The photoheterotrophic assimilation of valerate induces a cellular redox stress and an increased PHA production

The proteomic analysis showed the higher abundance of four proteins related to stress handling and redox homeostasis in the presence of valerate. Unfortunately, only one of the genes encoding for these proteins (stress protein, *rru*_A0894) was present in our mutant fitness dataset. The analysis of the fitness value for this gene revealed that it was essential in both conditions (Table 1). Beside proteins implicated in redox homeostasis, proteomic analysis also revealed a higher abundance of proteins involved in the metabolism of PHAs, such as phasins in the presence of valerate. One the two phasins quantified in the proteomic analysis (Rru_A2817) exhibited a large fold change of 48.24 compared to succinate condition. The second phasin (Rru_A3283) was found to be twice more abundant in the presence of valerate than under the succinate condition (fold change =2.06, *p*-value = 0.027, # peptides = 80). We also highlighted the higher abundance of the uncharacterized protein Rru_A2111 (fold change = 3.56, *p*-value = 2.24E-06, # peptides = 27) which was recently described as potential phasin by Narancic *et al* (41). Nevertheless, the exact role of these proteins remains unclear as they exhibit both a function in PHB granule mobilization and a role in increasing the number of PHB granules in a cell (42). Moreover, the polyhydroxyalkanoate depolymerase (Rru_A3356), responsible for the degradation of PHA, showed a lower abundance compared to the succinate condition (*Table* 1).

Conversely to the proteomic results, which suggest the involvement of PHA synthesis in the adaptation of *Rs. rubrum* to the growth with valerate as the sole carbon source, none of the genes coding for the abovementioned proteins were observed as essential for the photoheterotrophic assimilation of valerate in the genome-wide mutant fitness assay. This may be explained by the addition of a high concentration of bicarbonate ions in the medium used to perform this experiment. Indeed, both the PHA production and the CBB cycle are

known to act as electron sinks (15,34,36,37,43–46). This therefore suggests that PHA production may be not essential as long as another electron sink metabolism, such as the CBB cycle, is available. Moreover, the genes may also be redundant in the genome which means that the loss of a single gene will not lead to a specific phenotype. Interestingly, genes coding for enzymes involved in the conversion of acetyl-CoA into (S)-3-hydroxybutyryl-CoA were highlighted as essential for the phototrophic assimilation of valerate by *Rs. rubrum*. One of these enzymes, the 3-hydroxybutyryl-CoA dehydrogenase (Rru_A3079), is known to consume reduced equivalents (Fig. 2, see supplementary Table 1) and might thus take part in the handling of the redox equilibrium.

The higher abundance of phasins combined with a lower abundance of PHA depolymerase highlighted by the proteomic results suggests an increased production of PHA in valerate conditions. Moreover, the mutant fitness assays showed that the (S)-3-hydroxybutyryl-CoA dehydrogenase was essential in the presence of valerate. In order to validate the increased PHA production during valerate assimilation, the PHA content of the biomass was monitored along the growth curve under valerate and succinate conditions. As expected, an increased PHA production was observed during photoheterotrophic growth of *Rs. rubrum* in the presence of valerate (reaching up to 14.20±1.37 % of the biomass dry weight after 30 hours of culture) (Fig. 3A) whereas the PHA content was under the limit of detection under succinate condition (data not shown). The abundance of PHA produced under valerate was rather low. Indeed, Cerrone *et al.* performed PHA content analyses on *P. putida* and observed that this bacterium was able to accumulate from 19 to 24 % of PHA CDW in the presence of valerate as the sole carbon source (47). We hypothesized that PHA production was used as an electron sink and that its production could be curbed by other electron sinking processes. As such, the presence of bicarbonate ions and their assimilation through the Calvin-Benson-Bassham cycle could represent a major competitor pathway to PHA

306 production. Indeed, proteomic and mutant fitness assay data showed that the CBB cycle was
307 probably involved in the metabolism of valerate. Therefore, we attempted to reduce the level
308 of $HCO_3^-$ in the cultures in order to reduce the electron sink role of the CBB cycle as much as
309 possible. Cultures were started in valerate containing medium with a low concentration of
310 $HCO_3^-$ (3mM) which was only resupplied (up to 3mM in the medium) when a growth arrest
311 was observed. This resulted in a sequential growth behaviour restarting each time we added
312 bicarbonate ions in the medium (Fig. 3B). Each addition of $HCO_3^-$ was thus preceded by a
313 growth arrest and a reduced valerate uptake rate. Interestingly, each of the transient stationary
314 phases this procedure created was characterized by an increase of the intracellular PHA
315 content (Fig. 3B). By starving the CBB cycle of bicarbonate ions, *Rs. rubrum* accumulated up
316 to 22.57±5.89% of the cell dry weight after 70 hours of culture. Indeed, this content
317 significantly increased during the first transient stationary phase (T: 27h; PHA:10.86±2.58%
318 CDW; T:70h; PHA: 22.57±5.89% CDW, *p*-value<0.05). The second transient stationary
319 phase, which corresponded to the second bicarbonate starvation of the culture (T:101h), was
320 also accompanied by a significant (*p*-value < 0.05) PHA content increase of 133% rising from
321 7.98±1.94% (T101h) to 18.61±2.94% (T174h) CDW (Fig. 3B). Interestingly, the PHA
322 content obtained after both stationary phases was significantly higher (*p*-value <0.05) than the
323 PHA content observed in the presence of excess $HCO_3^-$ ($PHA_{excessHCO3}$-: 14.20±1.37%;
324 $PHA_{3mMfirst\ stationary\ phase}$: 22.57±5.89%; $PHA_{3mMsecond\ stationary\ phase}$: 18.61±2.94%, *p*-value <
325 0.05). In opposition to what is usually observed (48–52), PHA increased production is not
326 triggered through a feast and famine process, suggesting that PHA production could be linked
327 to an intracellular redox imbalance. Indeed, the depletion of bicarbonate ions in the medium
328 represents a non-favorable redox environment for *Rs. rubrum* cultivated in the presence of
329 valerate. The concomitant increase in the PHA content and its further mobilization after
330 $HCO_3^-$ repletion demonstrates that PHAs are produced by *Rs. rubrum* to deal with the redox

imbalance imputed to the photoheterotrophic assimilation of valerate as already hypothesized (15,44,46).

## 3.3 Acetyl-CoA and propionyl-CoA assimilation pathways highlighted as potential pathways involved in the photoheterotrophic assimilation of valerate

### 3.3.1 β-oxidation as a first step of the photoheterotrophic assimilation of valerate

The literature suggests that following its activation into valeryl-CoA, valerate (C5) is cleaved into acetyl-CoA (C2) plus propionyl-CoA (C3) through β-oxidation (21,53). As such, we searched our proteomic and mutant fitness dataset for β-oxidation related proteins or genes respectively. The mutant fitness analysis revealed the importance of the genes *rru_A1308*, *rru_A3801*, *rru_A1309* and *rru_A1310* coding for an acyl dehydrogenase, enoyl dehydratase, 3-hydroxyacyl-CoA dehydrogenase and acetyl-CoA-C-acyltransferase respectively for optimal fitness during valerate photoheterotrophic assimilation (significance threshold: -0.5; *rru_A1308*: fitness value Succ: 0.03, Val: -0.52; *rru_A3801:* fitness value Succ: -0.25, Val: -0.92; *rru_A1309*: fitness value Succ: -0.18, Val: -1.00 and *rru_A1310*: fitness value Succ: 0.10, Val: -0.72). Collectively, these enzymes may link the valerate uptake to the production of acetyl-CoA and propionyl-CoA. However, further investigations are required to understand the absence of change in the abundance or even the downregulation highlighted by the proteomic analysis for these enzymes (Rru_A1308: *p*-value: 0.16, fold change Val/Succ: 0.77; Rru_A3801:*p*-value: 0.51, fold change Val/Succ: 1.11; Rru_A1309: *p*-value: 0.0035, fold change Val/Succ: 0.65; Rru_A1310: 0.00075, fold change Val/Succ: 0.49).

### 3.3.2 Acetate related pathways are highlighted through proteomic analysis for the photoheterotrophic assimilation of valerate

The proteomic data revealed that several enzymes already associated with the acetate metabolism, such as the enzymes of the ethylmalonyl-CoA (EMC) pathway (Rru_A3062, fold change = 1.65, *p*-value = 0.05, #peptides = 9; Rru_A3063, fold change = 6.35, *p*-value = 0.00012, #peptides = 26; Rru_A3064, fold change = 2.53, *p*-value = 0.01, #peptides = 42) or the pyruvate ferredoxin oxydoreductase (PFOR) (Rru_A2398, fold change = 1.51, *p*-value = 0.04, #peptides = 125) (Fig. 2), were differentially regulated in the presence of valerate and could also be involved in the photoassimilation of valerate. The EMC pathway was shown to be essential for the photoheterotrophic assimilation of acetate by our group (11,12) as well as others (9,10), and it is now established that this pathway acts as an anaplerotic pathway during the phototrophic assimilation of acetate. PFOR is known to convert acetyl-CoA into pyruvate and has also been highlighted by previous proteomic analyses (2,11,12,54,55) in the presence of acetate. Besides proteins for which their implication in acetate assimilation has been well established, the proteomic analysis revealed the higher abundance of proteins belonging to the branched chain amino acid (BCAAs) biosynthesis and degradation pathways, which represent a more controversial assimilation pathway (Rru_A0468, fold change = 2.18, *p*-value = 0.05, #peptides = 9; Rru_A0469, fold change = 1.61, *p*-value = 0.01, #peptide =42; Rru_A2223, fold change =4.95, *p*-value = 0.016, #peptides = 24; Rru_A1946, fold change = 1.74, *p*-value = 0.04, #peptides = 2; MerR transcription regulator Rru_A1994, fold change = 1.75, *p*-value = 0.04, #peptides= 4) (Fig. 2). The implication of BCAA biosynthesis and degradation pathways have already been proposed as potential assimilation pathways in the presence of butyrate (14) or acetate (11,12). Recently, a sudden increase in light intensity highlighted that isoleucine biosynthesis may be implicated in high light intensity tolerance (15), further reinforcing the implication of this pathway in managing the redox balance as already proposed (56,57). This latter hypothesis is supported by the present redox issue observation but would require further dedicated research.

However, contrary to what was observed in the proteomic data, the genome-wide mutant fitness assay revealed that the ethylmalonyl-CoA pathway was not essential for valerate photoassimilation by *Rhodospirillum rubrum* (Fig. 2, see supplementary table 1). It could indicate that the observed upregulation of the enzyme of the EMC could be imputed to the side regulation of the EMC pathway due to the presence of acetyl-CoA following the cleavage of valerate, but that this pathway is not extensively used during valerate photoassimilation.

In order to clearly address the essentiality of the EMC pathway, we cultivated a Δ*ccr*:*km*$^{R}$ mutant strain (lacking the gene coding for the key enzyme of the EMC pathway, see Fig. 2) in the presence of valerate as the sole source of carbon. It revealed that the EMC pathway was not essential for the growth of *Rs rubrum* in the presence of valerate (Fig. 4A) confirming the genome-wide mutant fitness assay. The genotype of the mutant strain was confirmed using PCR analysis (see supplementary figure 1).

### 3.3.2 The methylmalonyl-CoA pathway is essential for the photoheterotrophic assimilation of valerate

The assimilation of valerate through acetyl-CoA and propionyl-CoA involves that a higher activity of the propionyl-CoA assimilation pathway (the methylmalonyl-CoA pathway-MMC) (58–60) should be observed under valerate conditions. However, enzymes of the MMC pathway were not upregulated under valerate conditions, as revealed by the proteomic analysis. Many researchers have already observed that propionyl-CoA could be formed from succinate (61–63), which could impair the detection of an increased abundance of enzymes of the MMC pathway. Interestingly, the mutant fitness analysis highlights that the two subunits of two proteins implicated in the propionyl-CoA assimilation pathway, the propionyl-CoA carboxylase and the methylmalonyl-CoA mutase (*rru*_A0052; *rru*_A0053; *rru*_A2479 and *rru*_A2480), are essential for optimal fitness for the phototrophic assimilation of valerate, but

not of succinate (Fig. 2, see supplementary table 2). These genes have already been shown to be essential for propionyl-CoA assimilation (58,64,65). These results suggest that the production of propionyl-CoA represents a major step for valerate assimilation. One hypothesis explaining the essentiality of MMC, but not of EMC, is that the propionyl-CoA assimilation pathway could represent an anaplerotic pathway for the TCA cycle as it yields succinate,EMC being no more essential to filling this role. In order to attest to the essential role of the methylmalonyl-CoA pathway in valerate assimilation, itaconic acid was used to inhibit the propionyl-CoA carboxylase (Rru_A0052/A0053, see Fig. 2) (64). Growth of *Rs. rubrum* in the presence of succinate as the main source of carbon and 20 mM of itaconic acid confirmed the absence of toxicity of itaconate, as already shown by Berg *et al.* (64). As presented in Fig. 4B, the addition of 20 mM of itaconic acid impaired the growth of *Rs. rubrum* in the presence of valerate, both for the wild-type strain and for the Δ*ccr::km*$^R$ strain, confirming that the methylmalonyl-CoA pathway is essential for the assimilation of valerate.

The assimilation of propionyl-CoA yielded succinyl-CoA that can be converted into oxaloacetate and α-ketoglutarate using the oxidative and reverse TCA cycle, respectively. The assimilation of this C3 compound thus acts as an anaplerotic pathway. This keystone role in metabolism is commonly assumed by the EMC pathway in *Rs. rubrum*, but EMC has been shown to be non-essential under valerate condition Our results also revealed that the pyruvate ferredoxin oxydoreductase, *rru*_A2398, was essential for phototrophic growth in the presence of valerate (Fig. 2, see supplementary table 3), indicating that acetyl-CoA could be converted into pyruvate, as this reaction is driven in this direction in an anaerobic environment (54). Moreover, we observed the essentiality of some enzymes of the BCAA biosynthesis pathway (Fig. 2, see supplementary table 3 and 4) for the photoheterotrophic assimilation of valerate, further corroborating the proteomic analysis and previous studies (11,12,14,15). The acetyl-CoA arising from the cleavage of valerate is thus redirected to other pathways than the EMC,

433 such as the BCAA biosynthesis pathway, PHA production or the PFOR. These pathways
434 could help balance the redox pool state. Whereas the implication of PHA production in redox
435 homeostasis is well accepted now, the involvement of the BCAA biosynthesis pathway as an
436 electron sink is still controversial. However, this pathway has already been highlighted during
437 the photoheterotrophic assimilation of other reduced VFAs, such as acetate (11), butyrate (14)
438 or even after a sudden increase in light intensity (15). Altogether, the data obtained
439 previously, and in this study, suggest that the BCAA biosynthesis pathway may act as another
440 electron sink.

441 Regarding the present results, the 3-hydroxyvalerate monomers observed during PHA
442 quantification could arise either from the condensation of acetyl-CoA and propionyl-CoA into
443 3-ketovaleryl-CoA and its further reduction into 3-hydroxyvaleryl-CoA or from the β-
444 oxidation of valerate in which 3-hydroxyvalerate constitutes one intermediary. However,
445 internal data show that the cultivation of *Rs. rubrum* in the presence of acetate and propionate
446 led to the production of PHA containing a reduced percentage of 3-hydroxyvalerate monomer
447 (unpublished data). Moreover, the cultivation of *Rs. rubrum* in the presence of hexanoate led
448 to the incorporation of 3-hydroxyhexanoate in the polymer, which is not observed in the
449 presence of acetate or butyrate, further suggesting that at least a part of the carbon source is
450 directly incorporated into the polymer(66).

451 **3.4 Polyhydroxyalkanoate characterization reveals the production of the**
452 **copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)**
453

454 The use of carbon sources with an odd number of carbon has been linked to the
455 production of copolymers of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) exhibiting
456 outstanding physicochemical properties(28,67–70). PHA quantitation has already revealed a
457 higher production of PHAs in the presence of valerate compared to succinate. Interestingly,

458 the PHAs produced are copolymers of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(HB-
459 *co*-HV)) with high monomeric percentage of 3-hydroxyvalerate (83.90±4.19% of the total
460 polymer) (Fig. 3A). Even though this HV monomer percentage has already been observed
461 using an engineered strain of *E. coli* in which the gene coding for the propionyl-CoA
462 transferase was introduced (71), we have now observed this percentage in a wild-type strain
463 (17,21,52). Moreover, it is interesting to note that cultivating *Rs. rubrum* in a bicarbonate ions
464 limiting condition, which allowed the total PHA content of biomass to be increased, had no
465 significant impact on the 3-HV content (Fig. 3B). The chemical and mechanical properties or
466 biodegradability of PHAs are dependent on their monomeric composition, as well ason how
467 they are concatenated (67,72,73). In this context, we attempted to control *Rs. rubrum* co-
468 polymer production by cultivating *Rs. rubrum* in the presence of acetate as the sole source of
469 carbon (124 mM C equivalent) and we added 10 mM of valerate (50 mM C equivalent) when
470 the culture reached mid-log phase ($DO_{680nm}$ ~ 2.0). Whereas the HV content was negligible
471 before valerate addition, the HV monomer accumulated quickly in the PHA polymer reaching
472 7.02±1.04% of CDW only 11 hours after the addition of valerate in the medium,
473 corresponding to a monomeric content of 14.51±2.41% of the total PHA (Fig. 5). This result
474 suggests that the production of PHA might be controlled by the sequential addition of the
475 suitable VFA in order to design the desired copolymer for defined applications.

476 **4. Conclusion**
477

478 We have demonstrated here that the presence of $HCO_3^-$ is mandatory for the
479 photoheterotrophic assimilation of valerate. We have shown that the requirement of $HCO_3^-$
480 was driven to the CBB cycle in order to regulate the redox balance (*i.e.* highly reduced carbon
481 source and phototrophic metabolism). We have highlighted that following its activation in
482 valeryl-CoA, valerate was cleaved into acetyl-CoA and propionyl-CoA. Whereas, the

ethylmalonyl-CoA pathway was not essential for the assimilation of valerate, a functional methylmalonyl-CoA pathway was mandatory. We observed polyhydroxyalkanoate production during photoheterotrophic metabolism of valerate that is undoubtedly used as an electron sink and that can reach up to 15% of the cell dry weight as PHA. Using a knowledge based optimization process, we have improved the yield of this production to achieve a PHA content of more than 22% of the biomass by cultivating the bacteria in bicarbonate ion limiting conditions, which further reduced the activity of another electron sink, the Calvin-Benson-Bassham cycle. This type of observation has already been emphasized by our research group following a sudden increase in light intensity. Finally, the characterization the PHA produced revealed an outstanding proportion of 3-hydroxyvalerate monomer content. Moreover, we have displayed the possibility of controlling the monomeric microstructural composition of PHA by sequentially addîng the wanted VFA in order to produce the most suitable polymer.

## 5. Data availability statement

The datasets generated for this study can be found on MassIVE repository (dataset identifier: MSV000085300)

(https://massive.ucsd.edu/ProteoSAFe/dataset.jsp?task=b5561aeb53f940248f6032205e12e014).

## 6. Figure legends

**Fig. *1:***

(A) Growth of *Rs. rubrum* under succinate low $HCO_3^-$ concentration (o) and valerate low (□) and high (Δ) concentration of $HCO_3^-$. (B) Growth of *Rhodospirillum rubrum* in the presence of succinate (o) and valerate (□) with a pulse of 10 mM bicarbonate ions. n=5

**Fig. *2*:**

Schematic representation of the central carbon metabolism with proteins implicated in valerate assimilation as highlighted by proteomic data. The colored squares indicate fold change between valerate and succinate condition ranging from red (proteins are less abundant in the valerate condition) to green (proteins are more abundant in the valerate condition). Non-significant proteins are represented by stripped markers. Peptide number used to identify the protein is represented next to the marker. Colored circles indicate essentiality of gene based on fitness values obtained during mutant fitness assay n=5

**Fig. *3:***

*Rs. rubrum* growth (upper figures) and PHA production (lower figures) observed in the presence of (A) valerate +50 mM $HCO_3$ and (B) valerate + progressive addition of 3 mM bicarbonate ions (vertical dotted lines). 3-hydroxybutyrate (dark grey) and 3-hydroxyvalerate (light grey) monomers content was determined through GC-MS analysis. n=5

**Fig. 4:**

(A) Growth of *Rs. rubrum* wild-type strain in the presence of succinate (o) or valerate (Δ) as the sole carbon source and Δ*ccr::Km*$^R$ strain in the presence of valerate (□) as the sole source of carbon.(B) Growth of *Rs. rubrum* wild-type strain in the presence of succinate (o) or valerate (Δ) as the sole carbon source and Δ*ccr::Km*$^R$ strain in the presence of valerate (□) as the sole source of carbon cultivated with the addition of 20mM of itaconic acid (B). n=5

**Fig. 5:**

*Rs. rubrum* growth and VFA consumption (upper figure) and PHA production (lower figure) observed in the presence of acetate (upper figure-full circle dotted line) and after a pulse of valerate (upper figure-open square dotted line-vertical dotted line). 3-hydroxybutyrate (dark grey) and 3-hydroxyvalerate (light grey) monomers content was determined through GC-MS analysis. n=5

## 7. Table legends

**Table 1:**

Proteins involved in the stress regulation and redox homeostasis highlighted by proteomic and mutant fitness analyses

## 8. Acknowledgement

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G.B.V., B.L., and R.W. designed the study. G.B.V. and S.Z. performed the *Rs. rubrum* cultivation experiments. The mutant library construction was designed by A.D. and conducted by B.L. The mutant fitness assay was designed by G.B.V. and performed by G.B.V. A.D. carried out the BarSeq analysis. G.B.V. performed the bioinformatic analysis. G.B.V. and B.L. designed and performed the proteomic analysis. G.B.V. wrote the manuscript with the help of S.Z., B.L., A.D. and R.W.

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Applied and Environmental Microbiology

| Peak Name | Locus Tag | Description | Proteomic analysis | | | Mutant fitness assay | |
|---|---|---|---|---|---|---|---|
| | | | *p*-value | Fold change Val/Succ | #Identified peptides | Fitness value Succinate | Fitness value Valerate |
| Stress handling and redox homeostasis | | | | | | | |
| Q2RW01 | Rru_A0893 | Stress protein | 7.17E-03 | 2.00 | 41 | N.A. | N.A. |
| Q2RW00 | Rru_A0894 | Stress protein | 3.87E-02 | 1.91 | 78 | -0.67 | -1.24 |
| Q2RUH6 | Rru_A1418 | Alkyl hydroperoxide reductase/ Thiol specific antioxidant/ Mal allergen | 2.40E-03 | 1.60 | 42 | N.A. | N.A. |
| Q2RTI5 | Rru_A1760 | Superoxide dismutase | 5.30E-04 | 2.09 | 51 | N.A. | N.A. |
| Polyhydroxyalkanoate metabolism | | | | | | | |
| Q2RXR6 | Rru_A0274 | Acetyl-CoA C-acetyltransferase | 0.51 | 0.95 | 95 | 0.5 | -2.4 |
| Q2RSI4 | Rru_A2111 | Uncharacterised protein-potential phasin | 2.24E-06 | 3.56 | 27 | -0.44 | 0.37 |
| Q2RQI1 | Rru_A2817 | Phasin | 3.80E-04 | 48.24 | 24 | -0.19 | 0.01 |
| Q2RPS1 | Rru_A3079 | 3-hydroxyacyl-CoA dehydrogenase | 0.38 | 1.19 | 17 | -0.3 | -1.2 |
| Q2RP67 | Rru_A3283 | Phasin | 2.79E-02 | 2.06 | 80 | N.A. | N.A. |
| Q2RNZ5 | Rru_A3356 | Polyhydroxyalkanoate depolymerase | 4.14E-03 | 0.58 | 3 | 0.02 | -0.28 |