

Butyrate metabolism under photoheterotrophic conditions in *Rhodospirillum rubrum* S1H

De Meur Q., , Wattiez R., Leroy B.

Department of Proteomics and Microbiology, Research Institute for Biosciences, University of Mons, Mons, Belgium
Contact: quentin.demeur@umons.ac.be

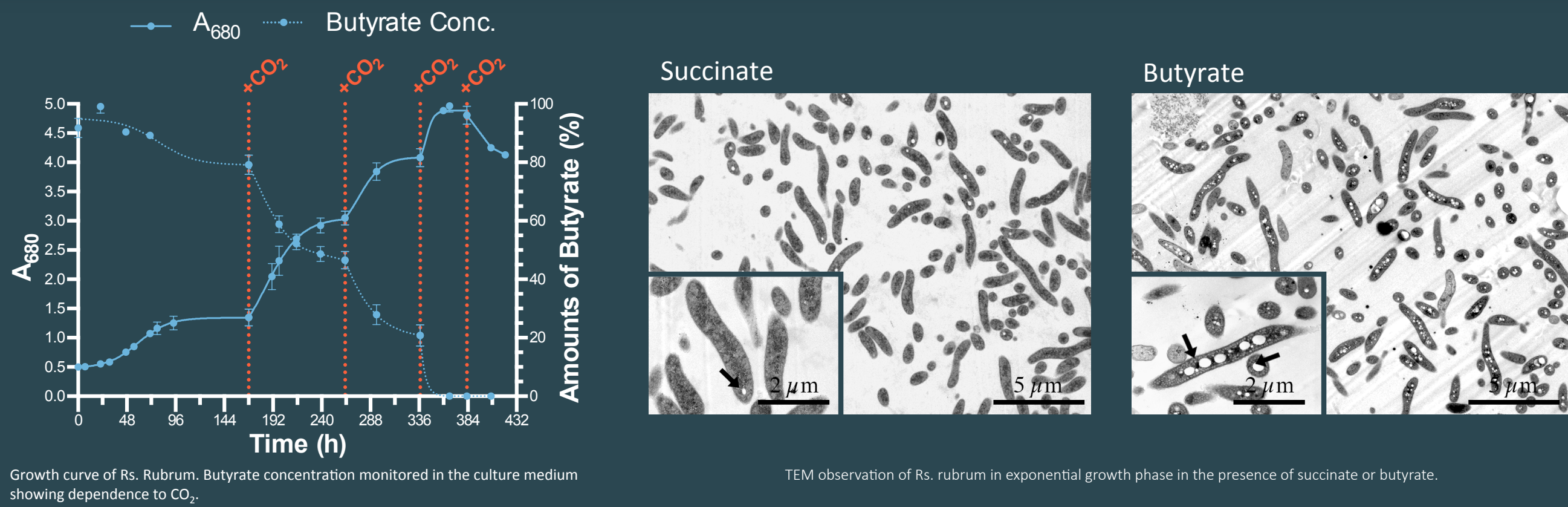
Purple non-sulfur (PNS) bacteria are well known for their remarkable metabolic versatility which allows them to occupy a broad range of environments. They are especially able to achieve a photoheterotrophic metabolism through which they can assimilate volatile fatty acids (VFAs). *Rhodospirillum rubrum* 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 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 (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.).



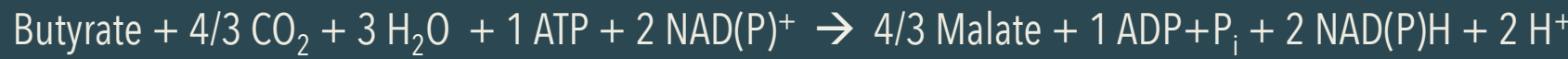
Biosynthesis and Degradation of the Valine as a new anaplerotic pathway

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:

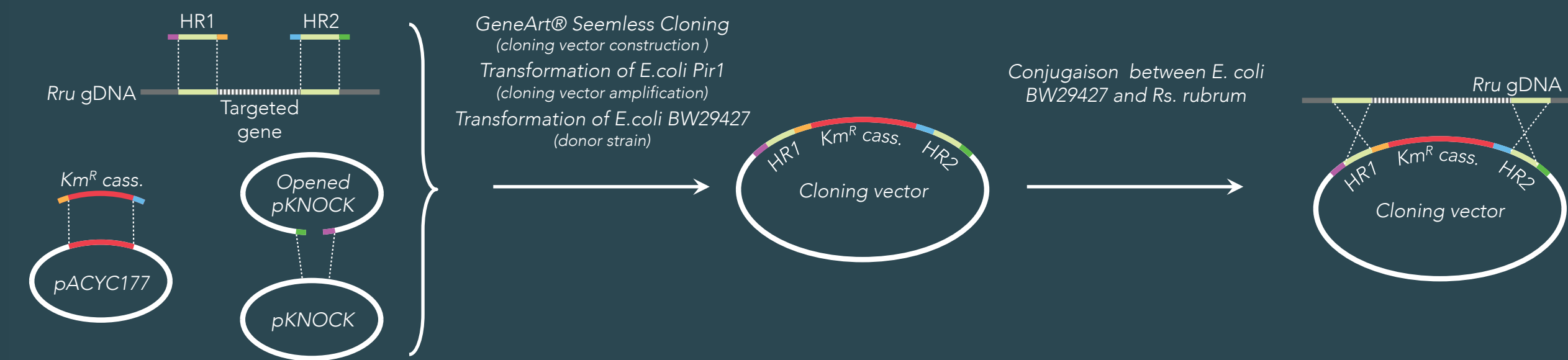


Surprisingly, a significantly higher abundance of enzymes involved in valine biosynthesis and degradation (VbSD) pathways is also observed. Acetyl-CoA formed from crotonyl-CoA could be converted into pyruvate that enters in the valine biosynthesis pathway to be converted in 2-oxoisovalerate. The latter 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 cinnamonensis* 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:

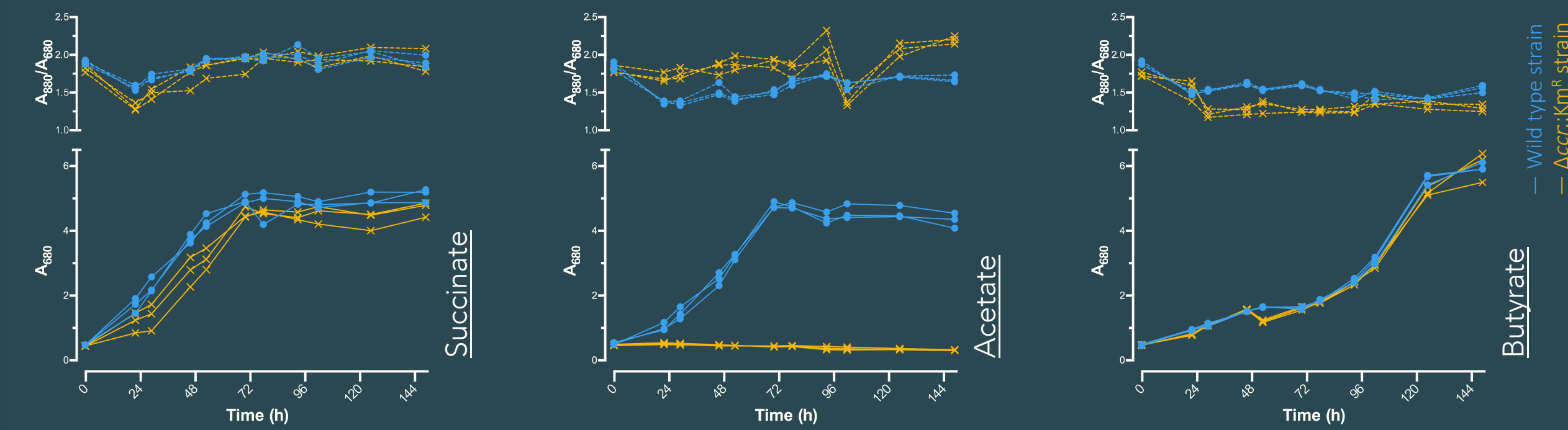


Validation throught mutagenesis experiment

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 Δ*hibadh*::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.

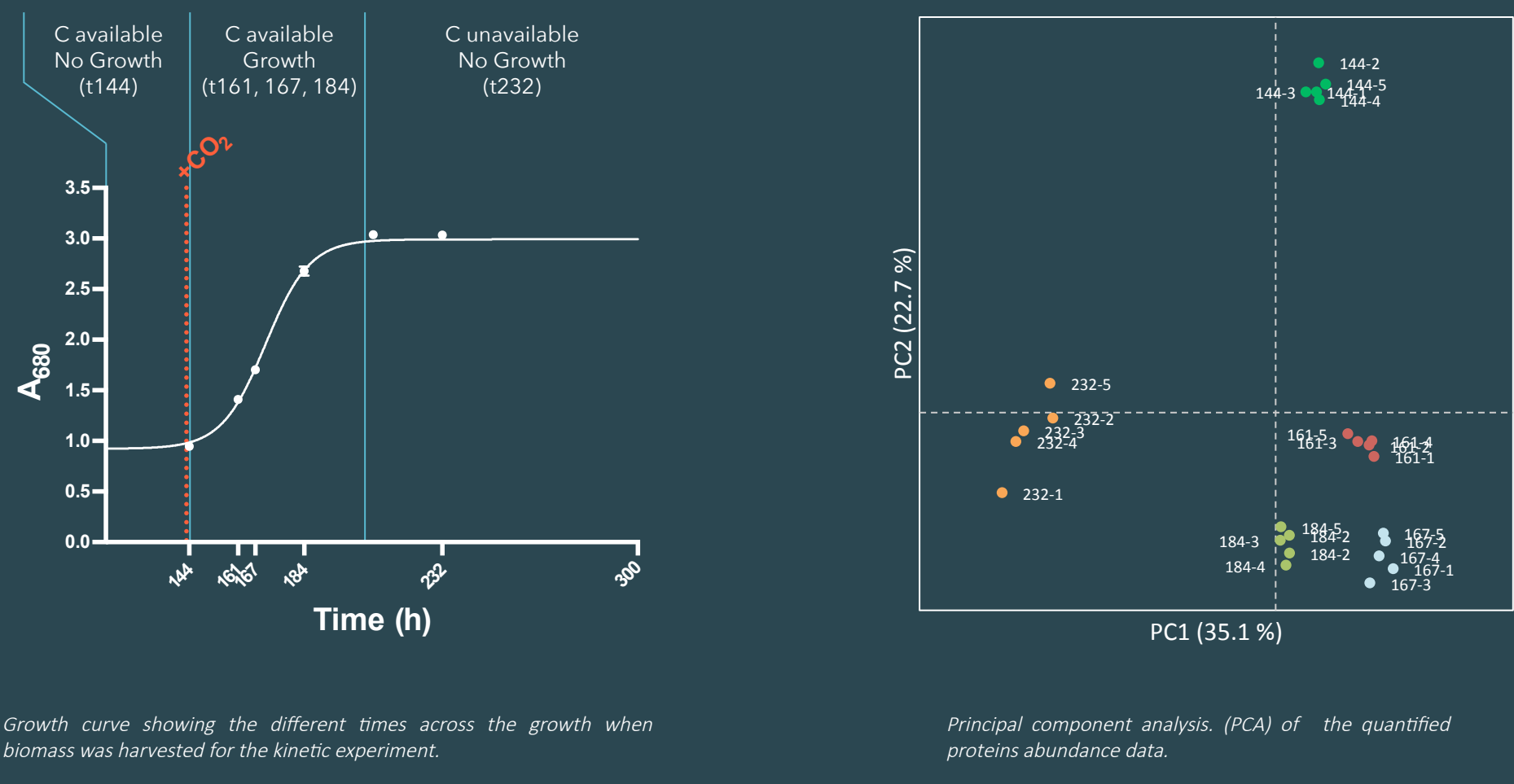
The various sequencing of *Rs. rubrum* S1H genome never highlighted multiple copies of the *ccr* gene. The duplication of the gene in our strain could then result from an adaptation to our culture conditions. Consequently, we have to compare our own strain to other S1H strains. Evidence of multiple copies in the different tested strains will confirm the crucial role of the *Ccr* in 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.

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.



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