Rhodospirillum rubrum S1H: Unravelling the Volatile Fatty Acids Assimilation in the MELiSSA Loop

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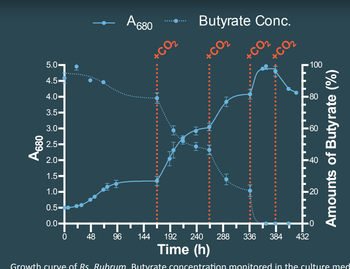
Purple non-sulfur (PNS) bacteria are well known for their remarkable metabolic versatility which allows them to occupy a broad range of environments. They are especially able to achieve a photoheterotrophic metabolism through which they can assimilate volatile fatty acids (VFAs). *Rhodospirillum rubrum* S1H was subsequently selected by the European Space Agency to colonize the second compartment (C2) of its bioregenerative life support system (i.e. MELiSSA loop) and to remove VFAs from the effluents produced by the liquefying first compartment (C1), avoiding accumulation of dead-end metabolites.

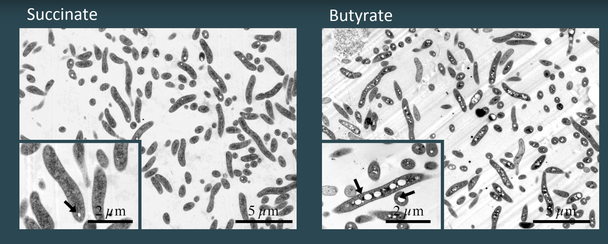
The ethylmalonyl-CoA pathway was recently highlighted in *Rs. rubrum* by our laboratory as being involved in the assimilation of acetate, the most abundant VFA produced by the C1. Below are reported our results for a better understanding of the photoheterotrophic metabolism of butyrate, the second most abundant VFA in the C1 effluent.

Bacterial Growth Analysis: Dependence to Carbonate

Bacterial growth analysis of *Rs. rubrum* showed a strong dependence on the presence of carbonate in the culture medium. It is hypothesized that butyrate assimilation induces a redox imbalance. Therefore, the CO_2 fixation is not likely used for an anabolic purpose but rather as an electron sink to recycle reduced cofactors. This hypothesis is supported by our proteomic analyses. Indeed, described in literature as being an electron sink, RuBisCO is clearly overexpressed in our experiments when butyrate is the unique source of carbon (Rru_A2400; 3,1x). Furthermore, transmission electron microscopy also highlights an increased accumulation of poly(3-hydroxybutyrate), well known as being another electron sink. Upon the same conditions, we also observe the upregulation of other carboxylases needed for butyrate assimilation as the crotonyl-CoA reductase (Rru_A3063; 2.9x) or the pyruvate-flavodoxin oxidoreductase (Rru_A2398; 1.4x.).



Growth curve of *Rs. Rubrum*. Butyrate concentration monitored in the culture medium showing dependence to CO_2.



TEM observation of *Rs. rubrum* in exponential growth phase in the presence of succinate or butyrate.

Biosynthesis and Degradation of the Valine as a new anaplerotic pathway

The proteomic aspect of the butyrate photoassimilation was investigated through mass spectrometry. Extracted proteins from five biological replicates were processed to be identified and quantified using SWATH acquisition on a UPLC-HRMS platform (Eksigent 2D ultra / AB Sciex TripleTOF 5600) following a label-free strategy. Two different approaches were used.

The first one compared the biomass harvested in butyrate conditions to the biomass obtained in presence of succinate or acetate. This first proteomic analysis resulted in the identification and quantification of 1752 proteins and allowed to highlight two probable anaplerotic pathways involved in butyrate assimilation.

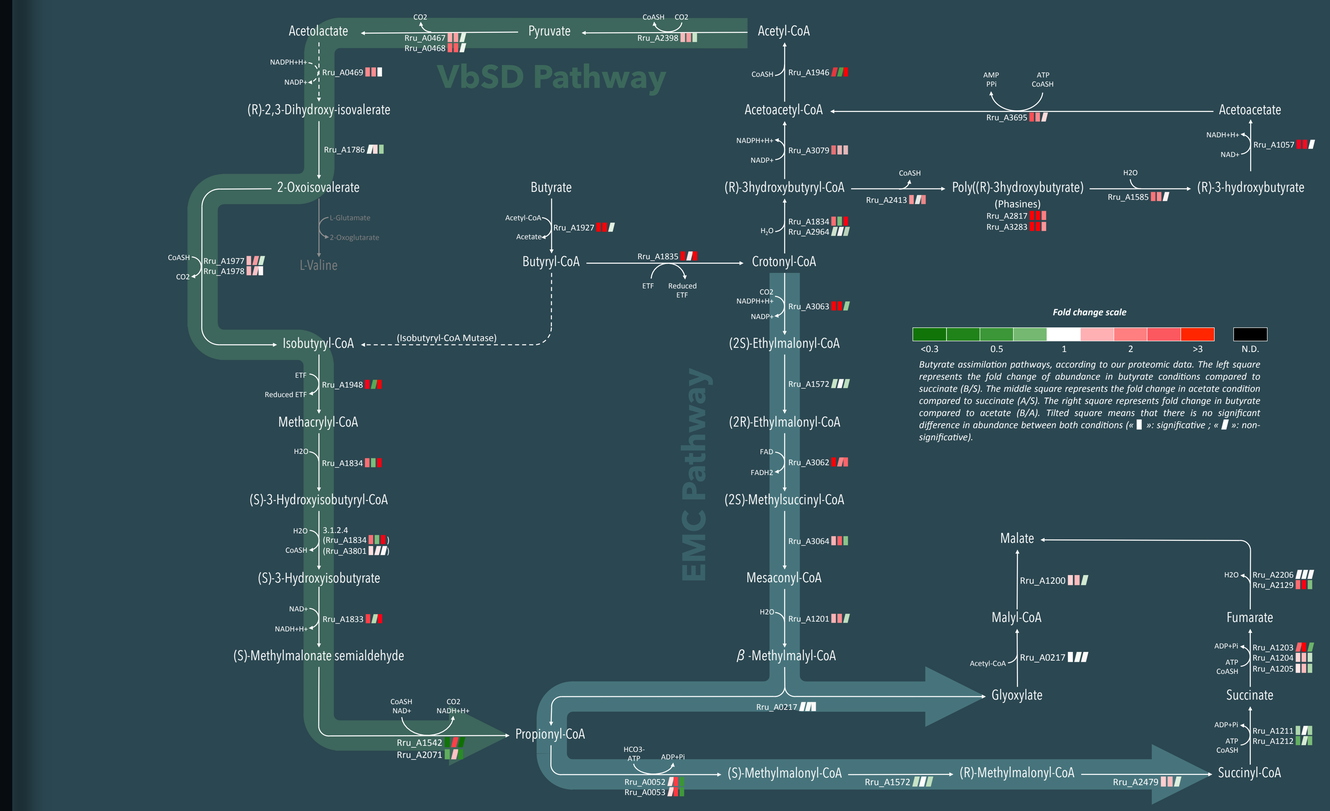
According to our proteomic data, butyrate is first converted to crotonyl-CoA, a central metabolite in butyrate assimilation. This crotonyl-CoA can be then converted into acetyl-CoA. In most organisms, the use of two carbon compounds like acetyl-CoA for anabolic purpose involves the glyoxylate cycle as a shunt to replenish the pool of biosynthesis precursor of the TCA cycle. *Rs. rubrum* is an isocitrate lyase negative organism, and thus lacks the key enzyme necessary to use the glyoxylate shunt. Alternative anaplerotic pathways are then expected to be used.

As reported for the acetate assimilation (Leroy et al., 2015), we also observe enzymes of the ethylmalonyl-CoA (EMC) pathway when butyrate is supplied as the carbon source. The crotonyl-CoA is converted into malate via the production of propionyl-CoA and glyoxylate. The global balance of the process is:

$$1\ \text{Butyrate} + 4/3\ CO_2 + 3\ H_2O + 1\ \text{ATP} + 2\ \text{NAD(P)}^+ \rightarrow 4/3\ \text{Malate} + 1\ \text{ADP+P}_i + 2\ \text{NAD(P)H} + 2\ H^+$$

Surprisingly, a significantly higher abundance of enzymes involved in valine biosynthesis and degradation (VbSD) pathways is also observed. Acetyl-CoA formed from crotonyl-CoA could be converted into pyruvate that enters in the valine biosynthesis pathway to be converted in 2-oxoisovalerate. The latter could then be converted in isobutyryl-CoA to finally produce propionyl-CoA through the valine degradation pathway. The direct conversion of butyryl-CoA in isobutyryl-CoA by a GTP-dependent isobutyryl-CoA mutase was already described in *Streptomyces cinnomonensis* and in *Cupriavidus metallidurans* CH34. However, this enzyme was not identified with a significant similarity in the genome of *Rs. rubrum*. The global balance of the VbSD pathway is:

$$1\ \text{Butyrate} + 1\ \text{ATP} + 4\ H_2O + 4\ \text{NAD(P)}^+ \rightarrow 1\ \text{Malate} + 1\ \text{ADP+P}_i + 4\ \text{NAD(P)H} + 4\ H^+$$

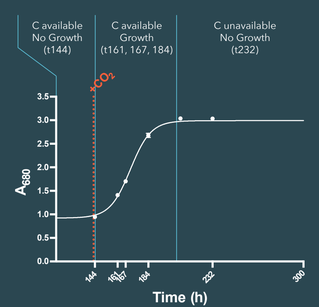


Butyrate assimilation pathways, according to our proteomic data. The left square represents the fold change of abundance in butyrate conditions compared to succinate (B/S). The middle square represents the fold change in acetate condition compared to succinate (A/S). The right square represents fold change in butyrate compared to acetate (B/A). Tilted square means that there is no significant difference in abundance between both conditions (x ■ x: significative ; x ■ x: non-significative).

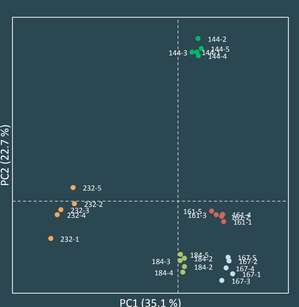
In order to determine if these two pathways, which present different global balance, are used simultaneously at different stages of the growth, a second proteomic approach was considered and consisted of a kinetic experiment of the butyrate photoassimilation. Different phases of growth in presence of butyrate were compared together to highlight if EMC and VbSD pathways are used simultaneously or successively. This second proteomic analysis resulted in the identification and quantification of 1804 proteins.

Since they produce key anabolic precursors, enzymes involved in the conversion of crotonyl-CoA to acetyl-CoA and pyruvate are steadily expressed during the growth. (Rru_A1834, 3079, 1946). Specific enzymes of the EMC pathway also exhibit similar abundances during the growth curve. Nonetheless, for all of them highest abundance were observed when butyrate is totally assimilated, probably to ensure metabolisation of depolymerised polyhydroxybutyrate (Rru_A3063, 1572, 3062, 3064, 1201, 0217).

If most of the enzymes involved in the VbSD pathway seem to be only weakly or even not regulated across the growth phase (Rru_A0467, 0468, 0469, 1786, 1977, 1978), the expression of some others is particularly activated during the second half of the growth. According to the global balance of the butyrate assimilation through this pathway, the presence of these enzymes in higher abundance could be linked to the low carbonate concentration in the culture medium.



Growth curve showing the different times across the growth when biomass was harvested for the kinetic experiment.



Principal component analysis. (PCA) of the quantified proteins abundance data.

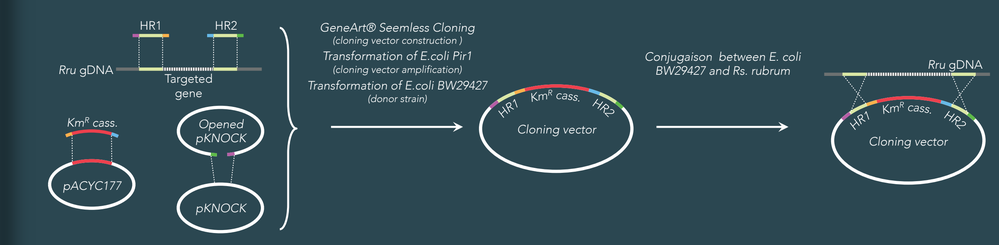
	#Pep	T144	T161	T167	T184	T232
Rru_A1834	6	99	89	80	100	79
Rru_A3079	6	87	86	67	100	99
Rru_A1946	3	100	79	58	81	76
Rru_A3063	6	65	48	43	53	100
Rru_A1572	4	59	83	37	100	82
Rru_A3062	2	63	60	65	91	100
Rru_A3064	6	79	62	52	59	100
Rru_A1201	6	81	78	70	80	100
Rru_A0217	6	64	64	60	72	100
Rru_A0467	6	92	100	79	87	87
Rru_A0468	6	95	100	85	79	91
Rru_A0469	6	100	99	85	87	86
Rru_A1786	6	100	94	75	90	93
Rru_A2398	6	78	75	66	100	67
Rru_A1948	4	100	87	57	84	63
Rru_A1833	6	95	78	48	100	91

Abundance of the main enzymes involved in the butyrate assimilation across the growth phase analysed by mass spectrometry. For a better understanding, relative abundances are presented here as a percentage of the maximal intensity registered for each protein.

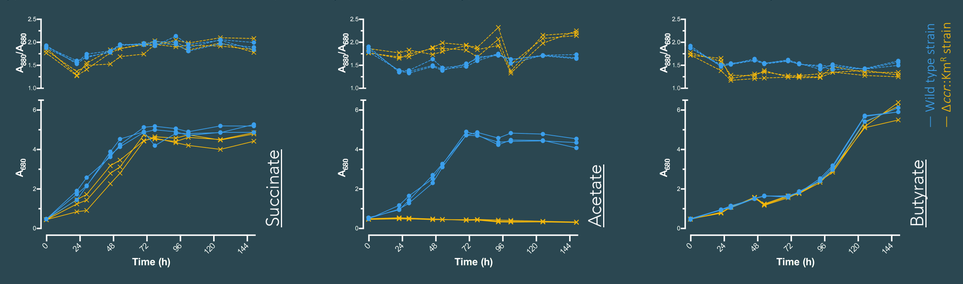
Leroy B., De Meur Q., Moulin Ch., Wegria G. and Wattiez R. (2015). **New insight into the photoheterotrophic growth of the isocytrate lyase-lacking purple bacterium *Rhodospirillum rubrum* on acetate**, Microbiology **161**, 1061-1072

Validation throught mutagenesis experiment

To provide evidence of the strict requirement of these pathways in the acetate or butyrate assimilation, we conducted two different mutagenesis experiments following the mutagenesis process below:

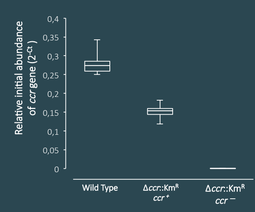


The resulting strains are Δ*ccr*::Km^R (targeted gene: Rru_A3063 — knockout for EMC pathway) and Δhibadh::Km^R (Targeted gene: Rru_A1833 — knockout for VbSD pathway). They are currently being characterized. Below, the preliminary results of the Δ*ccr*::Km^R phenotype characterization show an evident lack of growth when the provided carbon source is acetate. In butyrate condition, the mutant strain grows as well as the wild type strain, supporting our hypothesis of another assimilation pathway (VbSD pathway) used in addition to the EMC pathway.



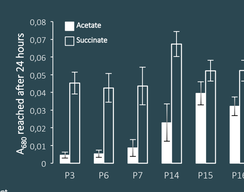
Multiple copies of *ccr* gene – Long term adaptation of strains used in MELiSSA?

During our mutagenesis experiment on the *ccr* gene, we highlighted a probable duplication of the targeted gene. Indeed, after the mutagenesis process we were still able to amplify a specific region of the *ccr* gene on several mutant strains that were resistant to kanamycin and for which we checked by PCR the correct insertion of the kanamycin resistance gene in the targeted region. According to qPCR experiments led on wild type strain, Δ*ccr*::Km^R *ccr*$^+$ strain and Δ*ccr*::Km^R *ccr*— strain, initial template DNA is at least 1.8x more abundant in the wild type strain than in the Δ*ccr*::Km^R *ccr*$^+$ strain. Consequently, two copies of the *ccr* gene should be present in the genome of our *Rs. rubrum* strain.

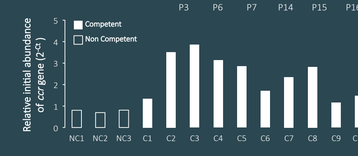


The various sequencing of *Rs. rubrum* S1H genome never highlighted multiple copies of the *ccr* gene. The duplication of the gene in our strain could then result from an adaptation to our culture conditions. Consequently, we have compared our strain to other available S1H strains, and the number of gene copy varied from 1 to 3.5 (data not shown).

One of these strains comes from a preliminary experiment that already led us to point a phenomenon of acclimatization. For this experiment *Rs. rubrum* was maintained under acetate condition (which is characterized by a dozens hours lag phase) for several growth phases (P1 to P16). The lag phase progressively tended to be shorter during a process of acclimatization through which *Rs. rubrum* became fully "competent" for acetate. This was translated by the measurement in the P16 biomass of the highest *ccr* copy number (i.e. 3.5 copies) observed by qPCR.



To assess the *ccr* copy number heterogeneity, the competent biomass was plated, monoclonal cultures were made from isolated colonies, and the amount of *ccr was* quantified by qPCR. Non acclimated strains underwent the same treatment. All the competent strains contained at least twice more copies of the gene than non-competent strains, and for most of them the gene is four times more abundant.



To confirm the link between *ccr* copy number and length of the lag phase in acetate condition, competent and no competent strains containing different amounts of *ccr* were submitted to acetate. Preliminary measurement (data not shown) highlighted an inverse relation: the higher the *ccr* copy number, the shorter the lag phase.
Consequently, acetate acclimatization could be linked to *ccr* copy number variation and maintaining cells in acetate condition seems to exert a selection pressure favoring cells with the highest copy number.

These results might question the genetic stability of MELiSSA organisms and their ability to face to unexpected environmental changes in the loop during space flight.

The photoheterotrophic metabolism of butyrate in Rs. rubrum seems to involve multiple pathways. Our proteomic analyses suggest that one fraction of butyrate is converted to acetyl-CoA while the other one is likely assimilated through the EMC pathway and the VbSD pathway. Further investigations, especially metabolites and fluxes analyses, will provide a better understanding of these assimilation pathways and their relative importance, as well as interactions between acetate and butyrate assimilation routes.

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