New insights in the butyrate photoassimilation in *Rhodospirillum rubrum*

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Purple non-sulfur (PNS) bacteria are well known for their remarkable metabolic versatility allowing 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). Over the last few years, the VFA metabolism has been of particular interest in biopolymer and bioenergy industry. Indeed, VFAs are cheap raw materials easily obtained by fermentation of organic waste and that can be used to produce environmental-friendly polymer or renewable energy like hydrogen or biofuel. In the European Space Agency (ESA) bioregenerative life support system, the liquefying first compartment (C1) also produces VFAs. To avoid any loss of carbon and accumulation of dead-end metabolites in the MELiSSA loop, *Rhodospirillum rubrum* was selected for its ability to photoassimilate VFAs from the C1 effluents.

New pathways were recently highlighted by our laboratory as being involved in the assimilation of acetate, the most abundant VFA produced by the C1, in *R. rubrum* (Leroy et al., 2014). In that way, the ethylmalonyl-CoA pathway was demonstrated as being the principal acetate assimilation pathway. More surprisingly, other potential pathways were also identified notably through an uncomplete branched-chain amino acids biosynthesis pathway as well as the glutaryl-CoA pathway. Below are reported our first results allowing to pave the way to a better understanding of the photoheterotrophic metabolism of butyrate, the second most abundant VFA in the C1 effluent.

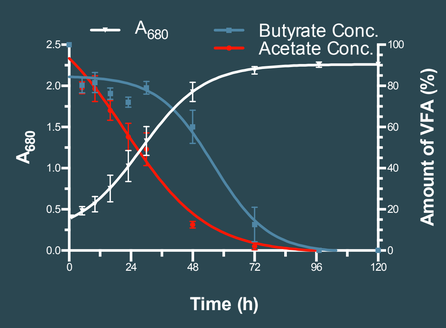
Valine degradation as a new assimilation pathway

The proteomic aspect of the butyrate photoassimilation was investigated through mass spectrometry. Extracted proteins from three biological replicates were identified and quantified on a UPLC-HRMS platform (Eksigent 2D ultra / AB Sciex TripleTOF 5600) following a label-free strategy.

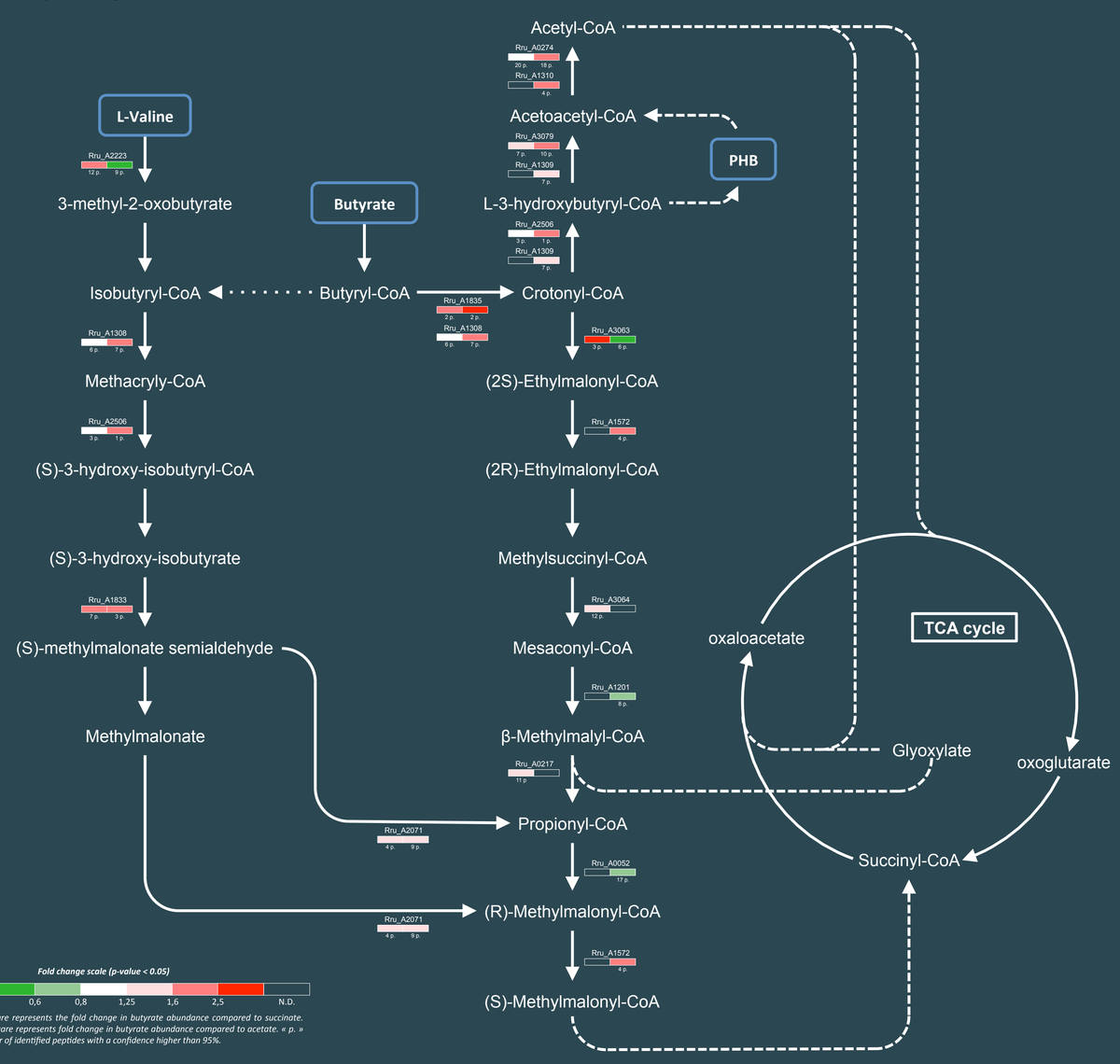
First, cultures of *Rhodospirillum rubrum* grown in the presence of butyrate as the unique source of carbone were compared to cultures grown in the presence of succinate (i.e., succinate being the control condition).

Secondly, in preliminary experiment, *Rhodospirillum rubrum* was cultivated in a mixture of butyrate and acetate at equivalent net carbon concentrations. The monitoring of VFAs via HPLC-refractometer analysis showed a sequential assimilation of acetate and butyrate. The butyrate is only assimilated when the acetate concentration is strongly reduced. To understand interactions between acetate and butyrate photoassimilation, the biomass harvested during butyrate assimilation was therefore compared with the one harvested during acetate assimilation.

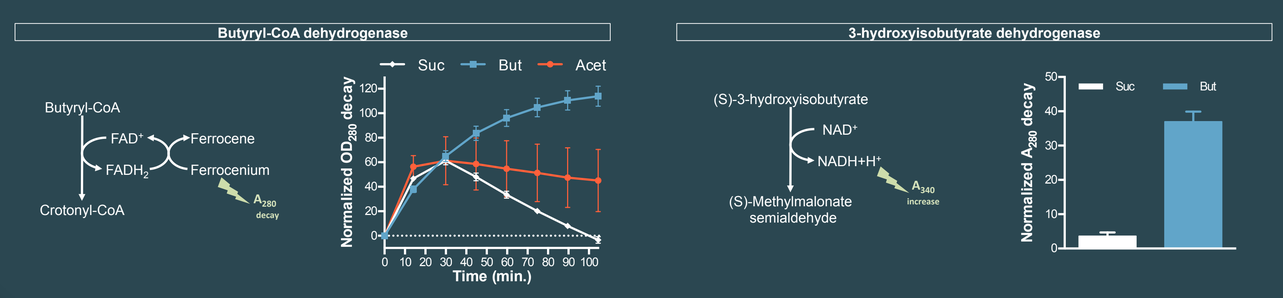
These proteomic analyses allowed the identification and quantification of 1,938 and 1,882 proteins in the first and second experiment, respectively.



Our proteomic data indicate that butyrate is converted to acetyl-CoA via the production of crotonyl-CoA. This first assimilation route is in common with the polyhydroxyalkanoate biosynthesis pathway. Therefore, it is likely that a fraction of butyrate could also be stored as a polymer of poly(3-hydroxybutyrate). Nevertheless, only a part of butyrate can be assimilated through this first pathway. Indeed, in most organisms, the assimilation of two carbon compounds involves the use of the glyoxylate cycle to replenish the pool of TCA cycle intermediates. However, *R. rubrum* is an isocitrate lyase negative organism meaning that this bacterium is not able to use this metabolic shunt. Butyrate is therefore expected to be assimilated through alternative anaplerotic pathways. Surprisingly, a significantly higher abundance of enzymes involved in the valine degradation pathway was observed when butyrate is supplied as the carbone source. Therefore, butyrate could be assimilated via this metabolic route and converted into isobutyryl-CoA to produce propionyl-CoA and succinate. Unfortunately, one of the key enzymes of this pathway has not been identified yet. The isobutyryl-CoA mutase has been already described in *Streptomyces cinnomonensis* to convert butyryl-CoA into isobutyryl-CoA. However, it was not identified with a significant similarity in the genome of *R. rubrum*.

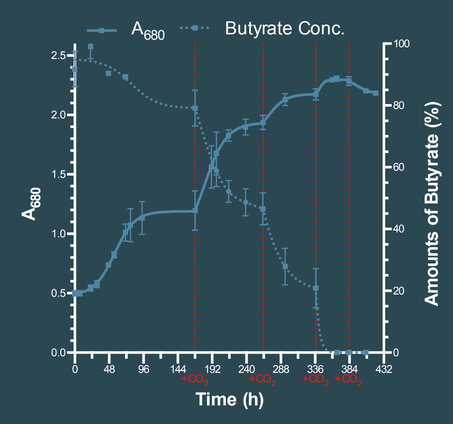


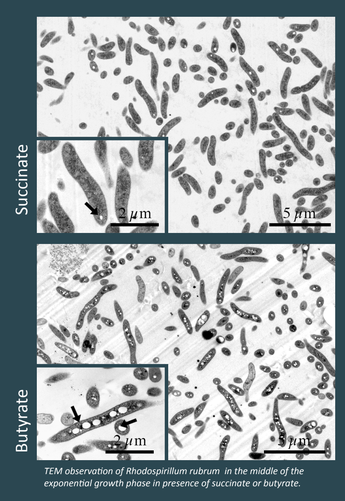
As a higher activity is not necessarily correlated with a higher abundance, enzymatic activities have been measured in cell free extracts of *R. rubrum* grown in the presence of succinate, butyrate or acetate. With regard to the valine degradation pathway, the 3-hydroxyisobutyrate dehydrogenase activity was measured and, as expected, is higher when butyrate is the carbon source. The activity of cell extracts from cultures grown with acetate was also assessed several times and showed opposite results (data not shown). The improvement of the assay will provide more accurate data. The butyryl-coA dehydrogenase activity was also measured in order to follow the conversion of butyryl-CoA into acetyl-CoA. It should be noted that some unexplained aspecificities were encountered. Although the butyryl-CoA dehydrogenase specific activity seems higher in butyrate, the monitoring of crotonyl-CoA formation is required to definitively confirm a higher activity in the presence of butyrate.



Bacterial growth analysis : dependence to carbonate

Bacterial growth analysis of *R. rubrum* showed a strong dependence on the presence of carbonate in the culture medium. In this study, it is hypothesized that butyrate assimilation induces a redox imbalance. Therefore, the CO2 fixation is not likely used for the anabolic purpose but rather as an electron sink to recycle reduced cofactors. First, 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 carbone (Rru_A2400; 3,2x, 23p). Upon the same conditions, we also observe the upregulation of another carboxylase, the crotonyl-CoA reductase (Rru_A3063; 2.3x, 3p). Secondly, the transmission electron microscopy also highlights an increased accumulation of poly(3-hydroxybutyrate), well known as being another electron sink.

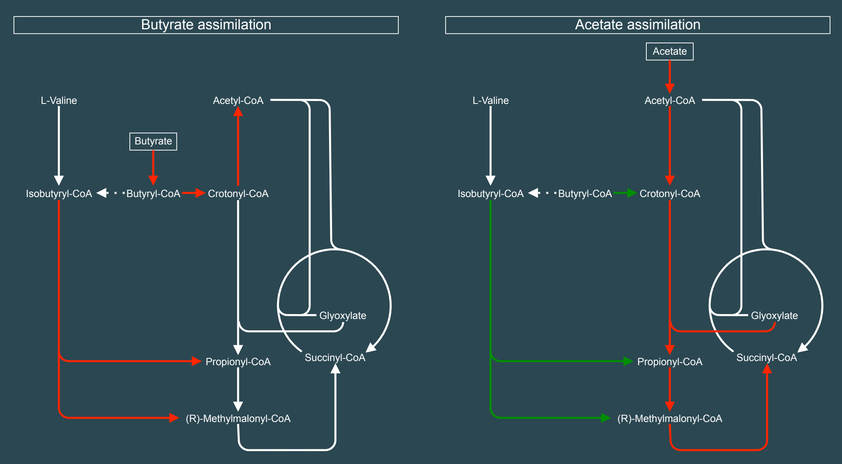




TEM observation of Rhodospirillum rubrum in the middle of the exponential growth phase in presence of succinate or butyrate.

Why does acetate inhibit butyrate assimilation ?

Acetate seems to have an inhibitory effect on the butyrate assimilation. According to our proteomic analysis and our previous study on acetate assimilation (Leroy et al., 2014), the acetate assimilation leads to a drastic reduction of the butyryl-CoA dehydrogenase abundance. This reduction counteracts the conversion of butyryl-CoA in acetyl-CoA. Moreover, acetate also seems to reduce expression of enzymes involved in the valine degradation pathway. Therefore, the metabolic adaptation to acetate seems to significantly reduce the butyrate assimilation through potential pathways highlighted in this work.



The photoheterotrophic metabolism of butyrate in *R. rubum* 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 valine degradation pathway. Further investigations, especially metabolites and fluxes analyses, will provide a better understanding of these assimilation pathways and theur relative importance, as well as interactions between acetate and butyrate assimilation routes.

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Leroy B., De Meur Q., Moulin Ch., Wegria G. and Wattiez R., **New insight in the photoheterotrophic growth of the ict- purple bacterium Rhodospirillum rubrum on acetate**. 2014, Microbiology (submitted)