Butyrate metabolism under photothermotrophic conditions in Rhodospirillum rubrum S1H

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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 photothermotrophic 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 photothermotrophic 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 carbon imbalance. Therefore, the CO2 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 (Ruu_A0400, 3.1x). Furthermore, transmission electron microscopy 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 butyrate-CoA reductase (Ruu_A2320, 2.9x) or the pyruvate-dehydrogenase isocarboxylase (Ruu_A2198, 14.4x).

**Biosynthesis and Degradation of the Valine as a new anaplerotic pathway**

The proteomic aspect of the butyrate photoassimilation was investigated through mass spectrometry of secreted proteins from five bacterial replicates were processed to be identified and quantified using UMAQ software. A total of 3752 proteins were identified. Trip/TOP 5002 following a label-free strategy. Two different approaches were adopted.

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 1572 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 then be converted into acetyl-CoA. In most organisms, the use of two carbon compounds like acetyl-CoA for anabolic purposes requires the glycolytic cycle as a shunt to replenish the pool of acetyl-CoA precursor of the TCA cycle. *Rs. rubrum* is an isolate lacking negative regulator, and thus lacks the key enzyme necessary to use the glycolytic shunt. Alternatively, anaplerotic pathways are then expected to be used.

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

\[ \text{Butyrate} + 4/3 \text{CO}_2 \rightarrow 1 \text{Malate} + 1 \text{ATP} + 4 \text{H}^+ \]

**Ethylmalonyl-CoA pathway**

\[ \text{Butyrate} + 4/3 \text{CO}_2 \rightarrow 1 \text{Malate} + 1 \text{ATP} + 4 \text{H}^+ \]

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:

1. **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, the mutagenesis process we were still able to amplify a specific region of the ccr gene on several months and was necessary to inactivate and for which was checked by PCR the correct insertion of the kanamycin resistance gene in the targeted region. According to mPCR experiments led on wild type strain, ΔΔ-kom ccr- strain and ΔΔ-kom ccr- strain, initial template DNA is at least 100 times abundant in the wild type strain than in the ΔΔ-kom 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 to compare our own strain to other S1H strains. Evidence of multiple copies in the different strains would be interesting for detailed study of the *Rs. rubrum* metabolism. These results might also question the genetic stability of MELiSSA organisms and their ability to face to unexpected environmental changes in the loop during space flight.

   - The photothermotrophic 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 ethylmalonyl-CoA pathway and the valine biosynthesis and degradation 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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**References**

