Life in changing environments

The intriguing cycles of Polyphosphate Accumulating Organisms

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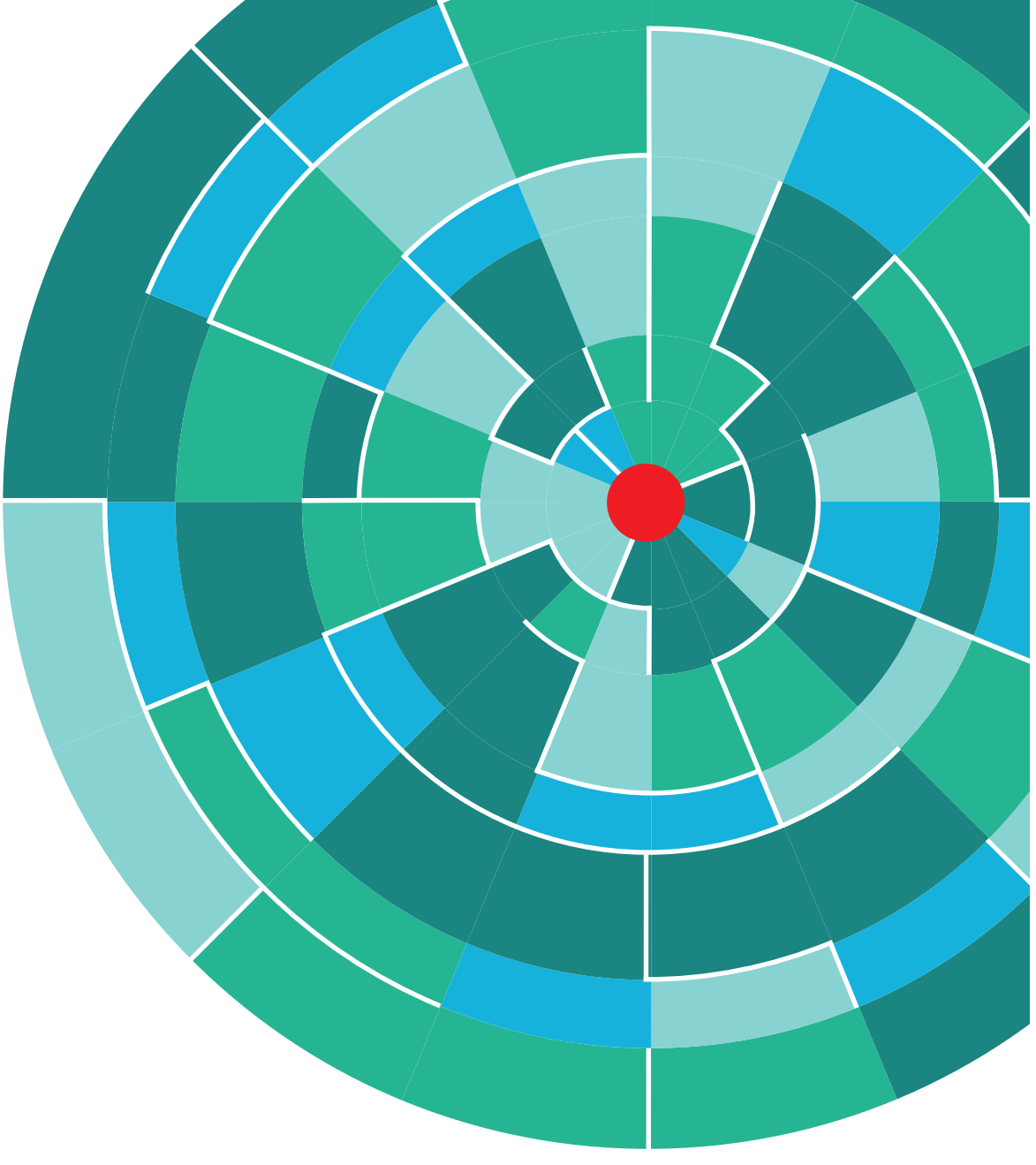

LIFE IN CHANGING ENVIRONMENTS

The intriguing cycles of Polyphosphate Accumulating Organisms

Leonor Guedes da Silva

Propositions

accompanying the dissertation

LIFE IN CHANGING ENVIRONMENTS

THE INTRIGUING CYCLES OF POLYPHOSPHATE ACCUMULATING ORGANISMS

by

Leonor GABRIEL GUEDES DA SILVA

1. Getting lost is what increases the chance of finding something new or unseen.

2. By simplifying reality, modelling is a powerful activity to understand complex problems, to connect observations, and to guide thoughts while building knowledge.

3. Accumulated polyphosphate is a key to unlock metabolic flexibility under anaerobic conditions. Chapter 2 of this dissertation

4. The winning strategy in one environment may not thrive in another environment. Flexibility and adaptability are therefore important to survive in changing environments. Chapter 3 of this dissertation

5. Alike increasing dilution rate in a bioreactor to accelerate the growth rate of a microorganism, pursuing challenges will also accelerate personal development and growth. However, too much will lead to wash out.

6. The biggest enemy of a researcher's time management is that one cannot plan the outcome of an experiment nor estimate the time needed for troubleshooting.

7. Feedback loops are essential to keep Life organized, so they must be equally crucial for organizations and relationships to maintain themselves in good health.

8. Lack of contextualization is a common problem in communication, which gets particularly aggravated by the increasing rush of our everyday life.

9. Curiosity is an important driver to explore the unknown, resulting in new knowledge and experiences that stimulate creativity, which ultimately lead to discovery and innovation.

10. A major scientific breakthrough happens when a mad scientist, a data magician and a visionary marketeer randomly meet in a bar and brainstorm while having drinks.

These propositions are regarded as opposable and defendable, and have been approved as such by the promotor prof. dr. ir. M. C. M. van Loosdrecht and copromotor dr. S. A. Wahl.

LIFE IN CHANGING ENVIRONMENTS

THE INTRIGUING CYCLES OF
POLYPHOSPHATE ACCUMULATING ORGANISMS

Dissertation

for the purpose of obtaining the degree of doctor

at Delft University of Technology

by the authority of the Rector Magnificus, Prof. dr. ir. T.H.J.J. van der Hagen,

chair of the Board for Doctorates

to be defended publicly on

Monday, 8 March 2021 at 12:30 o'clock

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Composition of the doctoral committee:

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Dr. S.A. Wahl	Delft University of Technology, *copromotor*
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Prof. dr. ir. H. J. Noorman	Royal DSM / Delft University of Technology
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Keywords: Metabolism, Dynamics, Polyphosphate Accumulating Organisms, Systems microbiology, Environment, Wastewater treatment

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Para a minha Mãe e o meu Pai

Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.
- Maria Skłodowska-Curie

Contents

PREFACE

Before delving into the rational, cold, emotionless realm of research, I would like to share a few words on the emotional side of being a human in science with you, my dear reader.

"The more I learn, the less I realize I know."
Socrates

This was the thought I shared with my supervisors sometime during my 2nd year of PhD. It seemed rational and logical, after all a philosopher had said it in the past, so it will not sound like I am in trouble. By then I was feeling overwhelmed with all the things I realized I did not know and I kept wondering *"how am I ever going to catch-up?"*.

I had a pile of articles and other doctoral dissertations to read on top of my desk and with each attempt of reading other people's scientific work I usually drowned in two thoughts:

- *"Wow, the author knows so much!"* Some seemed to know a lot about a specific topic, others seemed to know about a lot of topics. *"Why do I often feel like a know-nothing?"*
- *"Wow, the path from problem A to solution B is so logical and linear, they seem to get the experimental-design exactly right and to know what next steps to give. Why does everything seem to be so fuzzy for me?"*

I usually would not talk about this, because I was stuck in this thought *"I had a good performance during my past studies, so I should perform as well now"*. By now, some of you might be thinking and connecting this story to the Imposter Syndrome. I knew and thought about it too. Except, at a certain point I thought *"I don't have a syndrome, I'm the real imposter"* and I could come up with situations where I had just been lucky to prove it... Oh brain, why are you so evil?

Recently, I also became aware of the Dunning-Kruger effect.

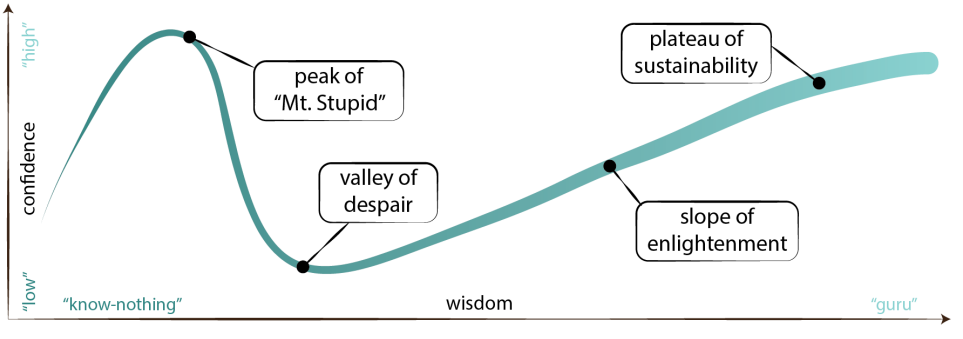

Figure 1: Dunning-Kruger effect. Figure adapted from `https://understandinginnovation.blog/`

This effect adds an interesting angle to the Impostor Syndrome, where actually feeling like an impostor is likely a good sign that you have finally fallen to right-side of "Mt. Stupid"

and you are ready to start your journey towards wisdom. It is often mentioned by PhD candidates that the 2nd and 3rd years are the most despairing. Looking at the description of the Dunning-Kruger effect, then I guess that is about the time when they finally fall from the peak of "Mt. Stupid" into the valley. Given that most PhD candidates are selected based on their (high) achievements on past studies, that peak is likely where they start their PhD journeys (oh yes, I used to be confident back then). However, besides this more negative-sounding interpretation of the curve, there is a very positive message in it too! Regardless of the emotional, self-confidence-related, roller-coaster-alike ride, if you keep going, you also keep getting wiser, and wiser. So the take-home message here is: just keep going, keep reading and learning, because eventually, after some (undefined) amount of time, you will certainly be wiser than before.

At this point, and given the topics addressed in this book, I feel I should make use of my wisdom and add a disclaimer: this simple model does not take into account time, nor (changing) environments!!

Another depiction of the "valley of despair" is the concept of "cloud" as described by Alon, 2009. Despite knowing about the cloud and the nurturing schema that should help me thrive in the cloud, the imposter feeling makes it difficult to self-identify when I am actually in the cloud (it could just be my incompetence, right?). This is the moment where reaching out to others can be a game-changer! When others join you in the cloud, then it becomes much easier to switch to a more constructive mindset.

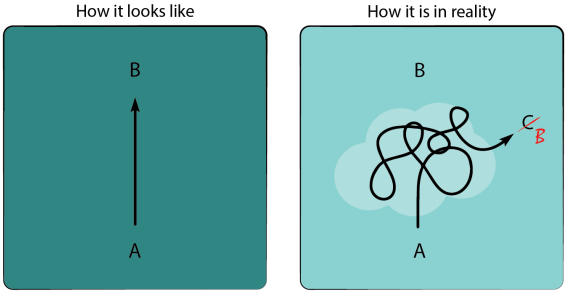

Figure 2: The objective (A to B) *versus* the nurturing schema of research featuring the "cloud" as proposed by Alon, 2009. This was also nicely presented in several TED talks by Uri Alon himself. Figure adapted from Alon, 2009.

As someone once told me, *"getting out of the cloud is a matter of mindset"*. One may choose to leave it towards point C, or D, or E,... and it is okay! The fact that you can now read this dissertation, despite outcomes written in its chapters not being the initially-planned point "B" is supporting evidence of it being okay. It is even okay that Chapter 5 is actually still in the cloud - anyone interested in joining? :)

So far, all processes described seemed linear. However, because of my experiences through this journey, I feel I should highlight that **RE**-search is not a linear process, but an iterative one. It seems trivial, I know! But perhaps current knowledge exchange systems (at least in biology) could improve to better accommodate for that iterative property of research? Food for thought.

Inspired by the main stars of this book, Polyphosphate Accumulating Organisms (PAOs), I would like to propose a simple model of iterative research in changing environments, which could also address some of the emotional aspects of being a human in science.

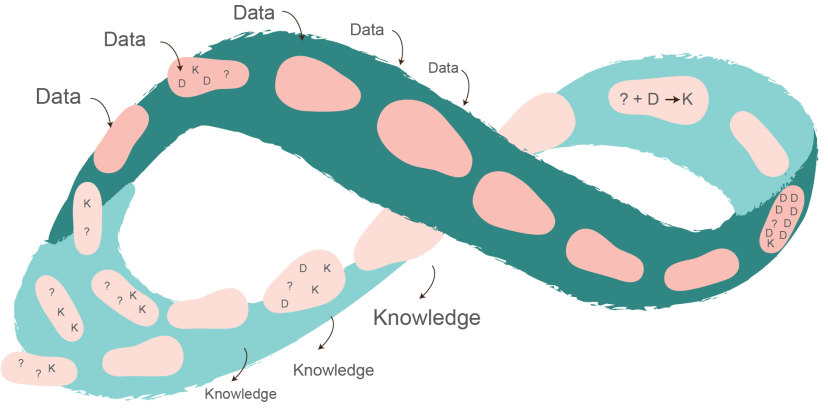

Figure 3: Research in changing environments: The intriguing cycles of Wisdom Accumulating Organisms (WAOs). **D:** data, **K:** knowledge, **?:** unknowns. Note that unknowns seem to be harder to secrete (also food for thought).

Enter the Wisdom Accumulating Organisms (WAOs). The goal of WAOs is to have increasingly more wisdom (knowledge + unknowns). Not as much as an individual organism, but more as a community of WAOs. They are fueled through experimentation and collection of **data** (D). They happily try to collect as much as they can (like hamsters) until they become completely stuffed. For the human, this is usually the overwhelming moment of *"how am I going to digest all this?"*. This is when changing environment becomes interesting. Without **data** to be collected, the strategy has to change and creativity kicks in. This is good, because **data** alone is not wisdom. The conversion of **data** into **knowledge** (K) then starts, where loads of **data** are connected and synthesized into insights. These in turn guide thoughts while building **knowledge**. However, an inevitable by-product of this conversion of **data** into **knowledge** are the **unknowns** (?). After squeezing the low hanging **knowledge** fruits from collected **data**, further squeezing will lead to increasingly higher amounts of **unknowns**. For the human, too many **unknowns** can lead to despair - *"Should I know this? How can I proceed without knowing this?"*. Fortunately, a healthy dose of **unknowns** is the realization that triggers and focuses the acquisition of more observations, more **data**. It is now a good time to change environment, and the cycle begins again.

And so, to the reader that is now starting or currently in a journey towards wisdom and to the future-supposedly-imposter-Leonor (yes, I should keep reminding myself):

"Worry less, and enjoy more!"

Leonor Gabriel Guedes da Silva
Rijswijk, February 2021

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SUMMARY

Cells are complex systems continuously exposed to changing, dynamic environments. Understanding how cells respond and adapt is of great interest not only from a fundamental viewpoint but also for the development of solutions for current challenges in medical, industrial and environmental research fields.

In this thesis, the bacterial community member *Candidatus* Accumulibacter phosphatis (hereafter referred to as Accumulibacter) from the functional group of Polyphosphate Accumulating Organisms (PAOs) was selected as study object due to their key-role in phosphorus removal at wastewater treatment processes and their adaptive metabolic strategies to thrive in fluctuating environments. These microorganisms are enriched in Enhanced Biological Phosphorus removal (EBPR) systems where they experience cyclic absence and presence of external electron acceptors (here, anaerobic and aerobic conditions, respectively). These fluctuations together with non-continuous availability of nutrients lead to intricate metabolic strategies. While the overall metabolic traits of these bacteria are well described, the non-availability of isolates has led to controversial hypotheses on which metabolic pathways are used - *structure*, when are these pathways active - *function*, and what mechanisms control the operation of these pathways - *regulation*.

A mismatch on the balancing of redox cofactors motivated an investigation on the redox cofactor preference of different oxidoreductases in the central carbon metabolism of a highly enriched Accumulibacter culture. In **Chapter 2**, a NADH-preferring acetoacetyl-CoA reductase engaged in polyhydroxyalkanoates (PHA) synthesis was found instead of the generally assumed NADPH dependency. This leads to re-thinking the ecological role of PHA accumulation as a fermentation product under anaerobic conditions rather than a stress response under unbalanced growth conditions.
The consequence of NADH dependency in the context of the anaerobic pathways functioning as sources and sinks of these redox cofactors was further analysed computationally in **Chapter 2** using the well-known stoichiometric modeling approach, flux balance analysis (FBA). By varying the acetate-to-glycogen consumption ratio different scenarios were simulated, which demonstrated *optima* using different combinations of glycolysis, glyoxylate shunt or oxidative and reductive branches of the TCA cycle. Thus, optimal metabolic flux strategies will depend on the environment (acetate uptake) and on intracellular storage compounds availability (polyphosphate/glycogen). With this analysis, it became apparent that the controversial discussion in literature is in fact caused by the flexible metabolism of *Ca.* Accumulibacter that allows for maintaining metabolic activity under varying environmental substrate conditions.

What will be the best metabolic strategy in a competitive environment where oxygen is periodically unavailable? This was the question addressed in the theoretical **Chapter 3**. A dynamic resource allocation modeling formalism was used to analyze the impact of selection pressures on metabolic function. Different optimal strategies could be predicted for gradually less stringent C-source availability selective pressures: Polyphosphate-AOs, Glycogen-AOs, Polyhydroxyalkanoate-AOs, and regular aerobic heterotrophs. Storage metabolism revealed itself as a selective advantage in situations where robustness and competitiveness are key, however at the cost of a lower biomass yield. The use of a meta-network in this model to simulate optimal metabolic strategies under different environmental conditions can be seen as an example of when *"Unity in biochemistry"* by A. Kluyver

meets *"Everything is everywhere, but the environment selects"* by L. Baas-Becking and how microbial ecosystems may be described by the energy allocation phenotype instead of a detailed description of each organism.

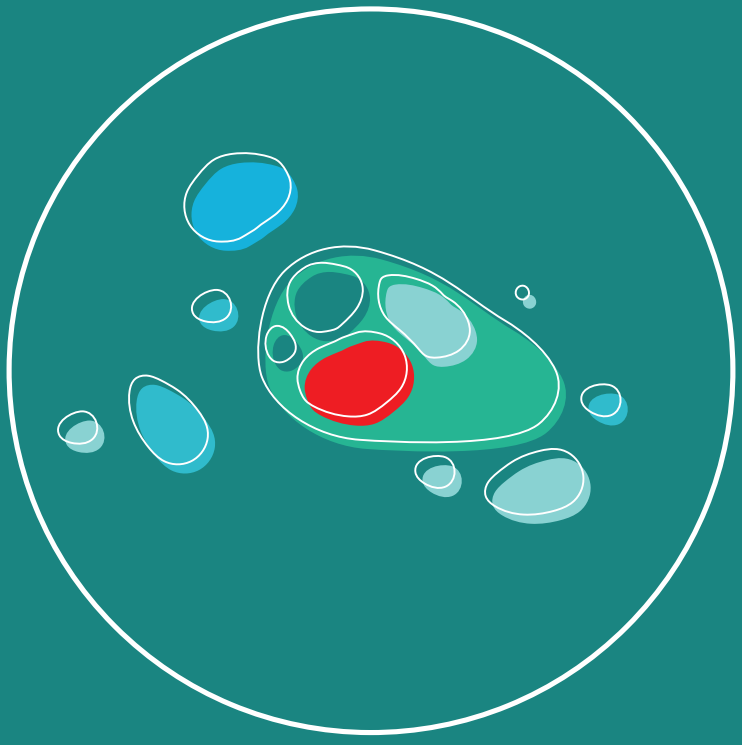
While the optimization problem in **Chapter 3** leads to an optimal metabolic flux distribution, the experimental approach used in **Chapter 4** reveals actual metabolic strategies. A stable isotope labeling experiment was conducted using ^{13}C-acetate to monitor intracellular switches of carbon flows of an enrichment culture of Accumulibacter upon changes in acetate and oxygen availability. The release of $^{13}CO_2$ to the off-gas points at the oxidation of labelled acetate anaerobically, which was consistent with an initial ^{13}C-enrichment of TCA cycle intermediates. However, an unexpected metabolic transition seems to take place during the anaerobic-feast phase as this enrichment of TCA cycle intermediates decreased after a few minutes. Furthermore, distinct anaerobic/aerobic ^{13}C-patterns in TCA intermediates hint to a changed glyoxylate shunt operation, suggesting a regulatory mechanism impeding this shunt's anaerobic activity unlike what is postulated in several biochemical models of Accumulibacter. The ^{13}C enrichment of upper glycolytic intermediates was only observed upon the anaerobic-to-aerobic switch, indicating a tight regulatory checkpoint of gluconeogenesis. This study demonstrates how ^{13}C tracing can generate insight on metabolic switches of microbial communities cultured under dynamic environmental conditions. From the insights generated, hypotheses are generated for further investigation of regulatory mechanisms governing the lifestyle of Accumulibacter, which may enable the design of novel control strategies to improve EBPR processes.

It has been postulated in previous studies that under anaerobic conditions a fully oxidative TCA cycle operates in these microorganisms. The observed ^{13}C-enrichment of TCA cycle intermediates in the initial period of the anaerobic phase from ^{13}C-acetate also supports this hypothesis. However, this highly debatable anaerobic TCA cycle activity sets a challenge since succinate dehydrogenation is commonly coupled to $FADH_2$ production, which needs a strong electron acceptor to be regenerated to FAD or high energy inputs to regenerate via reverse electron transport (RET). In **Chapter 5** the potential for Accumulibacter to regenerate FAD when there is no external electron acceptor was evaluated experimentally through enzymatic activity assays on membrane vesicles. While NADH dehydrogenase and succinate dehydrogenase activity was observed, the genome-based predicted NAD^+ reductase that could regenerate FAD did not show any appreciable activity under the conditions tested. Steps were proposed to further elucidate this putative FAD regeneration mechanism that may depend on electron bifurcation or proton motive force across the membrane. Such mechanism would grant additional flexibility to the anaerobic metabolism of Accumulibacter by alleviating or even eliminating the need for cycling glycogen.

While the bacterial community member Accumulibacter was the cellular system example studied here, this doctoral dissertation explored and combined systems biology methods to improve the mechanistic understanding of cells as structured metabolic systems with functional and regulatory frameworks, which respond and adapt to changing external conditions (environments). The developed and applied approaches are not only specific to Accumulibacter and can be applied to other cell systems and communities exposed to changing environments.

1 CHAPTER

General Introduction



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CELLS are the basic unit of life. Together, they are the building blocks of whole complex organisms such as a Human, or they can exist as single cells such as bacteria. For the past 3.8 billion years, these cells have been exposed to a plethora of environmental conditions, some of which do not occur anymore and others that are recurring and cyclic (*e.g.* seasons, day/night, tides, or nutrient cycles). Under such conditions, cells have developed highly efficient mechanisms – *i.e.* metabolism – for coupling energy sources (sunlight or chemical) to the many energy-requiring processes required to sustain life (Nelson and Cox, 2017). Nowadays, the study of cells as *metabolic* systems is of great interest not only from a fundamental viewpoint but also for the development of solutions for current challenges in medical, industrial and environmental areas (European Commission, 2018; Straathof et al., 2019).

This doctoral dissertation explores and combines methods to improve the mechanistic understanding of cells as structured metabolic systems with functional and regulatory frameworks, which respond and adapt to changing external conditions - *environment*. As a case-study, the bacterial community members *Candidatus* Accumulibacter phosphatis (hereafter referred to as Accumulibacter) from the functional group of Polyphosphate Accumulating Organisms (PAOs) were selected due to their key-role in biological phosphorus removal at wastewater treatment processes and their adaptive metabolic strategy to thrive in fluctuating environments.

MECHANISTIC UNDERSTANDING OF METABOLISM – *How do cells work?*

To understand how a cell operates as a system is far more complex than, *e.g.* a machine. Among several reasons is the fact that a cell is not man-made and so there is no schematic or circuit diagram readily available. Creating such schematic can thus be seen as gathering mechanistic understanding of a given system (Figure 1.1).

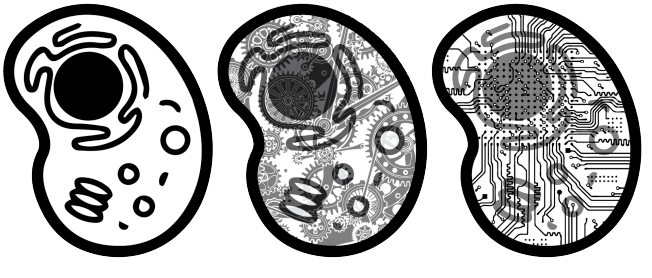

Figure 1.1: Impression of a cellular system regarded as a vesicle with organelles **(left)**, as a mechanical machine **(middle)** or as a circuit board **(right)**.

A cellular system is composed of many different types of molecules such as polymers like DNA, RNA, Proteins, Lipids, and their respective monomers among many other metabolites (Figure 1.2). The genome can be seen as the blueprint or the instruction's book of the cell as its DNA has, among other things, encoded all the potential functions that the cell can perform. Some of these instructions are transcribed, which then comprise the transcriptome. These RNA transcripts are single instructions to make tools such as proteins, which encompass the proteome. And finally, these proteins are used as catalysts in cellular activities such as conversion of nutrients into new building blocks - *metabolism*. All intermediates and end-products of these conversions are metabolites and together they form the metabolome.

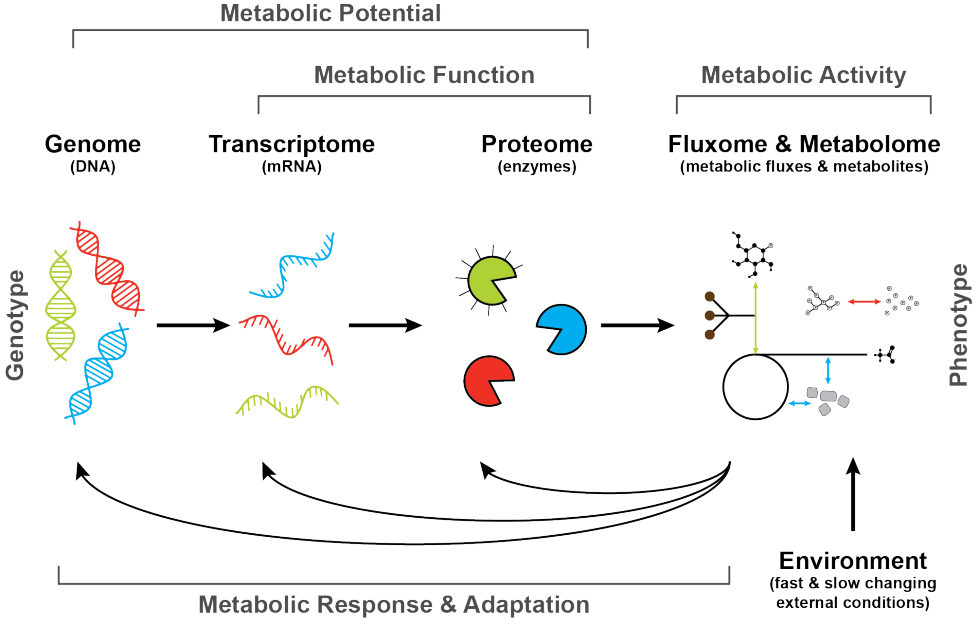

Figure 1.2: Schematic overview of the molecules and -omes of a cell system involved in metabolic mechanisms.

But, what determines which functions - *phenotype* - are selected and available from the genetic blueprint - *genotype*? Physical and chemical properties of the environment affect metabolic functions, which in turn relay these environmental cues back to all other "-omes" ("Beyond blueprints" 2012). In particular, the phenotypic shifts of Accumulibacter depending on the environmental conditions are the cornerstone of enhanced biological phosphorus removal from wastewater treatment. To mechanistically understand how cellular components function and interact, allowing cells to respond and adapt to fast and slow changing external conditions, a cybernetic systems approach will be used.

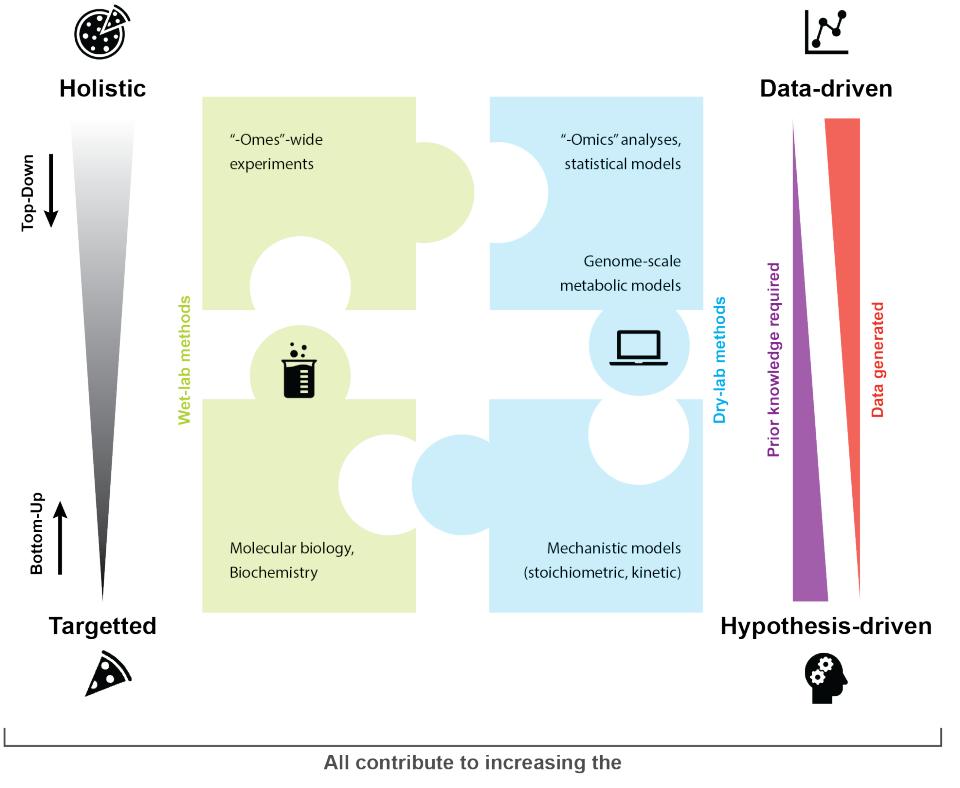

Figure 1.3: Systems biology approaches from "-omes"-wide experiments and analyses, targeted molecular and biochemical research, to computational and mathematical approaches aiming at generating mechanistic understanding of metabolism.

Nowadays known as Systems Biology, this approach combines two strategies: a top-down holistic approach where the complete "-omes" of a cell are studied separately or integrated in "-omics" analyses and a bottom-up which focuses on studying targeted components and how they interact using molecular biology, biochemistry and mechanistic models (Figure 1.3). Note that systems biology promotes the synergy between experimental analyses from *wet-labs* and modeling approaches of *dry-labs* to better understand how metabolic behavior emerges, and how it takes control of itself and other cellular processes while adapting to new environments (Heinemann and Sauer, 2010).

Molecular Biology & Biochemistry, a molecular-scale bottom-up approach

Biochemistry aims at understanding the different chemical reactions occurring in a cell, while Molecular Biology aims to study the composition, structure and interactions of the molecules underlying Biochemistry.

"From elephant to butyric acid bacterium – it is all the same."
From *"Unity in Biochemistry" by Albert Kluyver*

Supported by the notion that all living systems are somehow related, this view on biochemistry by A. Kluyver justifies the focus on careful characterization of metabolic and physiological processes so that later these can be used as framework for newly identified organisms (Singleton and Singleton, 2017).

Cellular characterization – *Who are they and what can they do?*

A newly identified cell type can be thoroughly characterized by phenotypic, chemotaxonomic, and molecular tests. Such characterization includes a description of their dominant phenotype, phylogenetic analysis based on 16S rRNA gene, cell shape, respiratory and denitrification capacity, nutritional type (*i.e.* carbon sources), pH and temperature *optima* and tolerance ranges.
The concept of *Candidatus* is used for well characterized but yet uncultured organisms (Garrity et al., 2002). This is the case for the bacteria understudy, *Candidatus* Accumulibacter phosphatis, whose characterization was thoroughly reviewed nearly two decades ago by Seviour and colleagues (Seviour, Mino, and Onuki, 2003). Polyphosphate Accumulating Organisms (PAOs) like Accumulibacter were accidentally discovered in treatment plants that contained an anaerobic zone at the entrance in otherwise fully aerobic activated sludge systems (Srinath, Sastry, and Pillai, 1959). As a result, microorganisms experienced time-varying presence and absence of external electron acceptors, *e.g.* O_2. In the absence of O_2, ordinary aerobic heterotrophs cannot produce energy from *e.g.* acetate, which gives a selective advantage to PAOs that can use their polyphosphate and glycogen storage for energy production. PAOs remove phosphate from the environment by accumulating it intracellularly as polyphosphate (Barnard, 1976; Fuhs and Chen, 1975; Levin and Shapiro, 1965; Seviour, Mino, and Onuki, 2003). The typical dominant phenotype observed in Enhanced Biological Phosphorus Removal (EBPR) processes is presented in Figure 1.2A. Microscopic observation of cells in these systems allowed to confirm the co-existence of polyphosphate (PP) and polyhydroxyalkanoates (PHA) granules inside the same cell, further clarifying *what these cells do*. In Figure 1.2B, an example of an electron micrograph of a thin section from an Accumulibacter cell at the end of the anaerobic phase (Welles et al., 2017).

As presented in Figure 1.4C fluorescence *in situ* hybridization (FISH) microscopic techniques (Crocetti et al., 2000; Hesselmann et al., 1999), allowed connecting the phylogeny of these bacteria (16S rRNA gene-based) with their observed dominant phenotype, *i.e.* polyphosphate and polyhydroxyalkanoate accumulation capacity (Liu et al., 2001). In simple, it connected the *who it is* to the *what it does*, serving to clarify the field and further research.

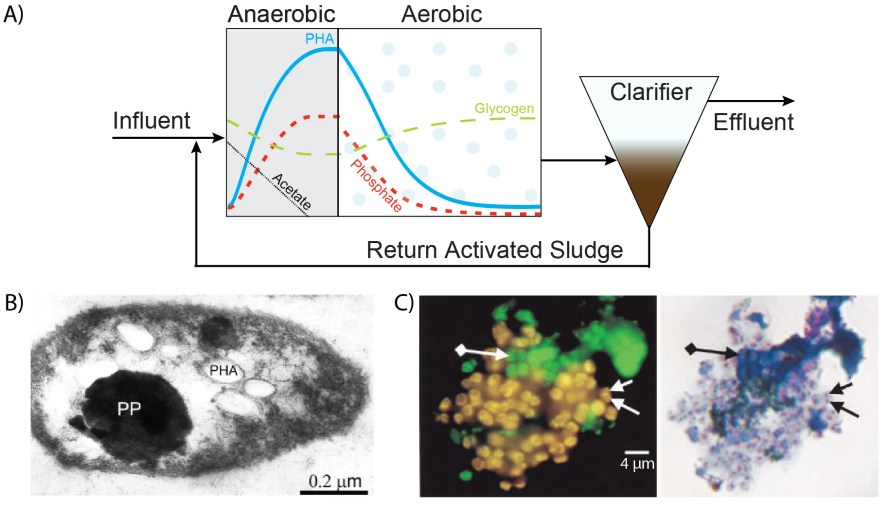

Figure 1.4: **A)** Dominant phenotype observed in EBPR processes. Anaerobically, phosphate (PO_4^{3-}) is released, PHA is accumulated, acetate and glycogen are consumed. Aerobically, acetate is no longer present, PHA is consumed, phosphate is taken up, glycogen and polyphosphate are replenished, biomass is synthesized; **B)** Electron microscope micrograph (scale bar 0.2 µm) of a thin section showing the polyphosphate (PP) and polyhydroxyalkanoates (PHA) granules inside an Accumulibacter cell at the end of the anaerobic phase (Welles et al., 2017). Glycogen was also observed but not stained in this micrograph; **C)** FISH micrograph (scale bar 4 µm) on the left showing yellow-labelled Accumulibacter cells surrounded by other green-labelled bacteria (general probe) and, on the right, same field showing the methylene blue-stained polyphosphate granules inside Accumulibacter cells (Crocetti et al., 2000).

To further assess the diversity within the Accumulibacter lineage, McMahon and colleagues (2007) designed a polyphosphate kinase (*ppk1*) primer set targeting total Accumulibacter. When analyzing several full scale sludges, the *ppk1* phylogeny allowed to distinguish between clades beyond the two types identified by 16S rRNA genes (He, Gall, and McMahon, 2007; McMahon et al., 2007). To date, fourteen (14) Accumulibacter clades have been described (Camejo et al., 2019) and are hypothesized to have metabolic and ecological differences (He and McMahon, 2011b). For example, Welles and colleagues demonstrated Accumulibacter types I and II perform kinetically different and even look different under phase contrast microscopy (Welles et al., 2015). On another example, ample evidence exists that Accumulibacter have the capacity to denitrify, *i.e.* to respire nitrate and nitrite into nitrogen gas, while cycling polyphosphate, however this capacity seems to differ between types and clades (reviewed in (Camejo et al., 2019)).

Without an isolate (Kang and Noguera, 2014), it becomes difficult to proceed with further specific characterization and one has to rely on noisier metabolic activity observations from microbial enrichments highly abundant in Accumulibacter. Concerning nutritional type (*i.e.* carbon sources), pH and temperature *optima* and tolerance ranges, the most comprehensive analysis can be found in the study by Lopez-Vasquez and colleagues (Lopez-Vazquez et al., 2009). However, as Accumulibacter can only be grown in mixed culture, from this study one can only conclude on which phenotypic types are most competitive in each situation and not specifically on their *optima* or tolerance. For example, while 20 °C seems to be the best temperature for obtaining an enrichment of Accumulibacter, it does not necessarily mean this temperature is their *optima*, it just means it performs better than others at that temperature.

STABLE ISOTOPE LABELING TO TRACK METABOLIC CONVERSIONS – *What functions do they perform?*

The use of labelled substrates to track connections between metabolic intermediates has revolutionized the study of metabolism. It allows to assess the metabolic origins of macromolecules and in some cases, it even allows to distinguish between alternative pathways and to identify regulated (delayed) reactions upon perturbations.

Stable isotopes of carbon (^{13}C), hydrogen (^{2}H), phosphorus (^{31}P), nitrogen (^{15}N) can either be distinguished from their most naturally abundant counterparts due to their different magnetic spin measured by nuclear magnetic resonance (NMR) or due to their different atomic weight, which can be measured by mass spectrometry-based (MS) techniques. These different characteristics is what allows to track isotopically labelled atoms through metabolism.

In vivo ^{13}C-NMR and ^{31}P-NMR techniques were applied to study phosphorus and carbon metabolism in aerobic sludge performing EBPR during both the anaerobic and the aerobic phases (Florentz, Granger, and Hartemann, 1984; Hesselmann et al., 2000; Maurer et al., 1997; Pereira et al., 1996). By supplying a ^{13}C labelled acetate or propionate it was possible to follow the flux of ^{13}C from substrate to PHA. In a second cycle, (intracellular) glycogen was labelled and ^{13}C was monitored from glycogen to PHA. In these experiments only the ^{13}C pattern in the different PHAs was measured and not in the metabolic intermediates of each pathway used. Observations clearly demonstrated the cycling of glycogen in Accumulibacter, however different glycolytic pathways (EMP or ED) were postulated by different studies. Moreover, different operations of the TCA cycle branches were inferred, giving rise to several discussions in literature as reviewed in (Zhou et al., 2010). These early studies comprise interpretations of ^{13}C patterns using simplified metabolic models (proposed before a sequenced genome was available), with (1) limited information on the reversibility of each reaction, (2) potentially missing reactions and (3) limited measurements of intermediates; these are common pitfalls of ^{13}C-labelling data analyses (Winden, Verheijen, and Heijnen, 2001). In **Chapter 4**, ^{13}C-acetate is used to trace ^{13}C through the metabolism of Accumulibacter and many metabolic intermediates were measured in time using MS-based techniques.

ENZYMATIC ACTIVITY STUDIES – *Which tools do they use?*

Enzymes are catalytic proteins essential to biochemical processes. They act in organized sequences of reactions - *pathways* - with the aim of converting nutrients into energy or precursors essential to sustain life. Enzymatic activity studies aim at determining the reaction kinetics of an enzyme and how these change in response to different experimental conditions (*e.g.* pH, temperature, cofactors, inhibitors). From these studies, one can get an idea for affinity, speed, inhibition or activation, which elucidate potential regulatory mechanisms. Enzymatic assays can be conducted with cell-free enzyme extracts (CFE) or with purified enzymes. CFEs are lysates of cells and typically the cytoplasmic proteins are separated from the membrane proteins during the CFE preparation. Thus, in a CFE, the enzyme of interest is studied alongside all other cellular macromolecules, which might increase the noise in the assay. A cleaner but far more laborious approach is to synthesize and purify the enzyme of interest (Gregorio, Levine, and Oza, 2019), to conduct the assay in isolation, *i.e.* a more controlled environment and much less noise.

Enzymatic assays can allow for unraveling which enzymes may be available in the different phases of cultivation of these microorganisms. Redox reactions are easy to follow by monitoring NADH or NAD(P)H production/consumption, and therefore of particular interest. Knowing the cofactor dependencies of enzymes allows for constraining stoichiometric metabolic models. For example, in **Chapter 2** we explored the activity of cytoplasmic enzymes in CFEs of Accumulibacter in the presence of either NAD(H) or NADP(H) to assess their cofactor preference and to better understand the coupling between metabolic pathways. This adds on to previous studies that were solely looking for absence/presence of enzymatic activity (Erdal, 2002; Erdal, Erdal, and Randall, 2005; Hesselmann et al., 2000). Also enzymatic assays with purified enzymes from Accumulibacter were performed, which investigated the kinetics of enzymes engaged in polyphosphate and polyhydroxyalkanoate synthesis (Mcmahon et al., 2002; Olavarria et al., 2021).

Membrane proteins are essential to assist the transport of polar compounds in and out of cells, but also to generate energy for cellular processes - *membrane bioenergetics*. The physiology of Accumulibacter relies upon a considerable exchange of small molecules and ions with the surroundings, thus membrane studies are very relevant to under Accumulibacter's physiology. To our knowledge, there are no studies available in which membrane enzymes of Accumulibacter have been assayed. This will be further addressed in **Chapter 5**.

METABOLIC INHIBITORS AND DNA-MANIPULATION METHODS TO UNDERSTAND METABOLIC FUNCTION – *Which tools are needed for what function?*

An interesting way to study the role of an enzyme or a pathway involves disturbing the system. The use of metabolic inhibitors allows blocking pathways at specific points to assess the impact of a given process to the overall phenotype.
Targeting enzymatic inhibitors in Accumulibacter enrichments have been used to elucidate specific membrane transport processes and to evaluate the putative activity of the TCA cycle in the absence of external electron acceptors (Burow et al., 2008; Louie et al., 2000;

Saunders et al., 2007). However, the use of inhibitors may trigger or target other cellular processes. Thus observations from this type of studies need to be interpreted cautiously (Oehmen et al., 2010).

Genetic engineering or DNA-manipulation methods allow for changing properties and/or understand a protein's function by inactivating or altering its corresponding gene (Yan and Fong, 2017). Site-directed mutagenesis allows targeting a gene and change a small part of it. For example, it would allow changing the affinity of an enzyme to a substrate or a cofactor. CRISPR allows to add, delete or modify complete genes, and it also allows to change gene promoters for higher/lower or controllable expression. With this technique, one could assess the functionality of a protein by adding its gene to a different cell host which does not possess such gene/protein - *heterologous expression*.
To our knowledge, Accumulibacter is still not genetically accessible, but two studies were performed that heterologously expressed genes of Accumulibacter in *E. coli*: polyphosphate kinase (Mcmahon et al., 2002), and acetoacetyl-CoA reductase (Olavarria et al., 2021).

THE *-omes* AND THE *-omics*,
A CELLULAR-SCALE TOP-DOWN APPROACH

"The premise and promise of systems biology has provided a powerful motivation for scientists to combine the data generated from multiple omics approaches to create a more holistic understanding of cells, organisms, and communities, relating to their growth, adaptation, development, and progression to disease." (Pinu et al., 2019)

The past two decades have been marked by accelerated development of *-omics* technologies used to analyse each of the *-omes* (Aizat, Ismail, and Noor, 2018). DNA and RNA sequencing methods are ever more affordable, protein analyses are under fast development and metabolite analyses can detect a broad range of metabolites. Once integrated, these different -omes can provide a snapshot of the cellular state at the moment of sampling in terms of potential functionality, *i.e.* which genes, transcripts, proteins and metabolites are present. However, it is difficult (if not impossible) to extract information on **1)** metabolic network structure - *i.e. connectivity/topology*, **2)** actual activity of each metabolic function and pathway - *fluxome*, and **3)** associated regulatory framework - *interactome* - from -omics datasets.

The use of -omics to study mixed microbial communities is referred to as *meta-omics*. Based on the *in silico* separation of -omes from different organisms, meta-omics have been widely applied to study both natural systems, *e.g.* gut microbiota (Zhang et al., 2019), and industrial processes, *e.g.* biological wastewater treatment (Rodríguez et al., 2015).

1

GENOMICS – *What instructions do cells have in their DNA?*

The genome can be seen as the "book of instructions" of a cell as its DNA encodes (directly or indirectly) all the potential functions - *tools* - that the cell can perform - *use*. These functions are actually performed by proteins, which in turn are encoded in DNA as a sequence of base-pairs (A, T, C, and G). Consequently, the measurement of DNA's sequence does not yield directly (*i.e.* without prior knowledge) the function of the encoded protein, and databases are used for mapping genes to functions – a step known as *annotation*. The more an organism or a specific protein is studied using molecular biology and biochemistry, the more complete is the information in the database and the better is its corresponding gene's annotation. Still, when an organism or a given protein has not yet been studied, annotations may be done by homology with existing information from other organisms. One might be able to identify correctly the function/enzyme encoded by a given unknown gene, but one cannot fully predict the enzyme's affinity to a given substrate, nor its promiscuity, neither how active it will be. For example, in **Chapter 2** we show how a seemingly good annotation by homology can lead to a misleading *posterior* metabolic analysis. Moreover, we may get an idea how a gene will be regulated, but not how the corresponding transcript, protein, and reaction will be regulated. Despite these limitations, an annotated genome is still a very important scaffold to support other -omics analyses and to derive hypotheses for further detailed investigation.

Genomics and genome scale metabolic modeling can be very useful for pinpointing which metabolic routes are available in the genome and that can be potentially used in cellular metabolism. Complemented with transcriptomics or proteomics, genomic information can also be used with the aim to find the regulatory mechanisms that govern metabolism. Thanks to short-read sequencing technologies, whole genome sequencing became cheap and accessible. However, this technology sequences small fragments of few hundred base-pairs obtained from genomes that may be as big as tens of thousands of base-pairs. In a simple analogy, assembling a genome is much like solving a very difficult puzzle with many small pieces, of which multiple might be identical or very similar, and some pieces might even be missing (Baker, 2012).

To facilitate this assembling process, the approach used in the past two decades has been reconstructing genomes based on an already assembled, closed, reference genome (Lischer and Shimizu, 2017). This works much like having the final image of the puzzle and then laying the puzzle pieces on top of the image - *mapping*. The downside of this approach is that if the pieces come from a slightly different genome picture, then some pieces may be discarded or misplaced, thus overlooking new or different genes and corresponding functionalities. In other words, these genome reconstructions tend to be biased towards the reference genome.

Recently developed long-read sequencing techniques have received much attention as they allow solving these puzzles with less and bigger pieces. However, their accuracy is not as good as with short-read sequencing, *i.e.* the bigger pieces are more error prone compared to short-read technologies. A combination of both technologies allows for solving

the puzzle in an easier and more accurate manner (De Maio et al., 2019; Goldstein et al., 2019); thus, it is expected that the number of genomes assembled *de novo* will increase in the future.

There are several metagenome-assembled genomes of Accumulibacter originating from enrichment cultivations with different types and clades (Barr et al., 2016; Camejo et al., 2019; Flowers et al., 2013; García Martín et al., 2006; Mao et al., 2014; Oyserman et al., 2016a; Skennerton et al., 2015). Most genomes are reconstructions from the first closed genome, a clade IIA strain of Accumulibacter (García Martín et al., 2006), except for the most recent published genome, a clade IC strain (Camejo et al., 2019), which has been *de novo* assembled using an hybrid approach of short-reads and long-read technologies, Illumina and Nanopore, respectively.

TRANSCRIPTOMICS – *What instructions are in use?*

While a complete genome of a cell reveals its metabolic capabilities, gene sequences alone do not necessarily tell when or where a gene is "in use". Bacterial cells can adapt to new environments by producing enzymes that allow cells to metabolize the available substrates or toxins present in the new environment. A classic example is the adaption of *E. coli* to a new environment with lactose within a few minutes. This is thanks to the lactose-induced expression of the genes in the *lac* operon leading to the synthesis of enzymes required to consume lactose. This kind of "on demand" enzyme synthesis allows cells to better manage their resources by not wasting them on unnecessary enzyme synthesis.

Transcriptomics has been widely employed to identify and quantify all transcripts of a cell's transcriptome revealing which genes are active in a given environment. The levels of each transcript can be relatively measured by either using reverse transcriptase quantitative PCR, microarrays, and (*next-generation*) RNA-seq. The analysis of gene expression patterns of cells in different or changing environments allows for the study of genetic regulation. Genes with similar expression patterns might be regulated by the same mechanism. Often, such genes are close together in the genome and their sequences are downstream of regulatory motifs that control their expression depending on environmental or cellular cues.

Given the rapid environmental changes imposed to Accumulibacter, several transcriptomics studies have been conducted with the premise that the effective physiology of these bacteria is due to highly dynamic gene expression (Camejo et al., 2019; He et al., 2010; He and McMahon, 2011b; Mao et al., 2014; Oyserman et al., 2016b).
To be noted, it has been observed that about half of the mRNA material obtained from an enrichment of Accumulibacter belonged to these bacteria indicating that this was the most active member in the community (Camejo et al., 2019), thus supporting the validity of studies performed in highly enriched cultures of Accumulibacter by culture-dependent methods. In another high-resolution time series metatranscriptomics study (Oyserman et al., 2016b), the authors clustered expression profiles of highly dynamic genes into several trend categories, which later were associated with EBPR-relevant patterns (*e.g.* "anaerobic

acetate contact", "redox transition", "aerobic"). Two regulatory motifs were identified: one motif upstream of genes upregulated during "anaerobic acetate contact" and another motif upstream of many genes/operons upregulated according to the "redox transition" pattern (*i.e.* transition between anaerobic and aerobic phases). While undoubtedly these findings explain why all these genes are co-expressed in Accumulibacter, the speed with which these cells switch their metabolism upon imposed EBPR-transitions cannot be explained by *de novo* synthesis of enzymes required for each new anaerobic/aerobic phase.

PROTEOMICS – *What tools are available?*

Transcripts may reveal which genes are active in a given moment, but the amount of mRNA can only explain part of the variation in protein abundances (Vogel and Marcotte, 2012). This is, transcripts may indicate how much protein can potentially be produced in the future but tell nothing about the past production of that protein and current levels. Furthermore, proteins can be subject to post-translational modifications such as phosphorylation and glycosylation, which renders them active or inactive. Thus, inferring protein activity from transcript levels can be deceiving. An alternative approach to build a genetic regulatory network based on proteomics has been demonstrated for *S. cerevisiae* in which, for example, co-expression artefacts at the transcript level could be avoided by measuring actual protein abundance changes (Garcia-Albornoz et al., 2020).

Current proteomics techniques are able to identify and quantify changes in proteins in a sample compared to a reference. Proteins are digested into small peptides that are analysed using powerful high-resolution mass spectroscopy devices. Peptide signals are translated into peptide aminoacid sequences either by peptide-spectrum mapping or by *de novo* peptide sequencing. Sequenced peptides are then mapped to translated genomic databases for identification of whole proteins and potentially also for taxonomic classification of the organism from whom those proteins originated (C Kleikamp et al., 2020; Mooradian et al., 2020).

It is important to note that careful sample preparation is needed if the objective is to detect proteins integrated in the membrane of cells due to their inherent characteristics, *e.g.* hydrophobicity, low solubility and low abundance (Yoneten et al., 2019). Typically, if not stated otherwise, proteomics studies are mainly analyzing cytoplasmic proteins.

Proteomics on Accumulibacter enrichments have been performed to investigate changes in their proteome between anaerobic/aerobic phases, but also between different clades (Barr et al., 2016; Wexler, Richardson, and Bond, 2009; Wilmes et al., 2008). Even though the metabolism of Accumulibacter is markedly different between anaerobic and aerobic phases, the observed proteome of Accumulibacter is very similar in both anaerobic and aerobic phases (Wexler, Richardson, and Bond, 2009; Wilmes et al., 2008; Wilmes and Bond, 2006). The stability of the proteins and mRNAs likely allows for these to be present in the next phase even though they were synthesized previously, based on previous *stimuli.* This creates a particular situation in which proteins characteristic from aerobic metabolism

are present anaerobically and *vice-versa*. Thus, to avoid energy wastage by costly protein turnover or by futile cycling due to the operation of opposing pathways, Accumulibacter must regulate their rapid metabolic response to alternating anaerobic/aerobic conditions, either by post-translational modifications of enzymes, allosteric regulation or by simple enzyme kinetic and thermodynamic regulation.

METABOLOMICS – *What raw materials are available to make cells?*

Metabolites are the building blocks of cells, *i.e.* they are the precursors to all the aforementioned macromolecules. Metabolites are also the result of the activity of these macromolecules. These metabolites also play an important role in metabolic regulation, either by directly interacting with enzymes but also indirectly by interacting with proteins involved in regulation at gene expression or protein translation levels. Metabolomics is the analysis of a cell's metabolome and it elucidates what is the cellular functional state, *i.e.* phenotype. Since metabolites are small molecules with vastly different physicochemical properties, identifying and quantifying all these substances is the main challenge of metabolomics, requiring many different analytical tools. Coupled methods are typically used to analyse metabolites, which are composed by a separation method, *e.g.* liquid (LC) or gas chromatography (GC), followed by a detection method such as mass spectrometry (MS) or nuclear magnetic resonance (NMR).

Metabolomics experiments can be either targeted or untargeted: Untargeted is a discovery-based method used for global detection and relative quantification of metabolites in a sample. Due to the diverse physicochemical properties of metabolites, sample preparation and analytical methods determine the subset of metabolites detected. Furthermore, unlike in proteomics, measured properties (mass-to-charge ratio and retention time) may not correspond to a unique metabolite. Validation of retention times and MS/MS fragmentation data using reference standards is often required for confident metabolite identification (Schrimpe-Rutledge et al., 2016). This validated approach is known as targeted metabolomics and it allows for absolute quantification. Again, the sample processing method and measurement device differs per group of targeted metabolites. For absolute quantitative analysis of intracellular metabolites, a method has been developed for the rapid quenching of metabolism, extraction of metabolites from cells, and subsequent quantification based on isotope dilution mass spectrometry, ID-MS (Mashego et al., 2004; Wahl et al., 2014; Wu et al., 2005). This method makes use of ^{13}C labeled cellular extracts as internal standards to account for losses during the sampling processing and minimize matrix effects during measurement. However, the method still requires adjustment for different organisms under different conditions (Canelas et al., 2008; Canelas et al., 2009; Lameiras, Heijnen, and Gulik, 2015; Taymaz-Nikerel et al., 2009; Walther et al., 2010).

To date, and to our knowledge, there is no metabolomics study performed with Accumulibacter enrichments. One possible reason is that metabolites are not species-specific therefore it is not possible to discern the source of each metabolite when analyzing a sample of a microbial community. Nevertheless, in an enrichment where Accumulibacter

comprises the vast majority of organisms, one can assume most metabolites measured originate from Accumulibacter. Such analysis will add another layer to the knowledge of these microorganisms; knowing the metabolite pool sizes during the anaerobic/aerobic phases allows for calculating mass-action ratios and detect putative regulatory mechanisms at metabolite or thermodynamic-level responsible for Accumulibacter's phenotype, such as performed previously, for example, for *S. cerevisiae* (Suarez-Mendez, 2015).

FLUXOMICS – *What transformations are occurring in cells?*

When regulation of metabolic pathways is required to be faster than protein turnover, the activity of enzymes can be fully or mildly activated or deactivated. Regulatory mechanisms in bacteria known to date include **1)** post-translational modifications (PTM) such as caused by the activity of isocitrate dehydrogenase kinase/phosphatase in the presence of glucose (LaPorte, Walsh, and Koshland, 1984), **2)** allosteric regulation as the inhibition of FBPase by ADP and G6P (Hines et al., 2007) or **3)** by inhibition directly at the active site such as postulated for β-ketothiolase in presence of high levels of free CoA (Senior and Dawes, 1973). The enzymes catalyzing the reactions of the central carbon metabolism of Accumulibacter seem to be present at all times according to several metaproteomic and targeted enzymatic studies and changes in metabolite levels - *metabolome* not always allow to derive conclusions on the intensity and direction of metabolic fluxes - *fluxome*. Fluxomics is the next approach to decipher what changes in Accumulibacter's metabolism in changing environments.

Fluxomics allows distinguishing metabolic fluxes through parallel pathways, *i.e.* different sets of reactions that use the same substrate and lead to the same product (Sonntag et al., 1993). It also allows to quantify flux through internal cycles, in particular, it allows to identify futile cycles that waste energy (Heerden et al., 2014).

Metabolic fluxes cannot be directly measured. In formal ^{13}C metabolic flux analysis, fluxes are estimated computationally from absolute quantification of metabolite concentration - *targeted metabolomics* - combined with the ^{13}C enrichment in time of metabolites upon stable isotope tracing using ^{13}C-labelled substrates (Wiechert et al., 2001). In a comprehensive review of ^{13}C fluxomics workflows, Niedenführ and colleagues discussed that such workflows are complex and difficult to standardize as these vary depending on the biological research aim, and on the experimental, analytical and modeling tools used (Niedenführ, Wiechert, and Nöh, 2015). Fluxomics has been applied to cells cultivated in both stationary and dynamic state. Sampling can be done in a single time-point or in a time-series. The proportion of ^{12}C-to-^{13}C in the substrate can change suddenly - *pulse* - or gradually in time or it can be constant throughout the whole experiment. Despite all the complexity and possible variations in fluxomics, qualitative interpretation of ^{13}C labelling patterns can provide information on the activity of one pathway relative to another, alternative metabolic routes and contribution of different nutrients to the production of each intermediate (Buescher et al., 2015).

This simple interpretation of ^{13}C labelling patterns is used in **Chapter 4** to identify carbon flow changes in the central carbon metabolism of Accumulibacter upon feast/famine and anaerobic/aerobic switches. These dynamic conditions select for highly adaptable and flexible organisms, whose storage metabolism allow for increased competitiveness (Van Loosdrecht, Pot, and Heijnen, 1997). The trade-off of this strategy is the relatively low growth yield but has the advantage that they are able to compensate for short-term disturbances in a cycle or a day (Maurer et al., 1997). Furthermore, the ability to, very quickly, switch around entire pathways from growth to storage and *vice-versa*, such as glycolysis to/from gluconeogenesis, entails a very interesting regulatory challenge, as parallel activity could lead to significant losses. Under investigation are the distinction of different flux modes through TCA cycle reactions, identification of putative futile cycles and identification of regulatory checkpoints. As mentioned earlier, previous stable isotope tracing experiments only assessed the positional labelling pattern imprinted in the storage polymers (glycogen, PHB and PHV) but did not analyze any intermediates in between, neither the *short-term* dynamics when switching from feast/famine and anaerobic/aerobic conditions.

INTERACTOMICS
– What are the interactions between the different cellular parts?

As mentioned earlier and depicted in Figure 1.2, all DNA, RNA, proteins, and metabolites can be found inside the cell and they interact with each other. Interactomics is the field looking at these interactions to understand how the cell responds and adapts to internal and environmental cues. As an example, recently a study was published which captured the proteome of *Saccharomyces cerevisiae* grown in different carbon sources and used a data-driven approach to derive an interaction-based network connecting transcription factors and chaperones involved in the response to changes in growth conditions (Garcia-Albornoz et al., 2020).

Despite not having been explored in this dissertation, such approach could be used to learn how Accumulibacter reorganize themselves to be able to cope with the longer-term effects of, for example, different carbon sources, seasonality, or (un)availability of specific ions and nutrients.

1

INTEGRATION OF MOLECULAR BIOLOGY & BIOCHEMISTRY WITH *-omics* INTO MATHEMATICAL MODELS

The success of systems biology is strongly dependent on the true integration of experimental observations and the development of mathematical models, which require iterative validation and refinement. (Abram, 2015)

After conducting experiments and collecting data, comes a moment for analysis and interpretation. If a problem is small, a Human brain is well capable of piecing together the different parts of the puzzle, actually creating a mental model that guides thoughts from problem to solution(s). Biological problems are big and complex and while several Human brains are needed to tackle them, they are not sufficient. Mathematical models can thus be seen as the written/formalized version of a mental model, which can scale and be solved computationally.
Models, regardless of their type and shape, are always simplifications of reality. They serve to understand complex systems and to, some extent, predict behavior and outcomes from given inputs. Biological models can be built for many different purposes like **1)** understanding the physiology or metabolic behavior of a cellular system in a given environment to **2)** better design or control processes and experiments of interest, which in turn generate **3)** data that can be again used to better understand the cellular system. Modeling is, in fact, an iterative process.
Models can be classified according to the amount/level of knowledge used to compute an outcome from given inputs: black-box, gray-box and white-box (Figure 1.5).

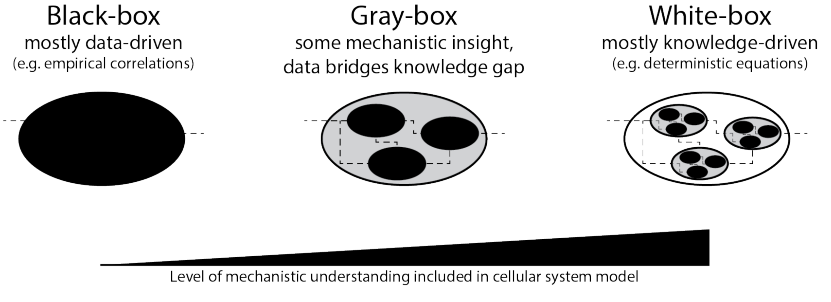

Figure 1.5: Black, gray and white-box models. Adapted from Smolders, 1995

A cell system can be modelled as a black-box where substrates are converted into products but internal processes are not explicitly modelled. This approach relies mostly on data to be calibrated. A gray-box approach would mechanistically describe some internal processes; however, each process would still be a black-box. For example, each different pathway is modelled as a lumped reaction instead of all the individual reactions involved.

A level further, in a "whiter" approach, each reaction in a pathway is modelled individually, but the regulation mechanisms involved in each reaction are not explicit. The level of description of the model can increase further until all mechanisms are modelled explicitly using first-principle physical and chemical rules in a white-box model.
White-box models are mostly hypothesis-driven and comprise explicitly written equations and relations such as ALEs (*e.g.* mass and energy conservation laws), ODEs, PDEs, or even stochastic equations. On the other hand, black-box models are mostly data-driven and make use of patterns extracted from large datasets to predict outcomes from given inputs. Techniques commonly used fall under the "umbrella" of machine learning techniques such as linear regression, ensemble learning, clustering, dimensionality reduction, neural networks, and more. A gray-model combines both hypothesis and data-driven approaches in a hybrid approach, which combines the advantages of both model types while minimizing their drawbacks. Metabolic models are typically gray-box models, using hypothesis-driven approaches to describe macro-processes, *i.e.* cell's surrounding and major pathways, that change considerably in combination with a data-driven approach to describe micro-processes, *i.e.* enzyme or metabolites activities, captured in extensive experimental testing.

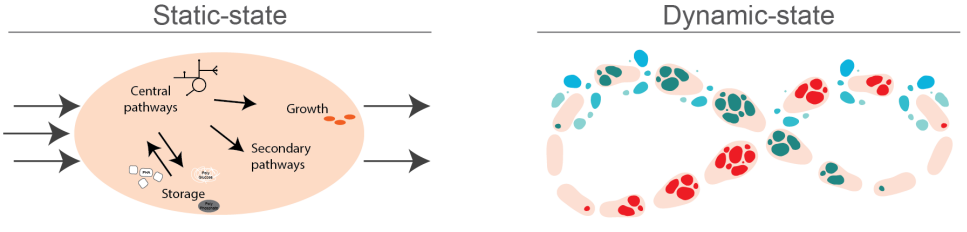

Figure 1.6: Cell systems can be modeled either at steady, constant conditions or under dynamic, changing conditions.

The complexity of metabolic models can range from stoichiometry, through functional, kinetic, and all the way to regulatory descriptions of cellular metabolism. Metabolic models can also be classified in either static or dynamic models (Figure 1.6). Static models are used to study the physiology of cells under (*pseudo*) steady-state conditions such as predicting the metabolic strategy in presence or absence of substrate or oxygen. On the other hand, dynamic models are used to study cellular mechanisms under dynamic conditions, such as the gradual adaptation of cells to a new environment that typically involves regulatory mechanisms and gradual changes in enzyme kinetics. While stoichiometric models are relatively easy to scale up to a genome-wide explicit description, the more complex mechanistic kinetic and regulatory models needed to describe systems in dynamic conditions are still challenging to scale.

STOICHIOMETRIC METABOLIC MODELS

For the past two decades, stoichiometric modeling has been mostly based on the constraint-based modeling (CBM) approach (Heirendt et al., 2019; Kauffman, Prakash, and Edwards,

2003; Lewis, Nagarajan, and Palsson, 2012). This method calculates metabolic fluxes from reactions' stoichiometry and intracellular metabolites' mass balances using simple algebraic linear equations (Figure 1.7). Imposed constraints on reaction flux values can be based on thermodynamic considerations (*e.g.* irreversibility of some reactions) and eventually biological or experimental information.

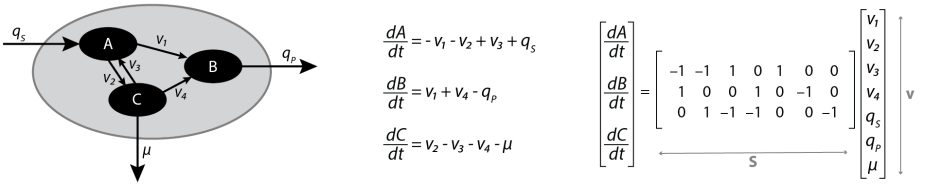

Figure 1.7: Simple model consisting in three metabolites (A, B, and C) with four reactions (v_i) and three exchange fluxes (q_j). The amount of A, B and C is described in time through mass balance equations. This system of ordinary differential equations can also be rewritten in matrix form. At steady state, the amounts of A, B and C do not change in time, thus $S \bullet v = 0$ and the system gets reduced to an algebraic linear equations system. Adapted from (Kauffman, Prakash, and Edwards, 2003).

The implementation of this modeling approach is relatively simple and there are several software packages readily available, such as COBRA (Heirendt et al., 2019), CellNetAnalyzer (Klamt et al., 2007), CBMPy (Boele, Olivier, and Teusink, 2012), Escher-FBA (Rowe, Palsson, and King, 2018), COPASI (Hoops et al., 2006), or JWS Online (Olivier and Snoep, 2004). CBM approaches can be applied to small *lumped* metabolic reaction networks up to genome-scale metabolic networks that include thousands of metabolites and reactions (Figure 1.8). In the past two decades, many genome-scale metabolic models (GEMs or GSMMs) have been developed for organisms across bacteria, archaea, and eukarya (reviewed in (Gu et al., 2019)). Despite the many efforts to automatically generate these large networks from genomic data, these still require manual curation to correct for stoichiometric inconsistency, cofactor dependency of certain enzymes, missing reactions, inadequate biomass reaction stoichiometry, among others. This is directly related with the quality of annotation of a sequenced genome, which greatly depends on the knowledge available on databases for the sequenced organism. Thus, for lesser known organisms like Accumulibacter, building such a genome-scale network still requires substantial manual curation. These endeavors will become simpler and more efficient as joint community efforts have been focused on developing standardized quality control tools for assessing genome-scale metabolic models (Mendoza et al., 2019) and version-control strategies that allow multiple modelers to curate different parts of one model simultaneously, *e.g.* MEMOTE (Lieven et al., 2020).

Different simple, lumped stoichiometric models have been developed to describe the metabolism of PAO (Arun, Mino, and Matsuo, 1988; Comeau et al., 1986; Mino, Tsuzuki, and Matsuo, 1987; Smolders et al., 1994b; Smolders et al., 1994a; Wentzel et al., 1986). For example, the models of Smolders can describe well the stoichiometry of Accumulibacter in laboratory-scale reactors, however their extrapolation to full-scale plants is limited. In full-

scale, substrates other than acetate may be present and in time-varying quantities, either coming from influent wastewater or from other microorganisms present. Furthermore, the metabolic flexibility of PAOs to switch between PAM and GAM is also not taken into account in this model since the lumped reactions predetermine the pathways used. These features were implemented later in the stoichiometric part of kinetic models: **1)** Propionate as carbon source (Oehmen et al., 2007b), **2)** effect of different carbon sources, pH and temperature (Lopez-Vazquez et al., 2009), and **3)** PAM-GAM switching (Acevedo et al., 2014). While the pathways needed for these phenotypes are contemplated in these models, the flux distribution between glycogen/polyphosphate storage, and growth still needs to be calibrated using experimental data, *i.e.* mechanistic understanding of what determines the flux towards these processes is still missing.
To our knowledge, to date there has been only one stoichiometric constraint-based model with over 300 reactions developed for EBPR metabolism, now known to be associated to Accumulibacter (Pramanik et al., 1999). This model used experimentally measured time-dependent profiles of glycogen, PHA and polyphosphate as inputs to predict intracellular metabolic fluxes assuming balanced growth and intermediates concentrations at steady-state using the dFBA approach. They also pre-defined the optimal metabolic strategy by minimizing/maximizing the different storage polymers according to what is expected throughout an EBPR cycle. It is important however to note that this model was built before the metagenome of Accumulibacter was available, thus it comprises a generic bacterial metabolic network presumed to match that of Accumulibacter. Furthermore, redox and energy cofactors used were assumed by homology with other organisms and **Chapter 2** explains thoroughly how this impacts metabolism and, by extension, metabolic analyses.

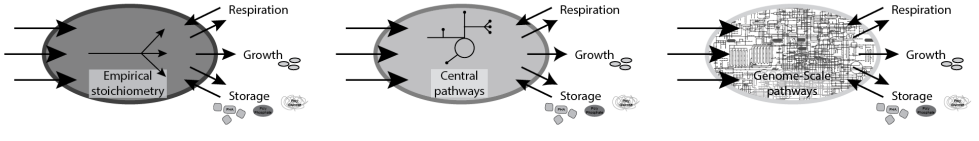

Figure 1.8: Connection between substrates and products involved in PAO metabolism. Unfortunately, measurement of these initial/end metabolites is not enough to distinguish between different metabolic routes.

The different types of constraint-based approaches are summarized in Table 1.1 are only some among the multitude of different formats for the optimization objective functions and constraints imposed on the model. Promising approaches integrating constraint-based modeling with large -omics datasets are now becoming available (reviewed in (Rana et al., 2020)). This integration improves the accuracy of constraint-based models by further constraining the space of feasible metabolic flux distributions based on experimental data. Successful approaches so far have included transcriptomic data (reviewed in (Machado and Herrgård, 2014)), proteome information (Bekiaris and Klamt, 2020; Sánchez et al., 2017), metabolomics (Niebel, Leupold, and Heinemann, 2019; Salvy et al., 2018; Soh and Hatzimanikatis, 2014) and even multi-omics (Hadadi et al., 2020; Salvy and Hatzimanikatis, 2020). Furthermore, by systematically integrating -omics data in these types of models, knowledgebases can be assembled for each organism, providing scientists with a living platform to generate and test hypotheses (Lu et al., 2019).

Table 1.1: Different types of constraint-based modeling approaches.

Metabolic Flux Analysis (MFA) When the number of independent measurements available is superior or equal to the number of degrees of freedom of the algebraic system of equations, then all remaining unknown metabolic fluxes can be simply calculated by solving the determined system of equations. This is the simplest type of constraint-based models. Examples:

- Anaerobic growth of yeast (Noorman, Heijnen, and Ch. A. M. Luyben, 1991)
- PAO-Anaerobic (Smolders et al., 1994b) and PAO-Aerobic (Smolders et al., 1994a)

^{13}C Metabolic Flux Analysis (^{13}C-MFA) When the network contains parallel reactions or pathways (internal cycles), not all fluxes can be determined directly from extracellular measurements. These fluxes can be solved by performing ^{13}C-isotope labelling experiments and by measuring the different fractions of labeled metabolites with mass spectrometry or NMR (Wiechert et al., 2001; Wittmann and Heinzle, 1999). This method requires advanced experimental procedures and statistical techniques to estimate internal fluxes from data. Examples:

- Anaplerotic node of *C. glutamicum* (Petersen et al., 2000; Winden et al., 2001)

Flux Balance Analysis (FBA) When systems are underdetermined because the number of independent measurements is smaller than the degrees of freedom of the network, optimization is applied to find a solution (Figure 1.7). This is typically the case when genome-scale networks are analyzed. These networks are extensively used for metabolic engineering applications such as yield or knockout predictions with the aim to optimize for the production of a metabolite of interest or minimize an undesired by-product. Models can be further constrained by the uptake rate of the carbon source (*e.g.*, glucose), irreversibility constraints (thermodynamics) and some limited reactions due to regulation/knockouts or low enzymatic activity. For example, proteome information can be used to indirectly set maximum fluxes based on the corresponding protein levels. Examples:

- *E. coli* (Orth, Thiele, and Palsson, 2010)
- *S. cerevisiae* (Sánchez and Nielsen, 2015; Gulik and Heijnen, 1995)

Flux Variability Analysis (FVA) This approach is complementary to FBA. If the system still has degrees of freedom after setting all constraints and optimization targets, then the obtained solution will not be unique. A solution space can be obtained by evaluating the minimum and maximum range of each flux that can still satisfy the constraints (Gudmundsson and Thiele, 2010).

Elementary flux mode analysis (EFM) When insufficient kinetic knowledge is available to constrain fluxes, another approach is to perform a computationally expensive calculation of all possible unique and minimal pathways that allow steady-state metabolic fluxes in the network (Klamt et al., 2007). The physiological state of a cell is then a weighted linear combination of elementary modes. This analysis also allows comparing different pathways in terms of their efficiency and without having to rely on *a priori* assumptions on kinetics. Examples:

- *E. coli* (Carlson, 2009)

Iterative FBA and Dynamic FBA (dFBA) Beyond a static FBA approach, a batch process or changing environments can be modeled as succession of steady state FBA simulations: constrained fluxes change from one simulation to another, while intracellular metabolite amounts remain in steady state. In essence, it predicts the optimal flux distribution for the given constraints in each single time interval but does not accurately predict metabolic transitions where regulatory processes take time (*e.g.* lag phase). Examples:

- *E. coli* (Mahadevan, Edwards, and Doyle, 2002; Varma and Palsson, 1994)

Thermodynamics-based Flux Analysis (TFA, TMFA, NET analysis) Often in FBA the flux distributions produced are thermodynamically infeasible. In TFA, thermodynamic constraints are set to further constrain the model and reduce the feasible solution space to thermodynamically feasible flux distributions (Henry, Broadbelt, and Hatzimanikatis, 2007; Kümmel, Panke, and Heinemann, 2006). Moreover, this approach can also be used for systematically checking metabolite data sets for consistency, or for resolving metabolite concentrations in different cellular compartments based on cell-averaged metabolome data.

Flux balance analysis with macromolecular crowding (FBAwMC) Metabolic fluxes in cells are limited by the available enzymatic capacity. In this approach, a global capacity constraint on the total cellular volume is imposed, thus presuming metabolic enzymes are limited by the available cellular space (Minton, 2001). In a situation of elevated macromolecular crowding, bigger (or higher mass) enzymes will be more penalized than smaller ones, thus resulting in metabolic shifts (*e.g.* overflow metabolism). Examples:

- Acetate overflow in *E. coli* (Beg et al., 2007; Vazquez et al., 2008)
- Crabtree effect in *S. cerevisiae* (Nilsson and Nielsen, 2016; Hoek and Merks, 2012)
- Warburg effect in cancer cells (Shlomi et al., 2011)

Metabolism and protein expression models (ME) Here the catalytic constraints are approximate stoichiometric relationships between enzyme abundance and catalyzed flux (Lerman et al., 2012; O'Brien and Palsson, 2015). Metabolic reactions and all processes required for the synthesis of functional proteins are also included in the network, including transcription rates of their respective genes. Consequentially, the feasible and optimal proteome and transcriptome of a cell is also computed. Examples:

- *E. coli* with limited enzyme capacity at high specific growth rates (O'Brien et al., 2013)

Resource balance analysis (RBA) Based on the mechanistic self-replicating model (Molenaar et al., 2009), this stoichiometric approach models explicitly the resource investment needed to produce ribosomes that produce enzymes, which catalyze metabolic reactions that in turn produce precursors for ribosomes, enzymes and other cellular macromolecules. This approach accounts for (individual) protein limitations by setting fluxes with hard constraints ($v = k_{cat} \times [E]$) based on apparent catalytic rates estimated from experimental data. Examples:

- *B. subtilis* (Goelzer et al., 2015)
- *E. coli* (Bulović et al., 2019)

MECHANISTIC METABOLIC MODELS

Stoichiometric models can provide insight on metabolic organization and optimal metabolic strategies for given environments, especially when combined with experimental efforts, either biomolecular, biochemical or -omics. However, they are only a step towards mechanistic understanding of a cellular system. Mechanistic models are thus needed to describe, for example, how a cellular system adjusts its machinery to keep itself stable and functional in either stable or changing environments.

Mechanistic enzyme kinetics modeling is widely used in systems biology to study dynamics of metabolism. A schematic explanation of this modeling approach can be found in Figure 1.9. The implementation of this approach requires initial value problem solvers for ODEs that are built-in MATLAB or can be found in the SUNDIALS suite (*e.g.* CVODE). These solvers are the cornerstone of many of the software packages readily available, such as JWS Online (Olivier and Snoep, 2004), PySCeS (Olivier, Rohwer, and Hofmeyr, 2005), COPASI (Hoops et al., 2006), ScrumPy (Poolman, 2006), AMIGO2 (Balsa-Canto et al., 2016), modelbase (Ebenhöh et al., 2018), or Tellurium notebooks (Medley et al., 2018). These different tools can be systematically evaluated and compared using benchmark problems gathered by the research community (Villaverde et al., 2015). To facilitate exchange and reusability of the developed models, the model parameters, processes, and kinetics can be described using the Systems Biology Markup Language (SBML) standards (Hucka et al., 2018). Associated simulation experiments can be encoded using the Simulation Experiment Description Markup Language (SED–ML) (Waltemath et al., 2011). Once in a standard format, models can be universally shared in BioModels, a repository for mathematical models, where models are curated, by the modeling community, to assess the quality of their description of biological processes and their reproducibility (Malik-Sheriff et al., 2019).

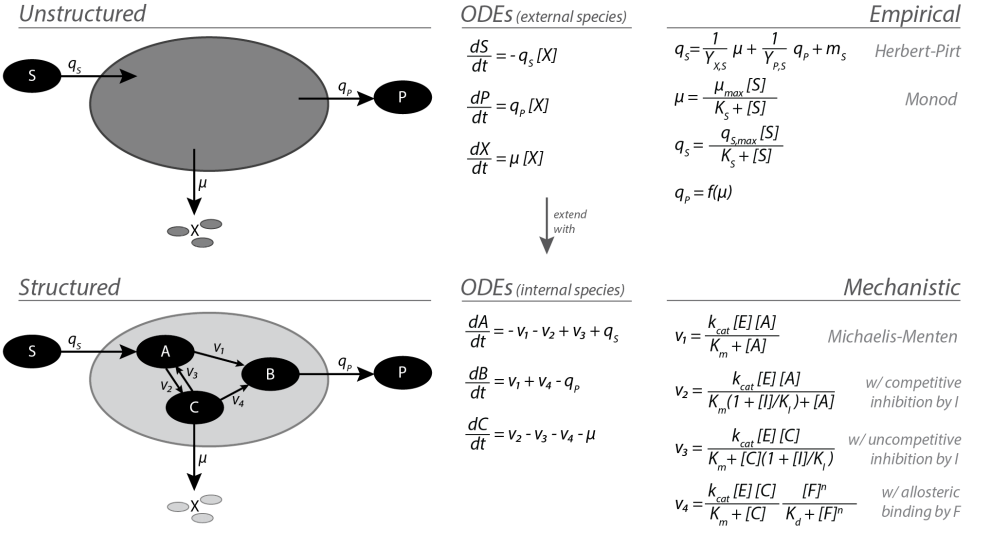

Figure 1.9: Examples of mechanistic metabolic models. **Top:** a "darker" *unstructured* gray-box metabolic model based on empirical relations. The amount of S, P and X is described in time by ordinary differential equations (ODEs) using empirical reaction rate relations obtained from experimental data. **Bottom:** a "lighter" *structured* gray-box metabolic model based on enzymatic kinetic mechanisms. In addition to the ODEs describing S, P and X in time, also the intracellular amounts of A, B and C are described in time through mass balance equations. Examples of different enzymatic reaction rate equations are shown. Please note that in both types of models, equations are composed of state variables (*i.e.* time-varying concentrations) and parameters. A challenging part of creating these models is their calibration, *i.e.* parameter estimation, which requires extensive amounts of data gathered from adequately designed experiments.

A selection of dynamic modeling approaches and respective examples are summarized in Table 1.2. Additional approaches are reviewed in (Ewald, Bartl, and Kaleta, 2017; Vasilakou et al., 2016).

Table 1.2: Different types of dynamic modeling approaches and respective examples.

Mechanistic enzyme kinetics models These models are based on time-varying description of metabolic processes through mechanistic insights on enzymatic kinetics (Figure 1.9). These kinetic models are able to describe the environmental conditions they were calibrated in but still depend on extensive re-calibration thus cannot predict the outcome of a new environmental condition. Examples:

- *S. cerevisiae* (Rizzi et al., 1997)
- *E. coli* (Millard, Smallbone, and Mendes, 2017)
- *P. chrysogenum* (Tang et al., 2017)
- Photosynthesis in plants (Matuszyńska, Saadat, and Ebenhöh, 2019)
- PAOs/GAOs (Santos et al., 2020; Smolders et al., 1995)
- PHA-AOs (Marang, Loosdrecht, and Kleerebezem, 2015).

Metabolic control analysis (MCA) MCA builds upon a sensitivity analysis: It describes the impact of each kinetic parameter on the pathway flux(es) within a metabolic network (Fell, 1992; Hofmeyr; Jan-Hendrik S., 2001). In other words, it allows to determine, quantitatively, the degree of control that a given enzyme activity exerts on the pathway flux and on the concentrations of metabolites at steady state. Examples:

- *E. coli* (Chassagnole et al., 2001)
- Lactic acid bacteria (Christensen, Hofmeyr, and Rohwer, 2018; Hoefnagel et al., 2002)
- Calvin-Benson cycle in plants (Arnold and Nikoloski, 2011)

Dynamic ^{13}C MFA In this approach, the explicit enzyme kinetics are replaced by piecewise linear functions obtained from simplified mass balances describing the ^{13}C-molar labeling content of metabolite pools (Abate, Hillen, and Wahl, 2012). This approach alleviates the difficult estimation of highly correlated kinetic parameters commonly experienced in explicit enzymatic kinetic functions. It also does not require that the mechanics of the reaction are known. This is particularly important for larger networks or less characterized organisms where such detailed knowledge is not yet available. In a nutshell, this is a hybrid approach that allows for gathering data-driven insights on cellular regulation upon changing environments without having the need for extensive prior knowledge on the system. Examples:

- *P. chrysogenum* (Jonge et al., 2014; Zhao et al., 2012)
- *S. cerevisiae* (Schumacher and Wahl, 2015; Suarez-Mendez, 2015; Heerden et al., 2014)

Self-replicating models Cell systems can be regarded as autocatalytic, self-replicating systems, *i.e.* a cell can produce from raw materials all the components required to replicate itself (Berkhout et al., 2013; Molenaar et al., 2009). In this modeling formalism, equations are used to describe in time the variation of metabolic intermediates, enzymes, ribosomes required to produce such enzymes, and other non-catalytic macromolecules. In this way, the model can balance the costs and benefits of the macromolecules that are produced together with the contribution of those macromolecules to the overall metabolism. Here, the total amount of macromolecules is constrained according to the concept of molecular crowding. The extracellular environment is assumed to be constant, thus only intracellular dynamics are taken into account. This leads to an increased number of equations and respective parameters, thus aggravating further the problem of adequate data availability for parameter estimation. The simplification of this approach by linearization is known as resource balance analysis (RBA) and it can be used to model a cell under static conditions.

Dynamic enzyme-cost FBA An extension of the self-replicating models with a detailed description of the costs of enzymes, both in terms of building blocks (aminoacids) and in terms of energy needed for polymerization (Waldherr, Oyarzún, and Bockmayr, 2015).

Conditional FBA cFBA integrates several approaches described above such as, FBA, dynamic FBA, self-replicating models, dynamic enzyme-cost FBA, resource balance analysis. In this approach, stoichiometric and mechanistic models are combined to estimate optimal phenotypes in changing, periodic environmental conditions (Rügen, Bockmayr, and Steuer, 2015). In this cFBA approach (Figure 1.10), the cell is regarded as an autocatalytic system where enzymes and ribosomes are explicitly modelled. Alike in traditional FBA, metabolites are assumed to be time invariant $S \bullet v = 0$ (*i.e.* static). Metabolic fluxes (v) are constrained by a simplified enzymatic kinetic function that takes the time-dependent amount of available enzyme and its respective time-invariant catalytic capacity (roughly estimated). Macromolecules such as DNA/RNA, enzymes, other non-catalytic proteins, and storage polymers are explicitly modeled and are considered time-dependent (*i.e.* dynamic). While the enzymes and storage polymers are actively engaged in reactions throughout the cycle, the remaining compounds are inert, and their synthesis is enforced by setting a so-called quota that scales with biomass synthesis. Lastly, biomass composition is constrained to be the same at the end and start of a cycle, thus simulating a cyclic system in steady state. Examples:

- Cyanobacterial growth in day/night cycles (Faizi et al., 2018; Reimers et al., 2017; Rügen, Bockmayr, and Steuer, 2015)

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Wastewater treatment plants are good examples of changing environments with non-continuous supply of carbon and energy sources. Not only do microorganisms experience cyclic variations inherent from how these processes are established, they also experience daily, weekly or even seasonal fluctuations (Maurer et al., 1997). Various gray-box models for Accumulibacter have been developed in the past decades to support the design and optimization of biological nutrient removal processes in wastewater treatment (compared and discussed in (Santos et al., 2020)). These models combine stoichiometry with kinetics to simulate time-variant concentrations of extracellular compounds and intracellular storage polymers. The stoichiometry can be derived from empirical Herbert-Pirt relations or from knowledge on metabolic pathways. The kinetics are based on empirical Monod-type growth relations and on Michaelis-Menten-type kinetic equations. Typically, these models have different sets of stoichiometric relations and kinetic equations to describe each metabolic phase: anaerobic feast/famine, aerobic, and assume an on/off switch between phases. Furthermore, knowledge on what influences the flux distribution through different pathways towards PHAs, glycogen, polyphosphate and growth is still very limited, thus the description of these processes is still data-driven and requires extensive calibration to different environmental conditions (Murnleitner et al., 1997; Santos et al., 2020).

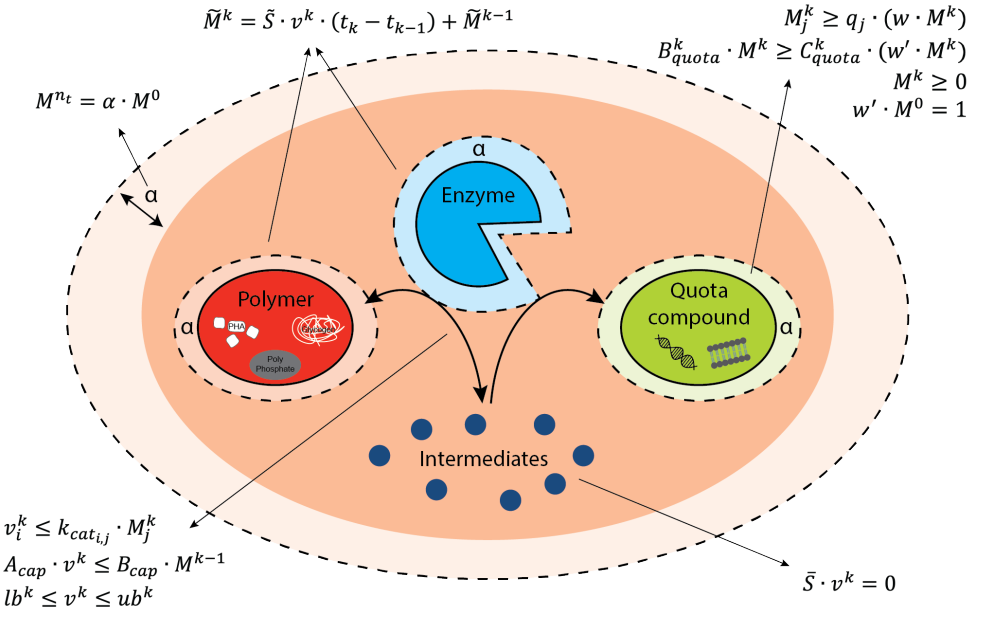


Figure 1.10: Schematic representation of the model structure of cFBA. A detailed explanation of these equations can be found in (Rügen, Bockmayr, and Steuer, 2015). In comparison, traditional FBA only balances intermediates assuming their amount is time invariant ($S \bullet v = 0$).

In **Chapter 3**, the conditional FBA modeling approach was used to study cellular growth in a periodic environment such as in a wastewater treatment process. Conditional FBA (cFBA) uses as optimization objective the *"maximization of biomass synthesis over a full cycle, constrained by the conditional interdependences of cellular metabolism"* (Rügen, Bockmayr, and Steuer, 2015). This allows to model the whole anaerobic/aerobic cycle without having to explicitly set the different cellular objectives in the different phases of the cycle as it would be needed in conventional FBA. The output of this approach is a prediction of the *dynamic* phenotype that has the highest fitness for the set cyclic environment. Specifically, for Accumulibacter, it mechanistically describes the optimal investment in storage polymers cycling, *i.e.* time-variant flux distribution through different pathways, needed to guarantee the survival and competitiveness of these bacteria in different environmental conditions.

While the approaches presented here are focused on describing a single cell type in changing environments, it is noteworthy to mention there are also several examples of modeling efforts invested into describing different functions in microbial ecosystems, *i.e.* multiple different cell types sharing the same environment (reviewed in (Succurro and Ebenhöh, 2018)). In this recent review, the authors present examples of modeling methods based on biochemical knowledge capturing multiple scales to understand emergent patterns in ecosystems and their dynamics. However, the authors defend that current models can achieve a much higher predictive power through simplicity and scalability, but also by recognizing possible synergies from integrating meta-omics in the efforts of unravelling the complex network of interactions in microbial ecosystems. For example, the reconstruction of community-level metabolic networks can be driven by metagenomic data to overcome the challenge of describing species that cannot yet be cultivated axenically (*i.e.* in isolation) (Henry et al., 2016).

1

RESEARCH AIM

In the past decades, both engineers and microbiologists have applied these experimental and computational approaches to increase understanding of the physiology of Accumulibacter. Robust protocols to obtain Accumulibacter enrichment cultures are established, but isolation of these bacteria has not been achieved so far. Still, an incredible amount of knowledge has been collected throughout the past four decades. However, the pathways used and their regulation under the different imposed conditions remain under discussion.

The aim of this research is to advance the *mechanistic* understanding of the functionality and regulation of storage and central carbon metabolism of the PAO Accumulibacter under dynamic conditions.

In the case of Accumulibacter, we would like to identify:

- which pathways are used to (re-)cycle glycogen, PHA and polyphosphate and how do they interact with respect to redox cofactors (re-)cycling - ***structure***;
- when are these pathways active and in which direction are they operating - ***function***; and
- what mechanisms control how these pathways operate - ***regulation***.

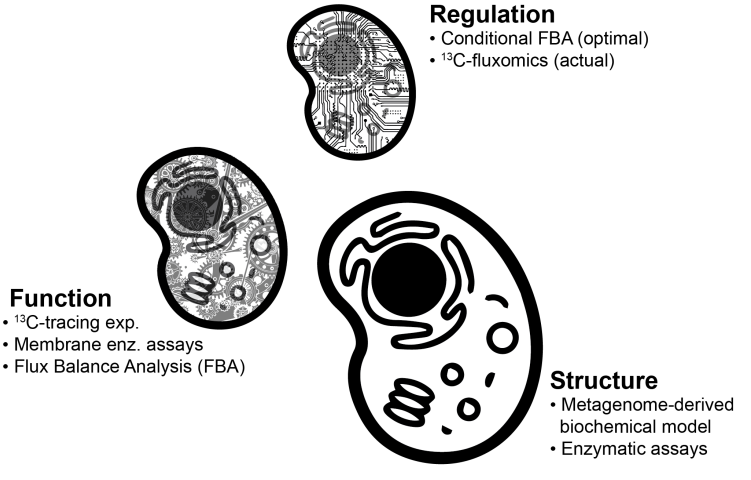


Figure 1.11: Theoretical and experimental methods were integrated to investigate different levels of the cellular system. While the bacterial community member Accumulibacter was the cellular system example studied here, the approaches here developed and applied can also be applied to other cell systems and communities exposed to changing environments.

OUTLINE OF THIS DISSERTATION

This dissertation contains six chapters that deal with the research aims stated above. In this introductory **Chapter** 1, a review containing both bottom-up, top-down and modeling approaches of systems biology are presented. It is also presented how these have contributed and can still be used to further advance the understanding of the metabolism of Accumulibacter under the fluctuating conditions applied at wastewater treatment processes. Given the iterative nature of a systems approach (Figure 1.12), the order of chapters 2 through 5 is not necessarily linear. Despite the effort made such that each chapter can be read independently, they are in fact complementary and together they give a superior view on the current systems understanding of Accumulibacter.

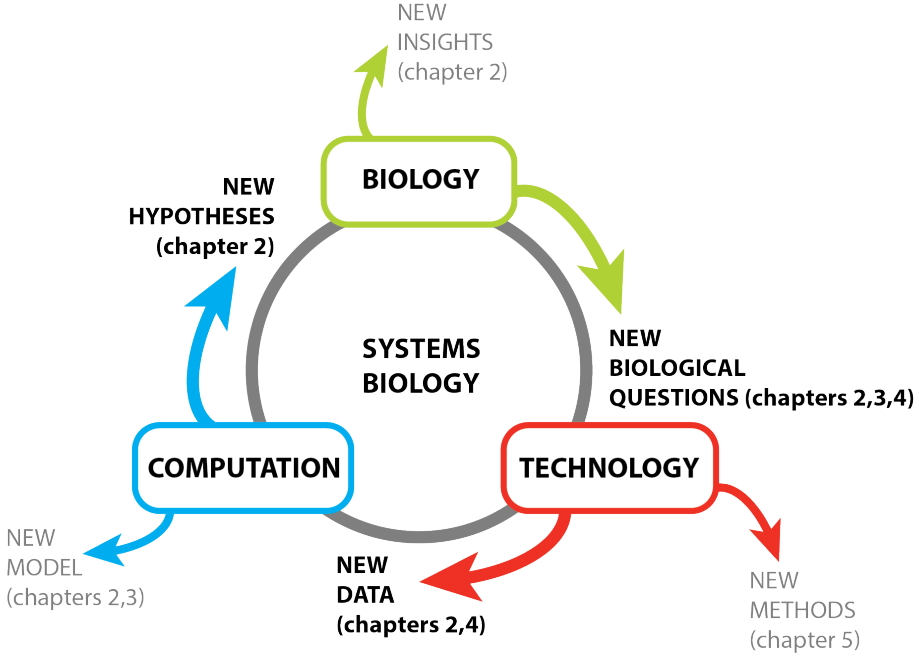


Figure 1.12: Outline of this dissertation from a systems biology perspective.

The central carbon pathways used under anaerobic conditions by Accumulibacter were reviewed in **Chapter** 2 and the identified knowledge gaps were elucidated using enzymatic assays - *structure*. With the obtained biochemical insights, an updated metabolic network was assembled to support a traditional flux balance analysis to study the different metabolic modes possible anaerobically given a defined proportion of acetate-to-glycogen consumed - *function*.

In **Chapter 3**, a conditional flux balance analysis modeling framework was used to simulate the optimal energy allocation strategy, *i.e. function*, employed by Accumulibacter when subjected to the defined fluctuating conditions under which they are selected (oxygen: anaerobic/aerobic, and carbon: feast/famine). Using the same framework and metabolic model, we tested other conditions under which other phenotypes are more likely to predominate over a polyphosphate-based metabolism.

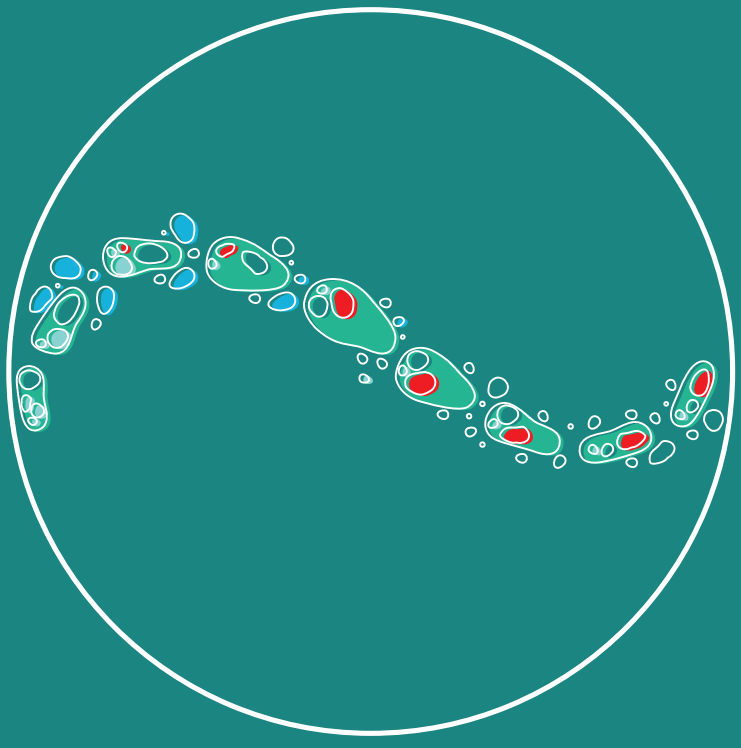
Under these fluctuating conditions, Accumulibacter are able to, very quickly, switch around entire pathways from growth to storage and *vice-versa*, such as glycolysis to/from gluconeogenesis, which entails a very interesting regulatory challenge, as parallel activity could lead to significant losses. In **Chapter 4**, a ^{13}C tracer experiment was conducted to explore carbon flow through central carbon pathways with a particular interest concerning the hypothesized anaerobic TCA cycle operation, *i.e. function*, and to identify putative regulated steps upon the metabolic switches of anaerobic famine-to-feast and anaerobic-to-aerobic - *regulation*.

In **Chapter 5**, we explored membrane bioenergetics in an Accumulibacter enrichment with the goal of finding a FAD regenerating mechanism that could enable anaerobic TCA cycle operation - *structure/function*. To this end, a protocol for membrane vesicles of an Accumulibacter enrichment was tested and assays were conducted to evaluate the activity of enzymes related with the operation of the TCA cycle and electron transport chain in the absence of an external electron acceptor.

Finally, **Chapter 6** contains an overview of the conclusions attained throughout the chapters of this dissertation and an outlook of the future challenges and opportunities following from this research.

2 CHAPTER

Metabolic flexibility of Accumulibacter



REVEALING THE METABOLIC FLEXIBILITY OF *Candidatus* ACCUMULIBACTER PHOSPHATIS THROUGH REDOX COFACTOR ANALYSIS AND METABOLIC NETWORK MODELING

Leonor Guedes da Silva, Karel Olavarria Gamez, Joana Castro Gomes, Kasper Akkermans, Laurens Welles, Ben Abbas, Mark C.M. van Loosdrecht, S. Aljoscha Wahl

Environmental fluctuations in the availability of nutrients lead to intricate metabolic strategies. *Candidatus* Accumulibacter phosphatis (hereafter referred to as Accumulibacter), a polyphosphate accumulating organism (PAO) responsible for enhanced biological phosphorus removal (EBPR) from wastewater treatment systems, is prevalent in aerobic/anaerobic environments. While the overall metabolic traits of these bacteria are well described, the non-availability of isolates has led to controversial conclusions on the metabolic pathways used.

Here, we experimentally determined the redox cofactor preference of different oxidoreductases in the central carbon metabolism of a highly enriched Accumulibacter culture. Remarkably, we observed that the acetoacetyl-CoA reductase engaged in polyhydroxyalkanoates (PHA) synthesis is NADH-preferring instead of the generally assumed NADPH dependency. This allows re-thinking the ecological role of PHA accumulation as a fermentation product under anaerobic conditions and not just a stress response. Based on previously published meta-omics data and the results of enzymatic assays, a reduced central carbon metabolic network was constructed and used for simulating different metabolic operating modes. In particular, scenarios with different acetate-to-glycogen consumption ratios were simulated, which demonstrated *optima* using different combinations of glycolysis, glyoxylate shunt or branches of the TCA cycle. Thus, optimal metabolic flux strategies will depend on the environment (acetate uptake) and on intracellular storage compounds availability (polyphosphate/glycogen).

This NADH-related metabolic flexibility is enabled by the NADH-driven PHA synthesis. It allows for maintaining metabolic activity under varying environmental substrate conditions, with high carbon conservation and lower energetic costs compared to NADPH dependent PHA synthesis. Such (flexible) metabolic redox coupling can explain PAOs' competitiveness under oxygen-fluctuating environments.

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IMPORTANCE

Here we demonstrate how microbial storage metabolism can adjust to a wide range of environmental conditions. Such flexibility generates a selective advantage under fluctuating environmental conditions. It can also explain the different observations reported in PAO literature, including the capacity of Accumulibacter to act like glycogen accumulating organisms (GAO). These observations stem from slightly different experimental conditions and controversy only arises when one assumes metabolism can only operate in one single mode. Furthermore, we also show how the study of metabolic strategies is possible when combining -omics data with functional cofactor assays and modeling. Genomic information can only provide the potential of a microorganism. The environmental context and other complementary approaches are still needed to study and predict the functional expression of such metabolic potential.

INTRODUCTION

Natural habitats of microorganisms are dynamic environments with intermittent supply of energy generating nutrients. Under these dynamic conditions, organisms are selected that can accumulate growth substrates when these are abundant to compensate for periods when these are exhausted (Van Loosdrecht, Pot, and Heijnen, 1997).

Enhanced biological phosphorus removal (EBPR) from wastewater is designed to make use of such physiological features by circulating activated sludge through alternating zones with or without an external electron acceptor here respectively defined as aerobic/anaerobic (see Figure 2.1A) (Seviour, Mino, and Onuki, 2003; Barnard, 1976). This environment selects for polyphosphate accumulating organisms (PAOs) like *Candidatus* Accumulibacter phosphatis (hereafter referred to as Accumulibacter). These bacteria thrive under these dynamic conditions thanks to a complex metabolic strategy encompassing the cycling of three common storage polymers: polyphosphate, glycogen and polyhydroxyalkanoates (PHA). Of these, polyphosphate stands out for allowing ATP generation for fast and competitive sequestration of organic matter (*e.g.* acetate) in the absence of an external electron acceptor (see Figure 2.1B).

In the past decades, a number of researchers have derived hypotheses about Accumulibacter's (anaerobic) physiology (Seviour, Mino, and Onuki, 2003; Seviour and McIlroy, 2008; Mino, Van Loosdrecht, and Heijnen, 1998; Oehmen et al., 2007a). One of the most important inconclusive discussions is about the source of reducing power for the anaerobic accumulation of polyhydroxyalkanoates (PHA) from volatile fatty acids (*e.g.* acetate, propionate), as reviewed by Zhou *et al* (Zhou et al., 2010). Most enrichment culture based experimental approaches were adequate to study the general physiology of Accumulibacter species, however the inherent population heterogeneity has not yet been sufficiently addressed; for example, only recently researchers have reported different Accumulibacter clades showing different kinetic characteristics leading to different metabolic operations (Welles et al., 2015). Furthermore, novel meta-omics approaches were used, which allowed

for Accumulibacter-targeted (culture-independent) analysis without the interference of other, non-Accumulibacter, sub-populations. A comprehensive overview of the physiological studies published on Accumulibacter can be found in the supplementary Table 2.4. In one of the metatranscriptomic studies (Oyserman et al., 2016b), Oyserman and colleagues highlighted the need for validating assumptions often made in metabolic models of Accumulibacter. In particular, they refined the discussion on reducing power sources

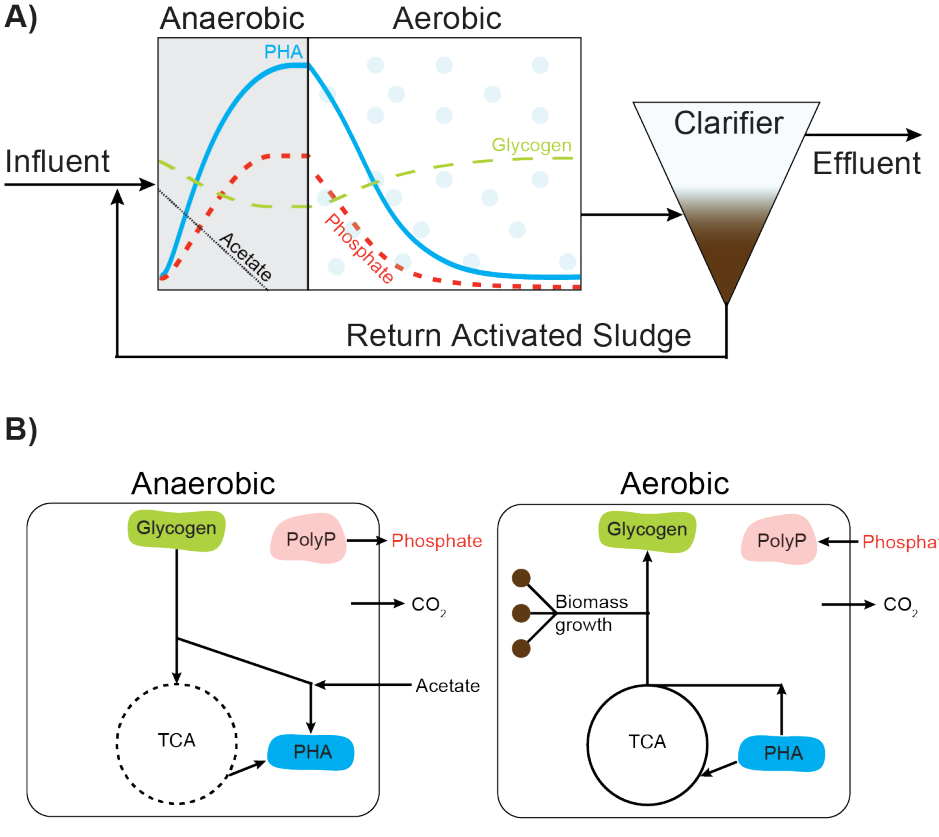


Figure 2.1: Schematic diagram of an EBPR process with nutrient/polymer profiles (A) and corresponding metabolic strategy (B). Recirculated activated sludge containing PAOs is mixed with influent wastewater in an anaerobic reactor. To compensate for the absence of an external electron acceptor, Accumulibacter use intracellular polyphosphate and glycogen reserves to generate ATP to take up organic carbon sources (*e.g.* acetate) and accumulate them in the form of polyhydroxyalkanoates (PHA). Phosphate is released at this stage. The TCA cycle is represented by a dashed line as it can have different operating modes depending on the NADH level. When oxygen becomes available, Accumulibacter make use of their PHA storage to grow, to replenish glycogen and polyphosphate. The re-accumulation of polyphosphate in growing biomass and subsequent purge of this biomass leads to the net removal of phosphate from wastewater. Adapted from (Oyserman et al., 2016a; McMahon and Read, 2013).
PHA: polyhydroxyalkanoates, **PolyP:** polyphosphate, **CO_2:** carbon dioxide, **TCA:** tricarboxylic acid cycle

and requirements by making a distinction between the different redox cofactors, NADH and NADPH. This distinction imposes a constraint between sources and sinks for each redox cofactor, which can only be alleviated by energy-consuming transhydrogenase(-like) mechanisms or using electrons for hydrogen production. However, in their analysis, the specificity of the involved oxidoreductases was postulated from analogy with other organisms, without experimental proof.

In Accumulibacter, reducing power is required for the anaerobic conversion of acetate to 3-hydroxybutyrate (3HB), the monomer of the reserve polymer poly-3-hydroxybutyrate (PHB). The intermediate reduction step is catalyzed by the enzyme acetoacetyl-CoA reductase (AAR). In aerobic PHA accumulating bacteria such as *Cupriavidus necator* and *Zoogloea ramigera*, the preferred electron donor cofactor of the AAR is NADPH (Madison and Huisman, 1999), which was the cofactor assumed by analogy for Accumulibacter (Oyserman et al., 2016b). However, AARs accepting both NADH and NADPH have been reported for *Azotobacter beijerinckii* (Ritchie, Senior, and Dawes, 1971; Senior et al., 1972) and *Allochromatium vinosum* (Liebergesell and Steinbüchel, 1992). A brief thermodynamic feasibility analysis of the coupling between glycolysis and either NADPH- or NADH-preferring AAR is present in the supplementary Figure 2.5.

So far and to our knowledge, the nature of the cofactor accepted by Accumulibacter's acetoacetyl-CoA reductase has not yet been experimentally established. In this study, we addressed this knowledge gap by measuring the NADH- and NADPH-dependent acetoacetyl-CoA reductase activities in a cell free extract from a highly enriched culture of Accumulibacter. We further extended the analysis to other key oxidoreductases involved in the metabolism of Accumulibacter. These results were then used to update Accumulibacter's anaerobic biochemical model and as constraints in a flux balance analysis (FBA) framework. Our simulations show how the use of different pathways and storage compounds lead to metabolic flexibility. Without this flexibility, Accumulibacter's anaerobic metabolism would become very restrictive and, under unpredictable environments, it could lead to situations where only a limited fraction of the externally available substrate could be consumed.

Results

Characterization of Accumulibacter enrichments

This study was carried out using two independent Accumulibacter enrichment cultures. SBR-1 contained the highest enrichment of Accumulibacter observed in our lab. However, this cultivation was operating close to a critical dilution rate which, unfortunately, resulted in washout (*i.e.* enrichment deterioration) after sampling. In SBR-2, conditions were adjusted to reduce the risk of washout, *i.e.* higher carbon source (chemical oxygen demand[COD]) load and higher solids retention time (SRT) were used.

For both cultures, anaerobic phosphorus (P) release per carbon (C) fed indicated that

the PAO present in the sludge are likely saturated with polyphosphate and that glycogen accumulating organisms (GAO) are not present (Welles et al., 2017). Furthermore, microbial characterization by FISH showed Accumulibacter from type I were the majority of the microorganisms across all biomass samples as it can be seen by the high overlap between the Accumulibacter-specific probe and the general Bacteria probe (micrographs available in Supplementary Figures 2.6 to 2.8). The *ppk1* gene analysis further specified Accumulibacter clades IC and IA were dominant in SBR-1 and SBR-2 respectively. The 16S rRNA gene analysis provided information on the *genus* of the most abundant subpopulations next to Accumulibacter (see Table 2.1 and Supplementary Figure 2.10).

Table 2.1: Process parameters and key performance indicators (KPIs) of the two independent enrichments of Accumulibacter.

Parameters	**SBR-1**	**SBR-2**
pH	7.6	7.6
Temp	20^{o}C	20^{o}C
Carbon source	210 mg_{COD}/L (63:37 $Acetate_{COD}$:$Propionate_{COD}$)	400 mg_{COD}/L (75:25 $Acetate_{COD}$:$Propionate_{COD}$)
Phosphate load per C-fed	0.1 Pmol/Cmol	0.1 Pmol/Cmol
Broth volume	1.5 L	1.5 L
Cycle time	Total – 6 h; Settling – 76 min; Anaerobic – 135 min; Aerobic – 135 min.	Total – 6 h; Settling – 30 min; Anaerobic – 112 min; Aerobic – 200 min.
Hydraulic retention time (HRT)	12 h	12 h
Solids retention time (SRT)	4 days	5.6 days
Aerobic SRT	1.5 days	3.1 days
KPIs		
Anaerobic P-release per C-fed	0.58 Pmol/Cmol	~0.75 Pmol/Cmol
Anaerobic-feast length	~41 min	~42 min
FISH	PAO Acc I	PAO Acc I
16S rRNA gene amplicon sequencing (dominant OTUs)	Ca_Accumulibacter, Rhodopseudomonas, Blastochloris	Start: Dechloromonas, Ca_Accumulibacter, *genus* from f__Hyphomonadaceae; End: Ca_Accumulibacter, Chryseobacterium, *genus* from o__Sphingobacteriales
ppk1 analysis	Dominant: clade IC	Dominant: clade IA

[a] Pmol, moles of phosphorus; Cmol, moles of carbon.

REDOX COFACTOR PREFERENCES OF KEY OXIDOREDUCTASES

For a better understanding of the metabolic mechanisms during the EBPR process, understanding the redox couplings are essential and have been a controversy in literature while direct biochemical evidence was missing. Therefore, it was essential to identify the cofactor specificity of relevant reactions and enable a validated model simulation. Enzymatic assays were performed using cell free extracts. While these assays cannot discriminate which organism has the respective activity, the performed assays still answer: **1)** if an activity is not observed for the whole community, it is also not present in Accumulibacter, **2)** if the cell extract shows a clear preference for NAD(H) or NADP(H) this is most probably also the

preferred cofactor of Accumulibacter which is highly enriched in the culture.
The enzymatic activity assay results display a clear preference which is sufficient to annotate a specific cofactor. These are shown in blue in the metabolic network in Figure 2.2. Further details can be found in Supplementary Section 2.6.4. The different assay results will be presented from the sinks (PHA) to the putative sources of reducing power:

- **Redox cofactor preference (NADH or NADPH) of the PHA synthesis.** The CFE from SBR-1 had a NADH preferring acetoacetyl-CoA reductase. The activity was ten times (10x) higher with NADH compared to NADPH as substrate. To further confirm this cofactor preference, the acetoacetyl-CoA reductase activity was assayed using CFEs from a second enrichment (SBR-2) and both directions of the reaction were monitored (*i.e.* NAD(P)H + acetoacetyl-CoA $\rightleftharpoons$ NAD(P)$^+$ + 3-hydroxybutyryl-CoA).

- **Stoichiometry of glycolysis – EMP or ED?** To discriminate between these pathways, the presence of glucose-6-phosphate dehydrogenase (G6PDH), the enzyme that catalyzes the first step of the Entner-Doudoroff (ED) pathway, was measured. This reaction is also common to the oxidative branch of the pentose phosphate pathway (oxPP). No activity was found in the CFEs from both SBRs. The biological positive control, a CFE from *P. putida* KT2440, showed activity with both NAD$^+$ and NADP$^+$, as expected (Olavarría, Valdés, and Cabrera, 2012). Therefore, Accumulibacter nor the community have an active ED or oxPP pathway leaving the Embden-Meyerhof-Parnas (EMP) pathway as glycolytic route. This raises the question: *what is now the source of NADPH?*

- **Alternative NADPH sources.** For many organisms oxPP is an important source of NADPH for biomass formation (Fuhrer and Sauer, 2009). This activity was not found in Accumulibacter, raising the question if NADPH could be provided by other reaction like the dehydrogenation of isocitrate by the isocitrate dehydrogenase (ICDH). This reaction was tested using either NAD$^+$ or NADP$^+$. The activity with NADP$^+$ was more than seventy times (70x) higher, indicating that isocitrate dehydrogenation is a source of NADPH in Accumulibacter.

Additionally, the activity of other oxidoreductases and anaplerotic routes was measured: **1)** The reduction of oxaloacetate to malate (catalyzed by malate dehydrogenase) had at least 55-times-higher activity when using NADH than when using NADPH. Because of the small activity observed when using NADPH, it was not possible to independently study the cofactor preference of the malic enzyme (oxidation of malate to pyruvate); **2)** The first step of the glyoxylate shunt, catalyzed by the isocitrate lyase (ICL), was also found to be active and to have an activity comparable to that of the positive biological control *Escherichia coli* grown in acetate (well known to make use of the glyoxylate shunt (Gottschalk, 1986)); **3)** Lastly, fumarate reductase (FR) and α-ketoglutarate dehydrogenase (AKGDH) were also tested, but their activities were very low compared to those of all other enzymes tested in our study.

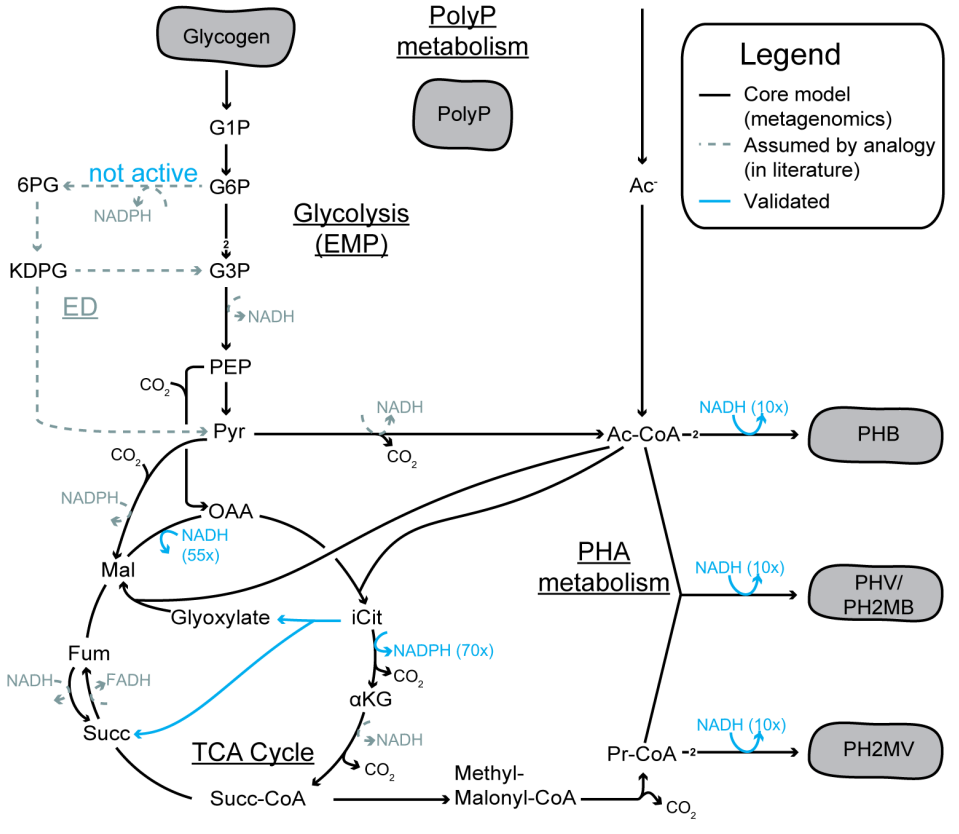


Figure 2.2: Central carbon metabolic network of Accumulibacter including redox cofactor preference of oxidoreductases assayed in this study. A simplified version of this network (Table 2.2) was used for the simulations. The values shown correspond to the factor difference between the preferred cofactor and the alternative one. This metabolic network is based on the ancestral genome reconstruction done by (Oyserman et al., 2016a). The arrows show the expected flux direction under anaerobic conditions. The untested cofactor preferences (in gray) remain as assumed by analogy with other microorganisms. Despite not being annotated in the genome, the ED pathway has been suggested as the route for glycogen degradation (Maurer et al., 1997; Hesselmann et al., 2000). A more extensive overview of evidence for each pathway can be found in Supplementary Table 2.4. **G1P:** glucose-1-phosphate, **G6P:** glucose-6-phosphate, **6PG:** 6-phosphogluconate, **KDPG:** 2-keto-3-deoxy-6-phosphogluconate, **G3P:** glyceraldehyde-3-phosphate, **PEP:** phosphoenolpyruvate, **Pyr:** pyruvate, **OAA:** oxaloacetate, **Ac-CoA:** Acetyl-CoA, **iCit:** isocitrate, **αKG:** α-ketoglutarate, **Succ-CoA:** succinyl-CoA, **Succ:** succinate, **Fum:** fumarate, **Mal:** malate, **Pr-CoA:** propionyl-CoA, **Ac$^-$:** acetate, **EMP:** Embden-Meyerhof-Parnas, **TCA:** tricarboxylic acid cycle.

ANAEROBIC STOICHIOMETRIC MODEL CONSTRUCTION

Based on the defined reaction network (Figure 2.2 and Table 2.2) the balance of reducing equivalents by Accumulibacter during anaerobic acetate conversion to PHAs was studied quantitatively.

Table 2.2: Stoichiometry used for flux balance analysis (in mol basis) to simulate Accumulibacter's *anaerobic* metabolism[a]. A comprehensive overview of the physiological studies published on Accumulibacter supporting this stoichiometric model can be found in the Supplementary Table 2.4.

Pathway	**Stoichiometry**	**Enzyme assayed**
Glycolysis (EMP)	$(Glycogen)_1 \rightarrow 2\ Pyr + 4$ electrons	G6PDH (ED)
Pyruvate to Acetyl-CoA	$Pyr \rightarrow Ac\text{-}CoA + 2$ electrons $+ CO_2$	-
Pyruvate to Oxaloacetate	$Pyr + CO_2 \rightleftharpoons OAA$	-
Oxidative TCA branch	OAA + Ac-CoA	ICDH
	$\rightarrow$ Succ-CoA + 2 CO_2 + 4 electrons	AKGDH
Reductive TCA branch (reverse TCA)	OAA + 4 electrons $\rightarrow$ Succ-CoA	MDH, FR
Glyoxylate shunt	2 Ac-CoA $\rightarrow$ Succ-CoA + 2 electrons	ICL
Succinate-propionate shunt	Succ-CoA $\rightleftharpoons$ Pr-CoA + CO_2	-
Ac-CoA* production	Ac-CoA + electron $\rightarrow$ Ac-CoA*	AAR
(precursor for 3HB, 3HV and 3HMB)		
Pr-CoA* production	Pr-CoA + electron $\rightarrow$ Pr-CoA*	AAR
(precursor for 3H2MV, 3HV and 3HMB)		

[a] Reversible reaction: $\rightleftharpoons$, irreversible reaction: $\rightarrow$. Note that these are lumped reactions, and each may involve several enzymatic steps. Please refer to Materials & Methods for a definition of Ac-CoA* and Pr-CoA*.
* means plus 1 electron.

When the NADPH-to-NADP ratio is in a more reduced state than the NADH-to-NAD ratio, the cofactors NADPH can be regenerated by donating their electrons to NADH using transhydrogenases without any energetic cost for the cell (Fuhrer and Sauer, 2009). This is usually not the case for $FADH_2$, which would require an input of metabolic energy to be re-oxidized using NAD^+. Currently, there is still no experimentally validated mechanism on how Accumulibacter could re-oxidize $FADH_2$ in the absence of an external electron acceptor (*e.g.* oxygen or nitrate). Therefore, for this simulation, we blocked this $FADH_2$ producing step in the TCA cycle (*i.e.* Succ-CoA to Fum) and for the remaining reactions we neglected the different types of redox cofactors by simply balancing "electrons"[1]. Since the $FADH_2$ producing step in the TCA cycle is blocked, only the oxidative branch (OAA to Succ-CoA *via* ICDH and AKGDH) and/or the reductive branch (OAA to Succ-CoA *via* MDH and FR) are possible (see Table 2.2).

In contrast to electron balances, the ATP balance cannot be used as there are still too many unknowns: **1)** how much ATP can be generated/consumed from efflux of ions (potassium, magnesium and phosphate) associated with polyphosphate hydrolysis; **2)** how much ATP is required for acetate uptake (*i.e.* to export the protons imported during acetate symport); **3)** how much is needed to upgrade redox cofactors (*i.e.* transfer electrons from NADH to $NADP^+$); and **4)** how much ATP is used for cellular maintenance. Therefore, ATP is not balanced in this simulation. Nevertheless, it is important to note that the different pathways used for redox balancing will lead to different levels of ATP generation. Thus, in cases of polyphosphate limitation, the metabolism could prefer pathways with higher ATP-yield.

[1] Note that each redox cofactor carries 2 electrons and, in some publications, these are simply referred to as [H].

Experiments show that the ratio between glycogen and acetate consumption by PAOs is variable, *e.g.* between high/low temperature (Erdal, Erdal, and Randall, 2005) or polyphosphate/glycogen availability (Welles et al., 2017; Zhou et al., 2008; Zhou et al., 2009). To reflect this variability, simulations were performed for a range of acetate and glycogen mixtures ranging from only acetate to only glycogen and results were compared to experimental data reported in literature.

For the anaerobic conversion of acetate to PHA, acetate needs to be reduced (please refer to Materials & Methods for a definition of Ac-CoA*). The Ac-CoA* *optimum* in Figure 2.3 corresponds to the situation in which most carbon is kept inside the cell. This optimum also corresponds to the maximum theoretical yield for PHB on acetate and/or glycogen of 0.89 $Cmol_{PHB}/Cmol_{consumed}$. Similar plots for Pr-CoA*, CO_2, alternative simulations and including error bars on the experimental data points can be found in Supplementary Figures 2.13 through 2.22.

The *optimum* and *minimum* lines were obtained by minimizing and maximizing CO_2 production, respectively. The feasible solution space (in yellow) lies in between these two cellular objectives, in which a mixture of the different redox balancing strategies mentioned above is used. The *minimum* carbon conserving strategy is the one that releases most CO_2 (see Supplementary Figures 2.15 and 2.16.). The majority of the experimental datapoints fall within the solution space possible with the network presented in Table 2.2. A few points fall outside this space, but are still within thermodynamically-feasible space, *i.e.* there may be alternative pathways active.

The required reducing power can come from either glycolysis (glycogen breakdown) or glyoxylate shunt (acetate oxidation) operation. The different modes are presented in Figure 2.4. These modes respectively form the extreme left and right values of the *optimum* line in Figure 2.3. If glycogen degradation can supply exactly the amount of electrons needed for the uptake and conversion of acetate, then only Ac-CoA* (as PHB) is produced and there is no need to use any part of the TCA cycle (Figure 2.4B, and highest PHB yield in Figure 2.3). The *optimum* can probably only be achieved with polyphosphate as ATP source. In a typical GAO-like metabolism, an excess of glycogen is degraded compared to the acetate consumed to supply all ATP needed. This leads to the generation of an excess of reducing power, which is used to produce Pr-CoA* (see Materials & Methods for a definition of Pr-CoA*) leading to PHAs more reduced than PHB (*e.g.* PHV or PH2MV), using the reductive TCA branch (Figure 2.4C). On the other hand, in a PAO-like metabolism the amount of reducing equivalents generated by glycolysis is usually lower than required to reduce all consumed acetate. In this situation, part of the acetate is oxidized to produce reducing equivalents. Such oxidation is possible *via* an active glyoxylate shunt which was also found from the enzymatic assays. The simulations show this as the optimal pathway to produce PHAs when glycogen is limiting (Figure 2.4A), *i.e.* it allows for higher carbon conservation (*i.e.* higher PHA yield) than the “horseshoe” TCA operation.

Discussion

Characterization of Accumulibacter enrichments

The discussion will be based on the observed high PAO enrichment of mainly Accumulibacter type I. Consequently, extrapolations of these results to Accumulibacter type II or GAOs are speculative until further validation using selective environments for their respective enrichments (Welles et al., 2015; Flowers et al., 2013; Welles et al., 2016; Acevedo et al., 2012; Wexler, Richardson, and Bond, 2009; Barr et al., 2016).

Updated biochemical (stoichiometric) model

The presented study shows that the metabolic traits of Accumulibacter enable a flexible metabolic operation under anaerobic conditions. This flexibility is made possible by their energy storage in polyphosphate and reducing power balancing, enabling the observed phenotypes: fast anaerobic acetate uptake and anaerobic PHB synthesis (decoupled from growth). The flexible reducing power balancing by Accumulibacter depends on the nature of the redox cofactors used – here, enzymatic assays were performed to define the redox cofactor preferences of the main oxidoreductases in Accumulibacter's central metabolism.

The key finding from these assays is the NADH-preferring PHA synthesis in Accumulibacter. This NADH-preference allows for a direct consumption of the NADH produced in most of Accumulibacter's reducing power sources. This also eliminates the need for NADH into NADPH conversion using the membrane-bound transhydrogenase (PntAB) driven by proton motive force, which was suggested in an earlier study (Oyserman et al., 2016b). Although there are previous reports showing NADH-driven PHB accumulation (Ritchie, Senior, and Dawes, 1971; Kim, Chang, and Kim, 2014; Haywood et al., 1988; Ling et al., 2018; Barycki et al., 2000), the here measured level of NADH preference of the acetoacetyl-CoA reductase from Accumulibacter is striking. Further characterization of this enzyme was undertaken by Olavarria and colleagues (Olavarria et al., 2021). This observation allows to re-think the role of PHAs: we hypothesize that depending on the environment where microorganisms thrive, PHA accumulation will play a role as carbon reservoir during aerobic metabolic over-flow (NADPH-driven accumulation) or as an anaerobic electron reservoir during scarcity of external electron acceptors (NADH-driven accumulation). Thus, for Accumulibacter, anaerobically, PHA is essentially a fermentation product.

A related finding was the absence of activity of the first step of the sometimes-implicated ED glycolytic pathway. These results match those observed by (Erdal, Erdal, and Randall, 2005) for PAOs and in (Filipe, Daigger, and Grady, 2001a) for GAOs, where no $NADP^+$ dependent glucose-6-phosphate dehydrogenase activity was found. The experimental findings are in line with the absence of key ED genes in the genome annotations of Accumulibacter and closest relatives *Dechloromonas aromatica* and *Azoarcus sp. EbN1* (Barr et al., 2016; García Martín et al., 2006). Furthermore, no enzymatic activity of the ED pathway with NAD^+ as electron acceptor (Olavarría, Valdés, and Cabrera, 2012) was observed.
These findings are in contradiction with earlier ^{13}C NMR studies (Maurer et al., 1997; Hesselmann et al., 2000), which indicated that the ED pathway was more likely the route

for glycolysis than EMP. Nevertheless, it has to be noted that these early studies comprise interpretations of ^{13}C patterns using simplified metabolic models, with limited information on the reversibility of each reaction and potentially with reactions missing; these are common pitfalls of the ^{13}C-labelling method (Winden, Verheijen, and Heijnen, 2001). Also note that these ^{13}C NMR studies were performed before the first draft genome of Accumulibacter was available (García Martín et al., 2006), and only the ^{13}C pattern in the different PHAs was measured and not in the metabolic intermediates of each pathway used.

While NADPH is not required anaerobically by Accumulibacter, this reducing cofactor must be produced aerobically to drive biomass synthesis. Here, isocitrate dehydrogenase was found $NADP^+$ dependent. This is consistent with the observation that most acetate consumers will use this conversion to produce NADPH for their anabolism (Gottschalk, 1986) and confirms the protein annotation found in (Barr et al., 2016). Since PHA accumulation is now known to be NADH-preferring and without another sink of NADPH, the latter might accumulate anaerobically and thereby inhibit the isocitrate dehydrogenase reaction. Alternatively, a soluble transhydrogenase could convert this NADPH into NADH and allow the oxidative branch of the TCA to be operational under anaerobic conditions.

Regarding the activity of other TCA oxidoreductases and anaplerotic routes: **1)** the oxidation of malate using NAD^+ was also found in the studies of (Erdal, Erdal, and Randall, 2005); **2)** the glyoxylate shunt was also found active as observed in previous assays done by (Erdal, Erdal, and Randall, 2005; Hesselmann et al., 2000; Burow, Mabbett, and Blackall, 2008) and metatranscriptomics/proteomics studies by (Wexler, Richardson, and Bond, 2009; Oyserman et al., 2016a; Skennerton et al., 2015; Wilmes et al., 2008; He and McMahon, 2011a), which is expected as this is the anaplerotic route that allows for microorganisms to convert C2 sources like acetate into C4 building blocks for anabolism (Gottschalk, 1986); and **3)** fumarate reductase and α-ketoglutarate dehydrogenase activities were very low compared to all other enzymes tested in our study and alike in the studies of (Erdal, Erdal, and Randall, 2005). It has to be noted that information on the Accumulibacter type was not found for all studies and there can be differences depending on the cultivation conditions. Additionally, there is one study suggesting the activity of an α-ketoglutarate:ferredoxin oxidoreductase – the enzyme was found in Accumulibacter's genome and proteome (Barr et al., 2016). This membrane-bound enzyme has not been assayed here as the simulations suggest an anaerobic operation mode *via* the glyoxylate shunt rather than a full or partial oxidative TCA cycle.

FLEXIBLE ANAEROBIC METABOLISM – ADJUSTMENTS DEPENDING ON THE ENVIRONMENT AND INTRACELLULAR STORAGE COMPOUNDS

As Zhou *et al.* suggested in their review, the flexibility of Accumulibacter's metabolism is likely the major reason why there is controversy in literature regarding how reducing power is balanced under anaerobic conditions (Zhou et al., 2010). Long-term exposure to constant conditions (*e.g.* pH, temperature, oxidation level of substrates, nutrient availability, counter-ions, SRT, settling time) will select for the best strategy for those conditions, but short-term perturbations of those set conditions have shown that Accumulibacter seems

to still be able to solve the redox balancing problem even if sub-optimally regarding carbon conservation (Welles et al., 2015; Zhou et al., 2008; Zhou et al., 2009; Erdal et al., 2008). Thus, based on our analysis, we observe that there is not one fixed overall stoichiometry for acetate + glycogen (+polyphosphate) → PHAs + CO_2 (+phosphate). Instead, there is a range of possible stoichiometries that in the end are defined by the relative proportions of each of the substrates, supplied to the system or produced by a side-population (*e.g.* acetate, glycogen, polyphosphate, other VFAs, oxygen, nitrate, H_2).

The simulations showed Accumulibacter can operate in three distinct modes (and combinations in between): **A)** when reducing equivalents from glycolysis (glycogen) are limiting compared to the acetate imported (Yagci's model for PAOs (Yagci et al., 2003)); **B)** when glycolysis (glycogen) supplies exactly enough reducing equivalents to convert all imported acetate into PHB (Mino's model (Mino, Tsuzuki, and Matsuo, 1987)); and lastly **C)** when glycolysis (glycogen) is producing an excess of reducing equivalents (alike Yagci's model for GAOs (Yagci et al., 2003)).

The anaerobic use of the glyoxylate shunt in a PAO-like metabolism to provide for the reducing equivalents needed for PHA accumulation is supported by the studies of (Burow, Mabbett, and Blackall, 2008; Yagci et al., 2003); However, as seen in Figure 2.3, it does not explain the higher levels of Ac-CoA* found (points above the red *optimum* line) when a shift to more reduced PHAs (Pr-CoA*) was expected. This indicates that there might be an alternative process that allows the cell to conserve extra carbon, in other words, accumulate more Ac-CoA* and less Pr-CoA*. It could be that **1)** fully anaerobic conditions were not attained when performing the experiments, or that **2)** another side population is providing electrons in the form of a more reduced organic substrate or even hydrogen gas (Lawson et al., 2015) or **3)** Accumulibacter has yet another, alternative way of balancing redox which has yet to be described and demonstrated.

Also for the scenario where glycolysis produces an excess of reduced cofactors as in a GAO-like metabolism, a few experimental data points fall outside the feasible solution space (points above the green *optimum* line in Figure 2.3); these could be explained in the case that Accumulibacter produces H_2 to solve an excess of NADH as described by (Oyserman et al., 2016b). While H_2 production extends the metabolic flexibility by alleviating the redox constraints, it also leads to the loss of electrons that cannot be used for energy production during the aerobic phase. Therefore, we expect H_2 formation only as a specific mechanism when redox cannot be balanced otherwise.

THE MULTILAYERED COMPLEXITY OF A PHYSIOLOGICAL ANALYSIS OF ACCUMULIBACTER

The experimental data gathered are likely also influenced by the intrinsic heterogeneity of Accumulibacter enrichments; clade differences, population heterogeneity or even different environment (*e.g.* depending on the position of the cell in a floc/granule). Therefore, experimental data points under the *optimum* line in Figure 2.3 likely represent mixtures of cells, each with a different metabolic mode (Figure 2.4). Furthermore, any kinetic limitation (*i.e.* pathway capacity) may also explain a sub-optimal, mixed phenotype.

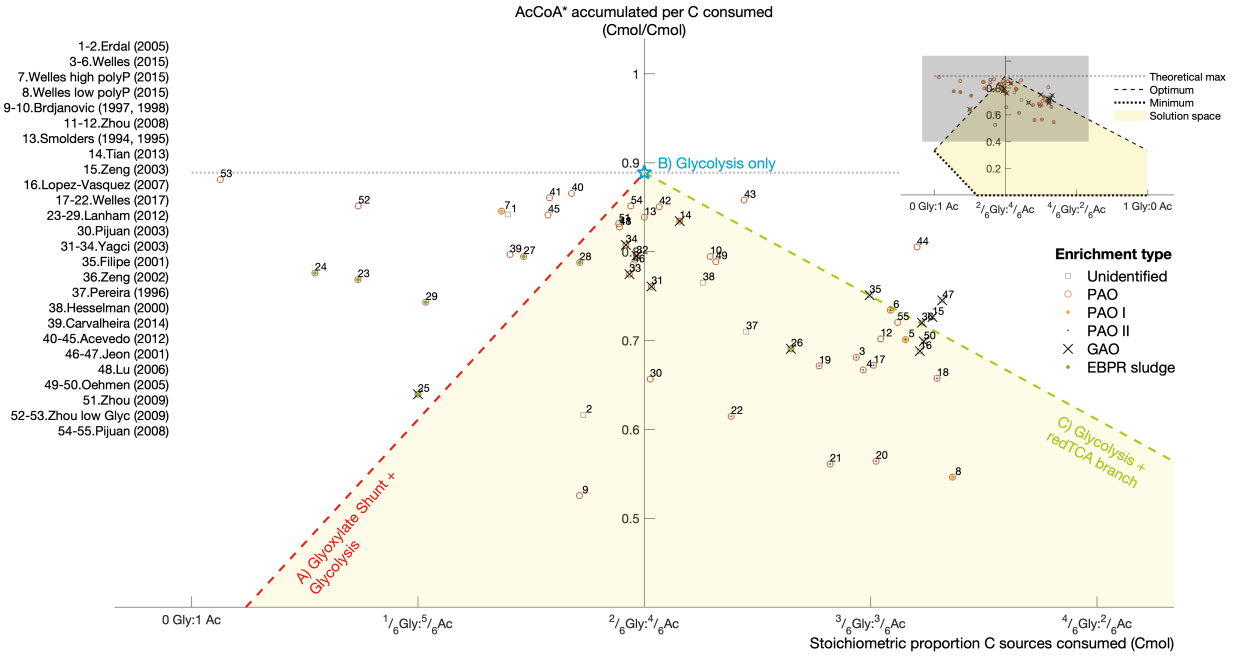


Figure 2.3: The amount of Ac-CoA* accumulated (PHB and PHV precursor) depends on the different proportion of glycogen to acetate consumed and on the available pathways (please refer to Materials & Methods for a definition of Ac-CoA*). The three *optima* redox balancing strategies are shown in Figure 2.4 ABC. From 0 to approx. 2/6 $Cmol_{glycogen}/Cmol_{consumed}$, it can be considered that bacteria are performing a polyphosphate-based metabolism (PAM, *optimum* line in red) and from approx. 3/6 to 1 $Cmol_{glycogen}/Cmol_{consumed}$, a glycogen-based metabolism (GAM, *optimum* line in green) is used. The *optimum* and *minimum* lines were obtained by minimizing and maximizing CO_2 production, respectively. The feasible solution space (in yellow) lies in between these two cellular objectives, in which a mixture of different redox balancing strategies is used. The *minimum* carbon conserving strategy is the one that releases most CO_2 (see Supplementary Figures 2.15 and 2.16.). Experimental datasets were retrieved from (Welles et al., 2015; Welles et al., 2017; Erdal, Erdal, and Randall, 2005; Zhou et al., 2008; Acevedo et al., 2012; Hesselmann et al., 2000; Yagci et al., 2003; Pereira et al., 1996; Smolders, 1995; Tian et al., 2013; Zeng, Yuan, and Keller, 2003; López-Vázquez et al., 2007; Lanham, 2012; Pijuan et al., 2004; Filipe, Daigger, and Grady, 2001a; Zeng et al., 2002; Carvalheira, 2014; Brdjanovic et al., 1997; Brdjanovic et al., 1998; Lu et al., 2006; Zhou et al., 2009; Pijuan et al., 2008; Jeon et al., 2001; Oehmen et al., 2005) and normalized to respect carbon and energy conservation principles as described in Materials & Methods. Similar plots for Pr-CoA*, CO_2, alternative simulations and including error bars on the experimental data points can be found in Supplementary Figures 2.13 through 2.22.

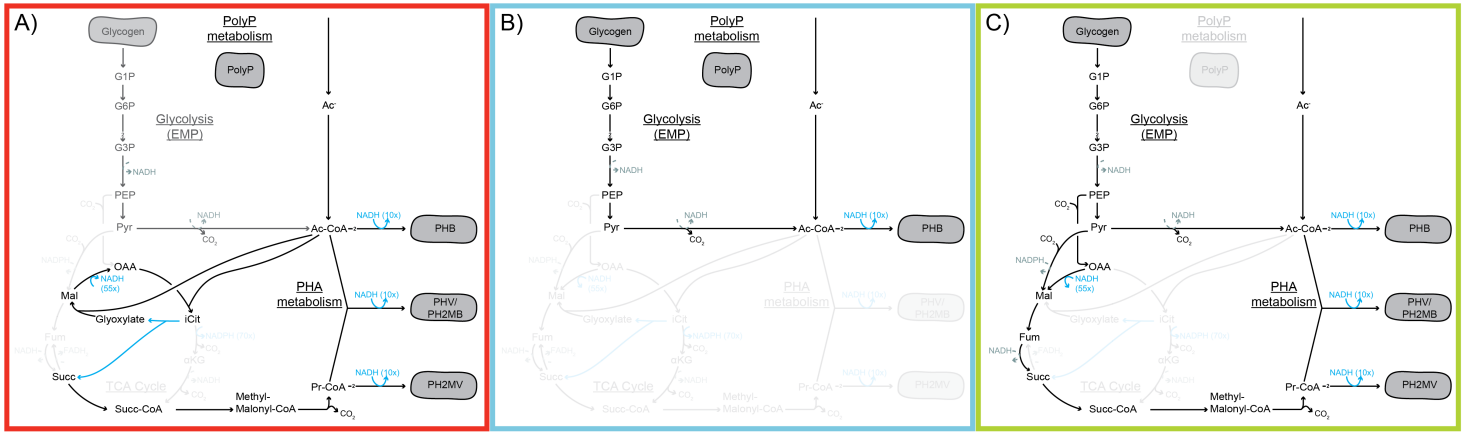


Figure 2.4: Different optimal redox balancing strategies for Accumulibacter under anaerobic conditions. **A)** When little glycogen is degraded and is not enough to reduce all acetate to PHB, the glyoxylate shunt as recommended by (Yagci et al., 2003) is the most optimal way to provide electrons required for the PHB pathway. **B)** When the stoichiometric amount of glycogen is degraded for acetate reduction to PHB, there is no need to operate any part of the TCA cycle to balance redox and only PHB is then produced, as proposed in (Mino, Tsuzuki, and Matsuo, 1987). **C)** When more glycogen is degraded than needed for acetate reduction to PHB, the reductive TCA branch can be used as proposed by (Yagci et al., 2003) for GAOs to sink electrons in more reduced PHAs (*e.g.* PHV and PH2MV). These flux distributions are unique, at the *optimum* (minimal CO_2 production) no flux variability is observed.

Additionally, if all enzymatic machinery is available, it seems possible that the same cell might change modes depending on the environment and its intracellular storage dynamics (further explored in **Chapter 4**). Particularly, availability of acetate (*e.g.* transition feast/famine) and/or polyphosphate storage as well as glycogen can trigger shifts in metabolic mode.

Despite the heterogeneity and noise, the experimental data outside the solution space in Figure 2.3 suggests this stoichiometric model is still incomplete to represent all experimental conditions. Two alternatives to expand the solution space could be **1)** adding a reaction for hydrogen production to regenerate NAD^+ in excess glycogen-to-acetate conditions or **2)** allowing for a fully operational TCA cycle to fully oxidize acetate to CO_2 in a glycolysis-limiting scenario. With these reactions, all experimental data can be explained (see Supplementary Figure 2.22). The first scenario is supported by the detection of hydrogen gas produced by an Accumulibacter enrichment (Oyserman et al., 2016b). For the second scenario, a full TCA cycle has been previously suggested based on ^{13}C NMR observations (Pereira et al., 1996) and stoichiometric analyses by (Zhou et al., 2009). Additionally, a novel protein has been proposed based on metagenomic analysis that could regenerate FAD without an external electron acceptor, which allows for full TCA operation under anaerobic conditions (García Martín et al., 2006); however, there is still no direct biochemical experimental evidence that indeed validates a full anaerobic TCA operation. Different alternatives to regenerate FAD are further explored in Chapter 5. Some indirect observation is presented in reference (Lemos et al., 2003). In that study, labelled propionate was used as substrate and ^{13}C enrichment was found in the PHV fragments that come from acetyl-CoA. This labeling pattern can be explained by a full TCA, but this is not the only metabolic route that could explain this observation.

Alternatively, it should also be considered that, like many (strict) anaerobes, Accumulibacter may use electron bifurcation mechanisms (Buckel and Thauer, 2013) *via* ferredoxin oxidoreductases to allow for alternative pathways in the central carbon metabolism.

CONCLUSION

In this study a network-based modeling approach was used to unravel the metabolic flexibility of Accumulibacter under dynamic conditions. The approach integrates findings and hypotheses derived from previous meta-omics studies as well as different physiological datasets and biochemical assays.

The developed metabolic model demonstrates the flexibility in metabolic function and could explain previous controversy in PAO literature. The NADH dependent PHA synthesis is both an efficient link to catabolic pathways as well as key for enabling metabolic flexibility. Depending on the exact history and cultivation conditions, Accumulibacter can exhibit different metabolic phenotypes; the metabolic network can handle different combinations of carbon sources (*i.e.* acetate and glycogen) adjusting the use of glycolysis (EMP), different

branches of the TCA cycle (incl. glyoxylate shunt) and potentially other pathways not yet considered. This poses a challenge to predictive EBPR modeling, as this implies that the overall anaerobic stoichiometry is not fixed, but variable, spanning continuously from polyphosphate to glycogen-based phenotypes.

This metabolic versatility is likely what allows Accumulibacter to be, in most situations, very close to a carbon conservation *optimum*, which is key to ensure their competitiveness in the dynamic environments of EBPR systems.

Materials & Methods

Reactor operation

Two independent Accumulibacter enrichments were obtained in sequencing batch reactors (SBR). The main operational conditions are described in Table 2.1 and were adapted from the SBR-S as described in Welles et al., 2015.

Cell free extracts preparation

Broth samples (10 mL) collected from the bioreactor during both anaerobic and aerobic phases were centrifuged (2,500 × *g*, 10 min, 4 °C) and the pellet was washed using 10 mL buffer (hereafter named Buffer 1X) containing 50 mM Tris at pH 8, 5 mM $MgCl_2$, 5 mM NaCl and 5% (v/v) glycerol, and the obtained suspension was centrifuged again (2,500 × *g*, 10 min, 4 °C). After centrifugation, pellets were kept at -20 °C for no longer than four days until further analysis. For the enzymatic assay, cellular pellets were suspended in 10 mL of Buffer 1X supplemented with 2 mM (L+D) 1,4-Dithiothreitol (DTT) and cOmplete^{TM} mini protease inhibitor cocktail (Roche). To avoid overheating and protein denaturation, cells were kept on ice while being sonicated until the cell suspension was homogenized (*i.e.* no granules visible). The resulting suspension was centrifuged (15,000 × *g* for at least 45 min, 4 °C). The obtained supernatant, a cell free extract (CFE), was used for the enzymatic assays.

Purification of Accumulibacter's Acetoacetyl-CoA reductase

A gene with high degree of similarity to *phaB* from Accumulibacter was cloned as described by Olavarria et al., 2021. Total metagenomic DNA was isolated from a lab-scale reactor of freshwater aerobic granular sludge operated as described by Graaff, Loosdrecht, and Pronk, 2020. This DNA was used as a template in a PCR reaction with primers designed according to the reference sequence of the *phaB* gene from Accumulibacter type II (*locus* CAP2UW1_-3919). The sequence of the primers used can be found in Supplementary Section S4. After constructing several plasmids with the amplified gene, cloning, and sequencing, the clone with the highest identity with respect to the reference sequence (colony number 6, 89% DNA identity, 95% amino acid identity) was subcloned into another vector envisioning over-expression in cells of *E. coli* BL21DE3, purification using His-tagging, and subsequent characterization (protein sequences available in Supplementary Section S4). The purity of the obtained acetoacetyl-CoA reductase was confirmed by SDS-page, and this purified enzyme was used for the enzymatic assays as control.

ENZYMATIC ASSAYS

The reaction mixtures used for each enzymatic assay are described in Table 2.3.

Activities were calculated using the initial rate of reduction of $NAD(P)^+$ or oxidation of NAD(P)H, which was obtained by following the changes in the absorbance at 340 nm, at 30°C. To control the contribution of putative background reactions, reaction mixtures were monitored that contained all the components except one substrate or without addition of CFE (Table 2.3). The measured rates were normalized to the total protein concentration in the respective CFE. The protein concentration was measured using the Bradford dye-binding protein assay (Bio-Rad), using known concentrations of bovine serum albumin as external standards.

MICROBIAL COMMUNITY CHARACTERIZATION

The microbial community present in our enrichments has been characterized by three orthogonal approaches as described below and the detailed results can be found in Supplementary Figures 2.6 through 2.10.

Fluorescence *in situ* hybridization (FISH) was used to qualitatively assess the presence of Accumulibacter in the enrichment cultures. All bacteria were targeted by the EUB338 mix (general bacteria probe) (Daims et al., 1999; Amann et al., 1990; Amann, 1995). Accumulibacter type I and II were targeted by the probes Acc-1-444 and Acc-2-444 (Flowers et al., 2009), respectively. Hybridized samples were examined with a Zeiss Axioplan-2 epifluorescence microscope.

To further confirm the specific Accumulibacter clade, the presence of the gene encoding for the polyphosphate kinase I (*ppk1*) present in Accumulibacter was tested as described in (Rubio-Rincón et al., 2017; Saad et al., 2016) using the primers ACCppk1-254F and ACCppk1-1376R targeting the *ppk1* gene from Accumulibacter-like bacteria (McMahon et al., 2007). The *ppk1* gene sequences obtained in this study have been deposited in the GenBank database under accession numbers MH899084-MH899086.

To identify putative side-populations, 16S rRNA gene amplicon sequencing was applied. DNA samples from cell pellets were extracted using the DNeasy UltraClean Microbial Kit (Qiagen, The Netherlands). Approximately 250 mg wet biomass was treated according to the standard protocol except an alternative lysis was implemented. This included a combination of 5 min of heat (65 °C) followed by 5 min of bead-beating for cell disruption on a Mini-Beadbeater-24 (Biospec, U.S.A.). After extraction the DNA was checked for quality by gel electrophoresis and quantified using a Qubit 4 (Thermo Fisher Scientific, U.S.A.). After quality control, samples were sent to Novogene Ltd. (Hongkong, China) for amplicon sequencing of the V3-4 region of the 16S rRNA gene (position 341-806) on an Illumina paired-end platform. After sequencing, the raw reads were quality filtered, chimeric sequences were removed and OTUs were generated on the base of ≥ 97% identity. Subsequently, microbial community analysis was performed by Novogene using Mothur & Qiime software (V1.7.0) (Schloss et al., 2009; Caporaso et al., 2010). For phylogenetical determination, the most recent SSURef database from SILVA (Quast et al., 2013) was used. The microbial communities in each enrichment were compared based on the ten (10) most abundant OTUs with a distinctive *genus, i.e.* with most reads assigned to it. The 16S rRNA gene amplicon data have been deposited in GenBank under Bioproject PRJNA490689.

Table 2.3: Origin of the cell free extract (CFE), buffer, substrates, controls and references (Fuhrer and Sauer, 2009; Satoh et al., 2003; Chohan and Copeland, 1998; Olavarria et al., 2015; Dean and Golding, 1997; Bologna, Andreo, and Drincovich, 2007; Giachetti, Pinzauti, and Vanni, 1984; Hillier et al., 1979; Bunik et al., 2005) used for each enzymatic assay.

CFE	Tested enzyme	Buffer	Substrates	Controls	Reference
SBR-1	acetoacetyl-CoA reductase (AAR)	1X, supplemented	200 μM acetoacetyl-CoA 200 μM NADH or 200 μM NADPH	No acetoacetyl-CoA; *E. coli* MG1655; *E. coli* MG1655 + p-*phaCAB*[*]	(Satoh et al., 2003)
SBR-2	acetoacetyl-CoA reductase (AAR)	1X, supplemented	40 μM acetoacetyl-CoA 100 μM NADH or 200 μM NADPH	No acetoacetyl-CoA; Isolated & purified enzyme	(Chohan and Copeland, 1998)
SBR-2	3-hydroxybutyryl-CoA dehydrogenase (3HBDH)	1X, supplemented	100 μM 3-hydroxybutyryl-CoA 1000 μM $NAD(P)^+$	No hydroxybutyryl-CoA	(Chohan and Copeland, 1998)
SBR-1	glucose-6-phosphate dehydrogenase (G6PDH)	1X, supplemented	2 mM glucose-6-phosphate 2000 μM NAD^+ or 200 μM $NADP^+$	No glucose-6-phosphate; No CFE	(Olavarria et al., 2015)
SBR-2	glucose-6-phosphate dehydrogenase (G6PDH)	1X, supplemented	4 mM glucose-6-phosphate 400 μM $NAD(P)^+$	No glucose-6-phosphate; No CFE	
SBR-2	isocitrate dehydrogenase (ICDH)	1X, supplemented	400 μM $NAD(P)^+$ 400 μM isocitrate	No substrate; No CFE	(Dean and Golding, 1997)
	malate dehydrogenase (MDH)	1X, supplemented	200 μM NAD(P)H 200 μM oxaloacetate		(Fuhrer and Sauer, 2009)
	malic enzyme (ME)	1X, supplemented	200 μM $NADP^+$ 200 μM L-malate		(Bologna, Andreo, and Drincovich, 2007)
	isocitrate lyase (ICL)	40 mM HEPES, pH 7 6 mM $MgCl_2$	4 mM isocitrate 280 μM NADH 45 units lactate dehydrogenase from rabbit muscle (Roche)	No isocitrate; No CFE; *E. coli* MG1655 grown on acetate; *E. coli* JW3975[*2] grown on glucose	(Giachetti, Pinzauti, and Vanni, 1984)
	fumarate reductase (FR)	50 mM HEPES, pH 7	15 mM fumarate 250 μM NADH	No substrate; No CFE	(Hillier et al., 1979)
	α-ketoglutarate dehydrogenase (AKGDH)	50 mM HEPES, pH 7 1 mM $MgCl_2$ 1 mM Thiamine pyrophosphate, 2.5 mM DTT	2 mM α-ketoglutaric acid 100 μM Coenzyme A 2.5 mM $NAD(P)^+$		(Bunik et al., 2005)

[*] Plasmid bearing the genes phaCAB from C. necator under the control of its native promoter. These genes encode for the enzymes of the pathway leading to polyhydroxybutyrate (PHB) synthesis and it was kindly donated by Dr. J.G. Cabrera Gomez from Universidade de Sao Paulo, Brazil.
[*2] Strain without isocitrate lyase from Keio collection (Baba et al., 2006): F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, ΔaceA782::kan, hsdR514

ANAEROBIC BIOCHEMICAL MODEL OF ACCUMULIBACTER

The metabolic network shown in Figure 2.2 is based on the ancestral genome reconstruction of Accumulibacter proposed by Oyserman et al., 2016a as well as experimental observations obtained here. Additionally, an extensive literature overview supporting this biochemical model of Accumulibacter can be found in Supplementary Table 2.4. There are more complex metabolic models, including genome-scale metabolic models available. For the purpose of this study, we focused on central carbon metabolism and lumped reactions capturing main functionalities.

FLUX BALANCE ANALYSIS (STOICHIOMETRIC MODELING)

To estimate the possible metabolic flux distributions Flux Balance Analysis (FBA) has been applied (Orth, Thiele, and Palsson, 2010). The flux distribution $\boldsymbol{\nu}_{opt}$ can be obtained by optimization:

$$\boldsymbol{\nu}_{opt} = \arg\min_{\boldsymbol{\nu}} \nu_{CO2_{prod}} \quad \text{subject to} \quad \begin{cases} N\boldsymbol{\nu} = 0 \\ \nu_{irr} \geq 0 \\ \nu_{Glyc_{deg}} = 1 - f \\ \nu_{Ac_{upt}} = f \end{cases} \tag{2.1}$$

$$\text{with} \quad f = 0...1 \frac{Cmol_{Acetate}}{Cmol_{C_{consumed}}} \tag{2.2}$$

where N is the stoichiometry matrix containing the balanced metabolites and reactions (see Table 2.2) and $\boldsymbol{\nu}$ is the vector containing all reaction fluxes. The simulation assumes that none of the balanced intermediates is accumulating inside the cell (steady-state assumption). This is justified by the short turnover time of the intracellular metabolites compared to the timespan of the experiment that is simulated. Inequality constraints were introduced for physiologically irreversible fluxes (ν_{irr}) that should always be positive. The consumption of acetate ($\nu_{Ac_{upt}}$) and glycogen ($\nu_{Glyc_{deg}}$) is varied between only acetate ($f = 1$) to only glycogen ($f = 0$). The respective experimental data is normalized to a summed consumption of 1 Cmol of C-source.

Commonly, maximization of biomass synthesis is used in FBA as an optimization objective. However, Accumulibacter only produces biomass aerobically and using the intracellular carbon reserves (PHAs) accumulated during substrate uptake in the anaerobic period. Biomass synthesis was thus assumed proportional to the carbon stored as PHA. The objective of maximal C storage was 'inverted' to minimal loss of carbon to the extracellular space, which for the used network is only CO_2 production ($\nu_{CO2_{prod}}$). Using this objective no sum of carbon in the different PHA pools is required, avoiding a putative bias towards one of those.

Furthermore, to prevent adding a stoichiometric constraint due to an assumption on the proportion of the different PHA polymers possible, *i.e.* poly-3-hydroxybutyrate (PHB), poly-3-hydroxyvalerate (PHV), poly-3-hydroxy-2-methylbutyrate (PH2MB), or poly-3-hydroxy-2-methylvalerate (PH2MV), the respective monomer amount is introduced, *i.e.* the reduced precursors Ac-CoA* and Pr-CoA* such that:

$$2\ \text{Ac-CoA}^* \rightarrow 3\text{HB}$$
$$\text{Ac-CoA}^* + \text{Pr-CoA}^* \rightarrow 3\text{HV (or 3H2MB)}$$
$$2\ \text{Pr-CoA}^* \rightarrow 3\text{H2MV}$$

The stoichiometric model was checked for conserved moieties, blocked reactions and non-identifiable reactions that form internal cycles. No conserved moieties nor blocked reactions were found, and the internal cycles found did not respect the reversibility constraints. After analysis, the network contained 9 independently balanced intermediates, 13 (lumped) reactions and, thus 4 degrees of freedom. ATP was included in the analysis but was not balanced (*i.e.* not a constraint).

To identify whether the solutions obtained from linear programming for different ratios of glycogen-to-acetate (see equation above) are unique, a nullspace analysis was performed. For this optimization, three extracellular rates are fixed: Uptake of glycogen and acetate and the objective, production of CO_2. When these rates are fixed, one degree of freedom remains, formed by one putative cycle comprising the reactions PDH, (reverse) PEPC, (reverse) oxTCA, TCAGOX, ATPnet which is an infeasible cycle given the thermodynamic (reversibility) constraints. Therefore, the flux solution for the *optimum* is unique.

COMPILATION AND NORMALIZATION OF STOICHIOMETRIC DATA

The results of the aforementioned simulations were compared to stoichiometric data reported in literature for several different Accumulibacter enrichments. Anaerobic-feast yields were used when available, otherwise the yields of the whole anaerobic phase were used. In case of missing compound rates (usually CO_2, PH2MV), these were estimated using electron and carbon balancing. For datasets with redundant measurements, a data reconciliation method was applied (Heijden et al., 1994). For all calculations, we assumed acetate and glycogen monomers were the only substrates and CO_2, PHB, PHV and PH2MV were the only products. In case yields were reported without the respective error, the error was calculated using error propagation. Here, the relative errors were assumed as follows: 5% for acetate and PHB measurements, and 10% for PHV, PH2MV and glycogen measurements. From the (reconciled) rates for PHB, PHV and PH2MV, the respective Ac-CoA* and Pr-CoA* rates were determined and normalized to the amount of consumed substrates, in Cmol.

DATA AVAILABILITY

FISH micrographs, a phylogenetic tree based on Accumulibacter's *ppk1* gene analysis, and a taxonomic distribution of microbial community based on 16S rRNA gene copy numbers can be found in Supplementary Figure 2.6 through 2.10. The *ppk1* gene sequences obtained in this study have been deposited in the GenBank database (`https://www.ncbi.nlm.nih.gov/`) under accession numbers MH899084-MH899086. The 16S rRNA gene amplicon data have been deposited in GenBank under Bioproject PRJNA490689. The AAR protein sequences, and enzymatic assays data are available in Supplementary Section 2.6.4.

The MATLAB (MathWorks, Natick, MA, USA) code and data used for the simulations are available in a repository in GitHub:

`https://github.com/cell-systems-engineering-tud/anaerobic-flex-pao`

All simulations were performed using MATLAB version R2018a (9.4.0.813654).

SUPPLEMENTARY INFO

S1. LITERATURE OVERVIEW ON ACCUMULIBACTER'S BIOCHEMICAL MODEL

Table 2.4: Overview of evidence found in literature studies on the biochemical model of Accumulibacter. Note: some studies refer to the general term "PAO" and not Accumulibacter. Furthermore, apart from the meta-omics analysis, all other evidence is originated from enrichments of Accumulibacter and thus may contain noise from side-populations.

Pathway	Enzyme(s)	Type of analysis	Findings and references
Acetate transport and activation	Na^+/H^+-Acetate permease symporter	Metaproteomics	(Wilmes et al., 2008)
	Acetyl-CoA synthetase (ACS)	Batch test + inhibition with Ap5A	Decreased acetate uptake (Hesselmann et al., 2000)
	Acetyl-CoA synthetase (ACS)	Metaproteomics, metatranscriptomics	Higher protein levels than ACK (Wilmes et al., 2008), highly expressed in anaerobic acetate contact (Oyserman et al., 2016b)
	Acetate kinase (ACK)	Batch test + inhibition with Ap5A	Did not affect acetate uptake (Hesselmann et al., 2000)
	Acetate kinase (ACK)	Metaproteomics	Lower protein levels than ACS (Wilmes et al., 2008)
	-	Metagenomics and metaproteomics	Present (Barr et al., 2016)
Phosphate transport	Low-affinity phosphate transporter (Pit)	Metagenomics, metatranscriptomics and metaproteomics	Found (García Martín et al., 2006; Skennerton et al., 2015); Present only in flocs (Barr et al., 2016); Two present, one high anaerobically and the other aerobically (Wilmes et al., 2008); highly expressed in high P conditions (Oyserman et al., 2016b)
	High-affinity phosphate transporter (Pst)	Metagenomics, metatranscriptomics and metaproteomics	Found (Skennerton et al., 2015); Present (Barr et al., 2016; He et al., 2010; Wilmes et al., 2008); highly expressed in low P conditions (Oyserman et al., 2016b)
Polyphosphate metabolism	PPi:PEP:AMP phosphotransferase PolyP:AMP phosphotransferase	Enzymatic assay	Active (Hesselmann et al., 2000)
	ppk1 (PolyP production) *ppx* (PolyP hydrolysis)	Metagenomics	Present (Skennerton et al., 2015)
	ppk1	qRT-PCR	Expression increased with acetate anaerobically and in early aerobic phase, decreased notably when all phosphate was taken up aerobically (He and McMahon, 2011a)

Full TCA cycle (ext. electron acceptor absent)	-	^{13}C NMR, TOGA-sensor	(Pereira et al., 1996; Louie et al., 2000) (Lemos et al., 2003) – propionate Very high $^{13}CO_2$ fraction suggests full TCA (Zhou et al., 2009), but low $^{14}CO_2$ fraction has also been reported (Bordacs and Chiesa, 1989)
"Horse-shoe" / Branched / Split TCA	-	^{13}C NMR	(Hesselmann et al., 2000; Erdal, Erdal, and Randall, 2005)
TCA cycle	All TCA cycle enzymes	Metagenomics, metatranscriptomics and metaproteomics	Found (Oyserman et al., 2016a; Barr et al., 2016; Oyserman et al., 2016b; García Martín et al., 2006; He and McMahon, 2011a; Skennerton et al., 2015; He et al., 2010; Wilmes et al., 2008; Flowers et al., 2013)
	2-Oxoglutarate: ferredoxin oxidoreductase (OGOR)		
	Fumarate reductase (FR)		
	Malate dehydrogenase (MDH)	Radiolabeled proteomics	Synthesized aerobically for Accumulibacter I, both anaerobic and aerobic for Accumulibacter I+II (Wexler, Richardson, and Bond, 2009)
	Isocitrate dehydrogenase (ICDH)	Radiolabeled proteomics	Synthesized mostly anaerobically (Wexler, Richardson, and Bond, 2009)
	Fumarate reductase (FR)	Batch test + inhibition with oxantel	Did not affect acetate uptake (Burow, Mabbett, and Blackall, 2008)
	Fumarate reductase (FR)	qRT-PCR	Expression increased with acetate anaerobically and in early aerobic phase (He and McMahon, 2011a)
	Succinate dehydrogenase (SDH)	Radiolabeled proteomics	Synthesized aerobically (Wexler, Richardson, and Bond, 2009)
	Other TCA enzymes except MDH		
	Succinate dehydrogenase (SDH)	qRT-PCR	Expression increased anaerobically in presence of acetate (Burow, Mabbett, and Blackall, 2008), expression increased in early aerobic phase (He and McMahon, 2011a)
		Batch test + inhibition with malonate	Decreased acetate uptake (Burow, Mabbett, and Blackall, 2008; Louie et al., 2000)
	Aconitase	Batch test + inhibition with fluoroacetate	Decreased acetate uptake (Burow, Mabbett, and Blackall, 2008; Louie et al., 2000);

		Batch test + inhibition with α-ketoglutarate	Decreased acetate uptake (Louie et al., 2000);
Methylmalonyl-CoA pathway	Methylmalonyl-CoA mutase	Metagenomics, metatranscriptomics and metaproteomics, qRT-PCR	Found (Skennerton et al., 2015; He and McMahon, 2011a; He et al., 2010; Wilmes et al., 2008); Induced anaerobically (He and McMahon, 2011a)
Glyoxylate shunt	Isocitrate lyase	Enzymatic assay	Active (Hesselmann et al., 2000) (this study)
			Not active, but still authors suggest the shunt is active (Erdal, 2002; Erdal, Erdal, and Randall, 2005)
	Malate synthase	Enzymatic assay	Active (Erdal, 2002; Erdal, Erdal, and Randall, 2005)
	Isocitrate lyase	Batch test + inhibition with 3-nitropropionate and itaconate	Decreased acetate uptake (Burow, Mabbett, and Blackall, 2008)
	Aconitase	qRT-PCR	Expression increased anaerobically in presence of acetate and aerobically (Burow, Mabbett, and Blackall, 2008; He and McMahon, 2011a)
	Isocitrate lyase		
	Isocitrate lyase	Metagenomics, metatranscriptomics and metaproteomics, radiolabeled proteomics	Present (Oyserman et al., 2016a; Skennerton et al., 2015; Wilmes et al., 2008; Wexler, Richardson, and Bond, 2009; He and McMahon, 2011a), highly expressed/synthesized in aerobic conditions (Oyserman et al., 2016b; Wexler, Richardson, and Bond, 2009)
	Malate synthase		
	Isocitrate lyase	^{13}C-Metaproteomics	Higher aerobically than anaerobically (Wexler, Richardson, and Bond, 2009)

Glycogen utilization	Glycogen debranching enzyme (Gde)	Metagenomics and metaproteomics	(Barr et al., 2016)
	UDP-glucose 6-dehydrogenase (ywqF)		
Glycolysis	-	^{13}C NMR	(Pereira et al., 1996; Satoh, Mino, and Matsuo, 1992)
	Glyceraldehyde-3-P dehydrogenase	Batch test + inhibition with iodoacetate	Decreased glycogen utilization (Burow, Mabbett, and Blackall, 2008)
Glycolysis – ED	-	^{13}C NMR	Most likely active (Maurer et al., 1997; Hesselmann et al., 2000)

	Glucose-6-P dehydrogenase	Metagenomics	Not found (García Martín et al., 2006)
	Glucose-6-P dehydrogenase	Enzymatic assay	Not active (Erdal, 2002; Erdal, Erdal, and Randall, 2005), (this study)
Glycolysis – EMP	Phosphofructokinase	Enzymatic assay	Active (Erdal, 2002; Erdal, Erdal, and Randall, 2005)
	All steps from glycogen to pyruvate	Metagenomics, metatranscriptomics and metaproteomics, radiolabeled proteomics	Found (García Martín et al., 2006; Oyserman et al., 2016b; Barr et al., 2016; Wilmes et al., 2008; Wexler, Richardson, and Bond, 2009; Flowers et al., 2013; He et al., 2010)
	Bifunctional PGK/TIM fusion protein	Metagenomics and metaproteomics	Present, more efficient than individual PGK and TIM (Barr et al., 2016)

Pyruvate -"related" pathways	Pyruvate dehydrogenase	Metagenomics and metaproteomics	Present (Barr et al., 2016; Oyserman et al., 2016a)
	Pyruvate:ferredoxin oxidoreductase (Pyruvate synthase)		
	Pyruvate dehydrogenase	qRT-PCR	Expression increased in early aerobic phase (He and McMahon, 2011a)
	Pyruvate synthase	qRT-PCR	Expression increased with acetate anaerobically but decreased without acetate (He and McMahon, 2011a)
	NADP-Malic enzyme	Metagenomics and metaproteomics	Present (Barr et al., 2016)
	Phosphoenolpyruvate carboxykinase	Metagenomics and metatranscriptomics	Highly expressed in anaerobic acetate contact (Oyserman et al., 2016b)
	Pyruvate kinase	Metagenomics, metatranscriptomics	Highly expressed in anaerobic acetate contact (Oyserman et al., 2016b)

Redox balancing enzymes	Complex Cytochrome b/b6 (acting as reverse quinol-NAD(P) reductase and allowing $FADH_2$ oxidation anaerobically)	Metagenomics	Found and proposed (García Martín et al., 2006)
			Found and pointed-out it was reported under the wrong gene-id (Flowers et al., 2013)
			Found (Skennerton et al., 2015)

		Metatranscriptomics and metaproteomics	Found (He and McMahon, 2011a) – but wrong gene id targeted (Flowers et al., 2013)
			No peptide hits (Barr et al., 2016) – possibly wrong gene id targeted?
		qRT-PCR	Expression increased anaerobically in presence of acetate (Burow, Mabbett, and Blackall, 2008) – but wrong gene id targeted (Flowers et al., 2013)
	Na^+-translocating NADH-quinone reductase	Metagenomics	Found (Flowers et al., 2013)
	RuBisCO (and other Calvin cycle enzymes)	Metagenomics, metatranscriptomics	Found (García Martín et al., 2006; Skennerton et al., 2015), not found for clade IA (Flowers et al., 2013), highly expressed in low P conditions (Oyserman et al., 2016b)
	Cytoplasmic Ni-Fe hydrogen dehydrogenase	Batch test, metatranscriptomics and metagenomics	Hydrogen gas production in closed anaerobic bottle with acetate and highly expressed in anaerobic acetate contact (Oyserman et al., 2016b)
	Membrane-bound hydrogenase		
	Membrane-bound transhydrogenase	Metagenomics, metatranscriptomics and metaproteomics	Present (Oyserman et al., 2016a; Barr et al., 2016; Oyserman et al., 2016b)
	Soluble transhydrogenase	Metagenomics	Present (Oyserman et al., 2016a)

Other	Glutamine synthetase	Radiolabeled proteomics	Synthesized mostly anaerobically (Wexler, Richardson, and Bond, 2009)
	Acetyl-CoA acetyltransferase (fatty acid β-oxidation)	Radiolabeled proteomics	Synthesized mostly anaerobically (Wexler, Richardson, and Bond, 2009)

S2. THERMODYNAMICS OF REDUCING EQUIVALENTS BALANCING IN ACCUMULIBACTER

To analyze the thermodynamic feasibility of how Accumulibacter balance reducing equivalents, the conversion of acetate to 3-hydroxybutyrate (3HB), the monomer of the reserve polymer poly-3-hydroxybutyrate (PHB), can be used as a starting point (for simplification, only acetate is considered as the carbon source ($C_2H_4O_2$, 4 e^-/C) and 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) as monomers for PHAs ($C_4H_8O_3$, 4.5 e^-/C and $C_5H_{10}O_3$, 4.8 e^-/C, respectively):

$$2\ \text{Acetate} + 2\ e^- \rightleftharpoons 3\text{HB}$$

To maintain the electron supply during acetate conversion to PHA, Accumulibacter have to harvest electrons elsewhere in their metabolism. To date, the following sources have been proposed (and thoroughly reviewed in (Zhou et al., 2009)):

a. Glycogen oxidation to acetyl-CoA and CO_2;

b. Acetate oxidation to CO_2;

c. 2 Acetate oxidation to propionyl-CoA and CO_2;

d. Glycogen oxidation to propionyl-CoA and CO_2.

Anaerobic acetate oxidation alone does not provide net energy and thus this option is only possible thanks to a special feature of Accumulibacter: energy generation from the mobilization of previously stored polyphosphate. On the other hand, glycogen oxidation does generate ATP. Glycogen oxidation is actually the strategy employed by Glycogen Accumulating Organisms (GAOs) to compete with PAOs for substrate, anaerobically. Accumulibacter also make use of glycogen, especially when phosphate is scarce in their environment and polyphosphate storage is limited (PAM/GAM switch, (Acevedo et al., 2012; Acevedo et al., 2014; Welles et al., 2015; Welles et al., 2016; Zhou et al., 2008)). Thus far, for the oxidation of glycogen, Embden-Meyerhof- Parnas (EMP) glycolysis has been confirmed several times to be the active pathway for the oxidation of glycogen into pyruvate (for evidence, see Supplementary Table 2.4). In this pathway, reducing equivalents in the form of NADH are produced in the reaction catalyzed by glyceraldehyde-phosphate dehydrogenase (GAPDH):

$$\text{GAP} + \text{phosphate} + \text{NAD}^+ \rightleftharpoons \text{3-phospho-D-glyceroyl phosphate} + \text{NADH}$$

In this study we found a direct link between the reducing equivalents source (glycolysis) and sink (PHA accumulation) – NAD(H) – by unravelling the redox cofactor preference of acetoacetyl-CoA reductase (AAR):

$$\text{Acetoacetyl-CoA} + \text{NADH} \rightleftharpoons \text{3HB-CoA} + \text{CoA} + \text{NAD}^+$$

For both NADH-producing and consuming reactions to run forward, the remaining reaction species are "responsible" to keep the reactions' Gibbs free energies ($\Delta_r G$) negative, *i.e.* feasible. Thus, we asked the question *"can GAP DH (glycolysis) and AAR (PHA formation) run simultaneously when using the same redox cofactor couple, NAD/NADH?"*. In Figure 2.5

one can see the reaction catalyzed by AAR has a rather broad feasible range (gray area), since one of its products, 3HB-CoA is simultaneously removed and polymerized into the solid state. Therefore, the ratio CoA/Acetoacetyl-CoA is likely what defines the direction of this reaction. The reaction catalyzed by GAPDH has a rather narrow feasible range (yellow area). Nevertheless, when overlapped, one can see the feasible range in which both GAPDH and AAR can operate simultaneously lies well within any of the typical values for the ratio NAD/NADH found in different types of cells under different conditions. Therefore, both reactions can run simultaneously.

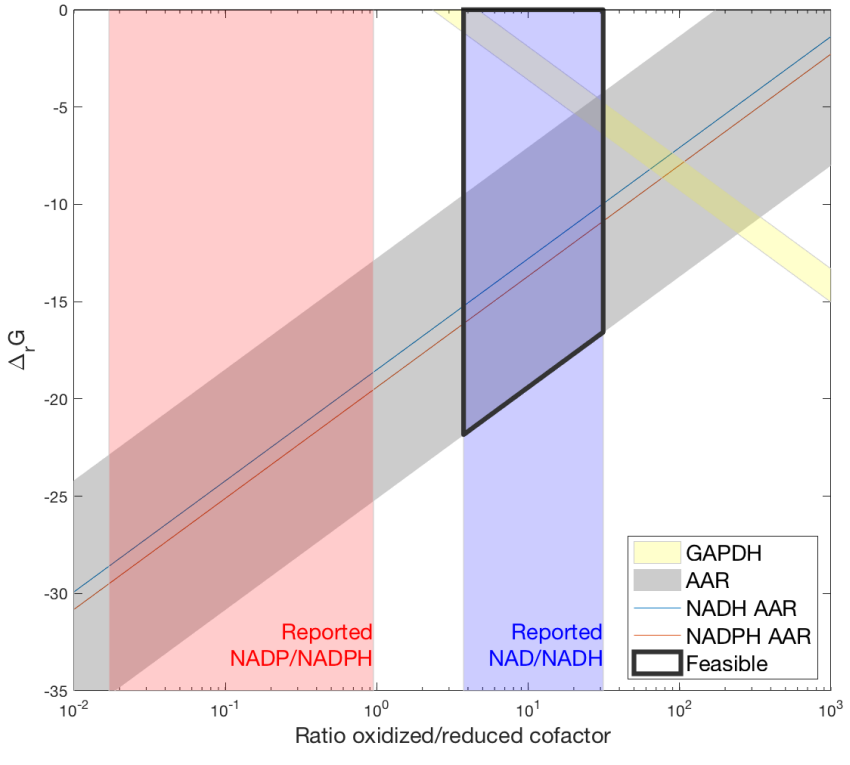


Figure 2.5: Thermodynamic analysis of the simultaneous operation of GAP dehydrogenase (GAPDH) and acetoacetyl-CoA reductase (AAR). Standard Gibbs free energies ($\Delta_r G^0$) were obtained from eQuilibrator (Flamholz et al., 2012) and the typical values for the ratios NAD(P)/NAD(P)H from (Spaans et al., 2015). For the reaction catalyzed by GAP dehydrogenase, the ratio [1,3-biphosphoglycerate]/[GAP] was assumed to be 0.1 and [Pi] was varied from 50 to 100 mM based on assumptions made for other microorganisms and assuming that in PAOs, anaerobically, this value is likely higher than for a normal microorganism. For the reaction catalyzed by acetoacetyl-CoA reductase, the ratio between [3HB][CoA]/[AcAcCoA] was assumed to vary between 0.1 (more negative $\Delta_r G$) and 10 (less negative $\Delta_r G$).

S3. MICROBIAL CHARACTERIZATION

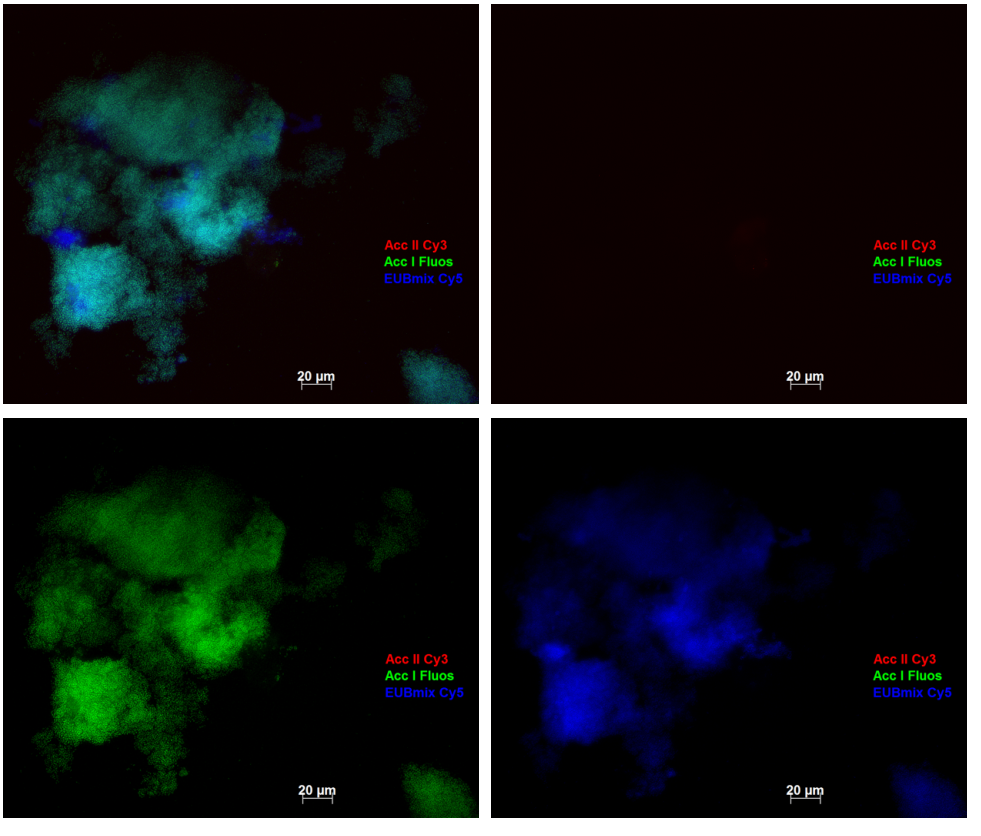


Figure 2.6: Standard fluorescence *in situ* hybridization (FISH) images from the microbial community present in SBR-1: **Top-left** All fluorescence channels merged; **Bottom-left** Cells hybridized with Accumulibacter clade I probe (Acc-1-444); **Top-right** Cells hybridized with Accumulibacter clade II probe (Acc-2-444); and **Bottom-right** Cells hybridized with the general bacteria EUB338 probes. This community was also tested with GAOmix (data not shown) and no hybridization was observed.

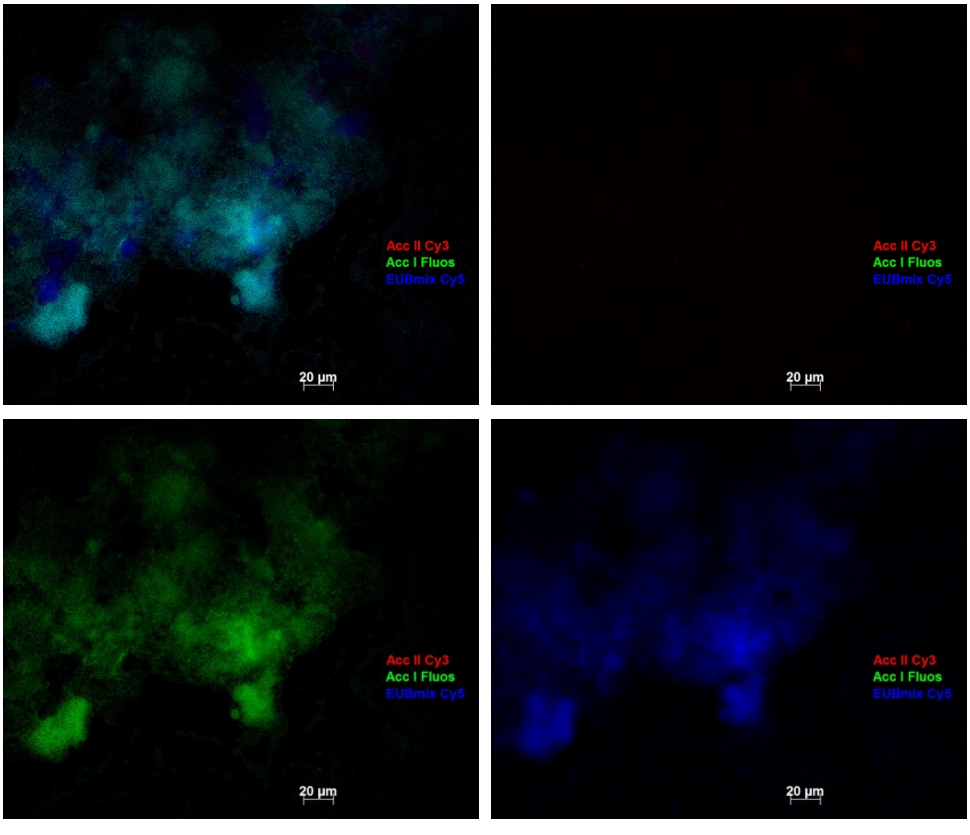


Figure 2.7: Standard fluorescence *in situ* hybridization (FISH) images from the microbial community present in SBR-2 at the start of the experiments: **Top-left** All fluorescence channels merged; **Bottom-left** Cells hybridized with Accumulibacter clade I probe (Acc-1-444); **Top-right** Cells hybridized with Accumulibacter clade II probe (Acc-2-444); and **Bottom-right** Cells hybridized with the general bacteria EUB338 probes. This community was also tested with GAOmix (data not shown) and a negligible amount of GAOs was observed.

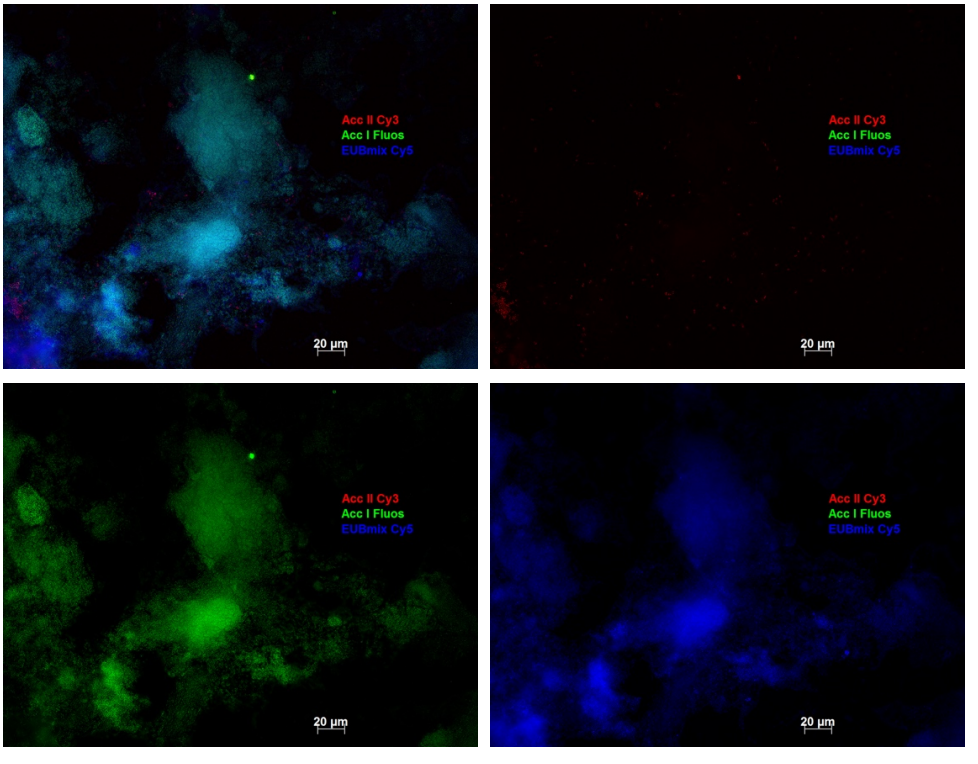


Figure 2.8: Standard fluorescence *in situ* hybridization (FISH) images from the microbial community present in SBR-2 at the end of the experiments (approx. 2 months later): **Top-left** All fluorescence channels merged; **Bottom-left** Cells hybridized with Accumulibacter clade I probe (Acc-1-444); **Top-right** Cells hybridized with Accumulibacter clade II probe (Acc-2-444); and **Bottom-right** Cells hybridized with the general bacteria EUB338 probes.

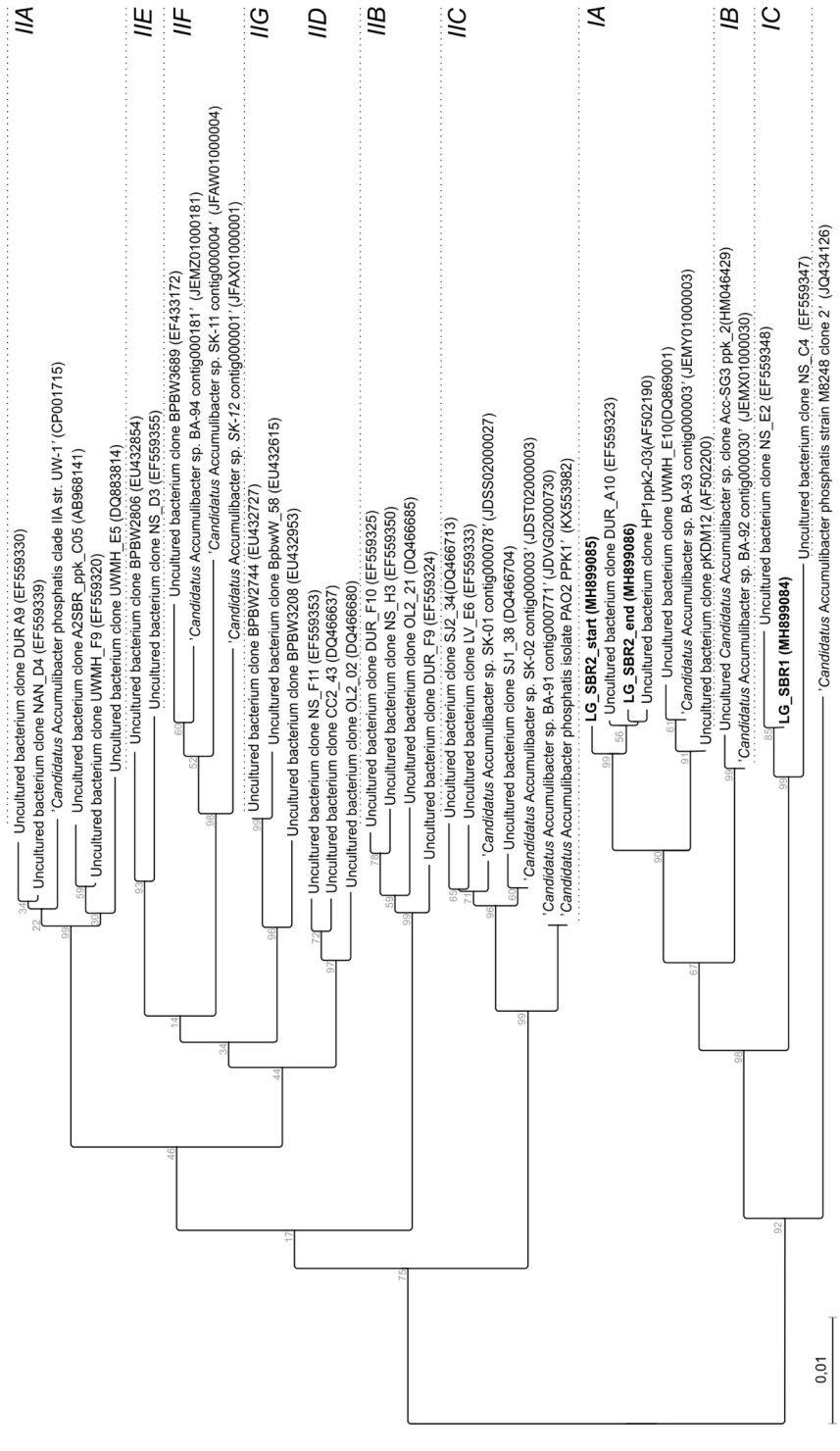
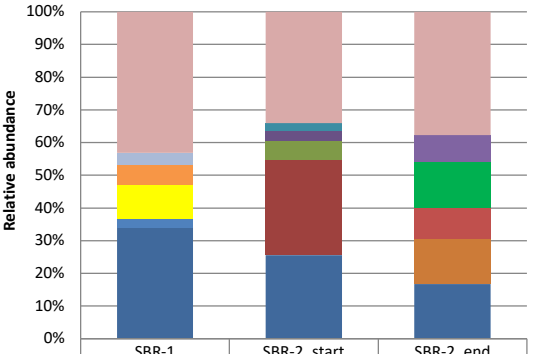


Figure 2.9: Phylogenetic tree based on Accumulibacter's *ppk1* gene analysis. Both SBR-1 and 2 contained Accumulibacter type I, but clade IC and IA, respectively.



	SBR-1	SBR-2_start	SBR-2_end
others	43.2	34.1	37.7
Lachnotalea	3.6		
Blastochloris	6.2		
Rhodopseudomonas	10.3		
Aquimonas			8.3
Chryseobacterium			14.0
Flavobacterium			9.6
Rhodobacter	2.7		
genus from o__Sphingobacteriales (2)			13.7
Lactobacillus		2.4	
genus from o__Sphingobacteriales		2.7	
genus from f__Hyphomonadaceae		6.0	
Dechloromonas		29.3	
Candidatus_Accumulibacter	34.1	25.5	16.7

Figure 2.10: Taxonomic distribution of microbial community based on 16S rRNA gene copy numbers. The 16S rRNA gene amplicon libraries have been deposited under project PRJNA490689.

S4. ENZYMATIC ASSAYS WITH CELL FREE EXTRACTS OF TWO INDEPENDENT ENRICHMENTS OF ACCUMULIBACTER

PRIMERS USED TO AMPLIFY *phaB* GENE FROM ACCUMULIBACTER

PAOphaB_BamUpCrt: tcgataGGATCCatgacgcaacgtgttgctttggttacg

PAOphaB_XbaDwCrt: tgtgaaTCTAGAttactgatagtagaggccaccacag

PROTEIN SEQUENCES CORRESPONDING TO REFERENCE AND CLONED GENE

> Acetoacetyl-CoA reductase [EC:1.1.1.36]
(*Candidatus* Accumulibacter phosphatis, KEGG CAP2UW1_3919)
MTQRVALVTGAMGGIGTAICQELAKAGHKVVAAYHPEFDKPEEWTKAMAEAGFNDFICVAGDVSDYDSCVALTAEAEAKAGPIDILV NNAG-
ITRDKMFARMELAQWNAVISTNLSSLFNMTKQVSAKMAERGWGRIINISSLNGLKGQAGQTNYSAAKAGVIGFTKALAAEVAA KGVTV-
NAICPGYVATPMVMAIKPEILQGIVDTVPMKRLAKPEEIGGACAYLASDIAGFMTGSTMNICGGLYYQ

> colony6
MTQRVALVTGAMGGIGTAICQELAKAGHKVVAAYHPEFDKPAEWTKAMEEAGFKDFICVAGDVSDYDSCVALTAEAEAKAGPIDILV NNAG-
ITRDKMFAKMELAQWNAVISTNLNSLFNMTKQVSAKMAERGWGRIINISSLNGLKGQAGQTNYSAAKAGVIGFSKALAAEVAA KGVTVN-
SICPGYVATPMVMAIKPEILQSIIATVPMKRLAQPEEIGGACAYLASDLAGFMTGSTMNICGGLYYQ

PHB PATHWAY

From the PHB pathway, the AcAcCoARed and the reverse reaction, 3HBCoA dehydrogenation, from a microbial community enriched with Accumulibacter were assessed, as a result the specific activities were determined as can be seen in Table 2.5.

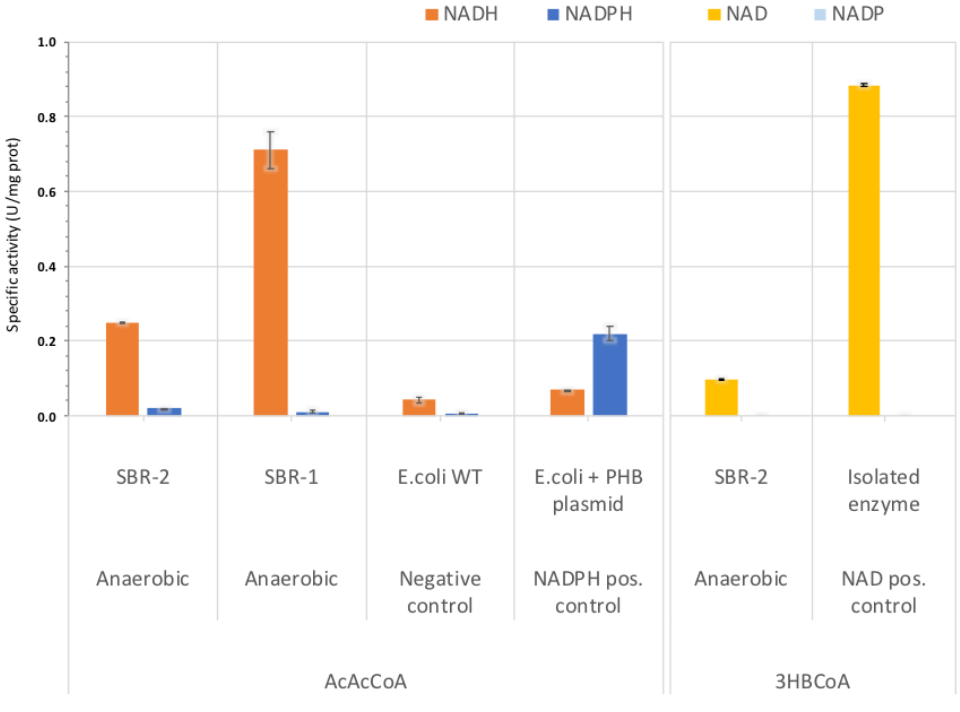


Figure 2.11: Accumulibacter acetoacetylCoA (AcAcCoA) reductase (step in PHA synthesis pathway) specific activity during the anaerobic phase. Note that in the assay with CFE from SBR-2 the used concentration of AcAcCoA was five times (5x) lower, hence the lower specific activity when compared to that of SBR-1. Specific activities during the aerobic phase are comparable and thus not shown here (see Table 2.5). Controls: **(1)** *E. coli* WT was used as negative control (still, a minor activity with NAD(H) is expected since this enzyme is also involved in fatty acids synthesis and is present in *E. coli*), **(2)** *E. coli* with a PHB plasmid from *R. eutropha* was used as NADP(H)-positive control, and **(3)** Purified enzyme from Accumulibacter (overexpressed and purified in *E. coli*)

Table 2.5: AcetoAcetyl-CoA reduction and 3-hydroxybutyryl-CoA dehydrogenation average specific activities (U/ mg protein) in a cell free extract from a microbial community enriched in Accumulibacter with NAD(P)H and NAD^+ / $NADP^+$, respectively, during anaerobic and aerobic phases. Standard deviations were calculated based on at least two (2) technical replicates. Due to absorption of acetoacetyl-CoA at 340nm, absorbance was also measured at 310 nm for comparison but it had no effect in the conclusion regarding the cofactor specificity of this enzyme. Refer to Table 2.3 for the used conditions in each assay. The clear cofactor preferences are marked in **bold**.

Substrate	Type CFE	CFE	Cofactor	Sp. activity NAD(P)H 340nm (U/mg prot)	SD	Sp. activity $NAD(P)^+$ 340nm (U/mg prot)	SD	Sp. activity NAD(P)H 310nm (U/mg prot)	SD
AcAcCoA	Anaerobic	SBR-2	**NADH**	**0.249**	**0.003**			0.35	0.01
	Anaerobic	SBR-1	**NADH**	**0.71**	**0.05**				
	Negative control	*E.coli* WT	**NADH**	**0.042**	**0.008**				
	NADPH pos. control	*E.coli* + PHB plasmid	NADH	0.0691	0.0008				
3HBCoA	Anaerobic	SBR-2	**NAD**			**0.098**	**0.001**		
	NAD pos. control	Isolated enzyme	**NAD**			**0.884**	**0.003**		
AcAcCoA	Aerobic	SBR-2	**NADH**	**0.2**	**9E-16**			**0.199**	**0.003**
	Aerobic	SBR-1	**NADH**	**0.8**	**0.1**				
3HBCoA	Aerobic	SBR-2	**NAD**			**0.226**	**0.009**		
AcAcCoA	Anaerobic	SBR-2	NADPH	0.020	0.001			0.053	0.009
	Anaerobic	SBR-1	NADPH	0.010	0.004				
	Negative control	*E.coli* WT	NADPH	0.0060	0.0004				
	NADPH pos. control	*E.coli* + PHB plasmid	**NADPH**	**0.22**	**0.02**				
3HBCoA	Anaerobic	SBR-2	NADP			5E-15	5E-15		
	NAD pos. control	Isolated enzyme	NADP			8E-14	2E-14		
AcAcCoA	Aerobic	SBR-2	NADPH	0.005	0.002			0.024	0.007
	Aerobic	SBR-1	NADPH	0.012	0.002				
3HBCoA	Aerobic	SBR-2	NADP			0.02	0.01		

GLYCOLYSIS – ED PATHWAY?

In Table 2.6, the specific activities determined with NAD and NADP for the G6PDH for the anaerobic phase, the aerobic phase and the biological controls can be found.

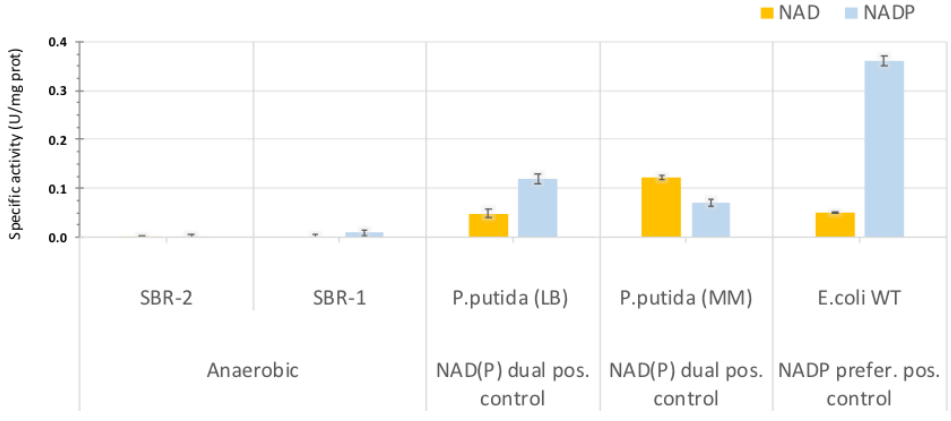


Figure 2.12: Glucose-6-phosphate dehydrogenase (G6PDH) specific activity (U/mg protein) in a microbial community enriched in Accumulibacter with NAD(P)$^+$ during anaerobic and aerobic phase. Error bars represent standard deviation (four (4) technical replicates). CFEs from SBR-1, *Pseudomonas putida* grown in LB, *P. putida* grown in MM and *E. coli* WT were all assayed together except the SBR-2 CFE which was assayed separately at a later stage.

Table 2.6: Glucose-6-phosphate dehydrogenase (G6PDH) average specific activity (U/mg protein) in a microbial community enriched in Accumulibacter with NAD(P)$^+$ during anaerobic and aerobic phase. Standard deviations are calculated based on, at least, two (2) technical replicates. CFEs from SBR-1, *Pseudomonas putida* grown in LB, *P. putida* grown in MM and *E. coli* WT were all assayed together except the SBR-2 CFE which was assayed separately later. Refer to Table 2.3 for the used conditions in each assay.

Type CFE	CFE	Cofactor	Specific activity (U/mg prot)	SD
Anaerobic	SBR-2	NAD	0.002	0.001
	SBR-1	NAD	0.003	0.005
NAD(P) dual pos. control	*P. putida* (LB)	NAD	0.049	0.01
NAD(P) dual pos. control	*P. putida* (MM)	NAD	0.122	0.004
NADP prefer. pos. control	*E. coli* WT	NAD	0.051	0.002
Aerobic	SBR-2	NAD	0.07	0.01

Anaerobic	SBR-2	NADP	0.003	0.002
	SBR-1	NADP	0.009	0.006
NAD(P) dual pos. control	*P. putida* (LB)	NADP	0.12	0.01
NAD(P) dual pos. control	*P. putida* (MM)	NADP	0.07	0.008
NADP prefer. pos. control	*E. coli* WT	NADP	0.36	0.01
Aerobic	SBR-2	NADP	0.014	0.004

Furthermore, the GAP dehydrogenase was studied with NAD$^+$ and NADP$^+$ for both phases

using a microbial community enriched in Accumulibacter and the results can be found below in Table 2.7. However, no signal was found with $NADP^+$, neither for Accumulibacter nor for the *E.coli* WT control, as expected.

Table 2.7: Glyceraldehyde phosphate dehydrogenase (GAPDH) average specific activity (U/mg protein) in a microbial community enriched in Accumulibacter with $NAD(P)^+$ during anaerobic and aerobic phase. Standard deviations are calculated based on three (3) technical replicates. CFEs from SBR-1 and *E. coli* WT were assayed. Refer to Table 2.3 for the used conditions in each assay.

Type CFE	CFE	Cofactor	Specific activity $NAD(P)^+$ 340nm (U/mg prot)	SD
Anaerobic	SBR-1	NAD	0.32	0.03
Aerobic	SBR-1	NAD	0.20	0.01
NAD pos. control	*E.coli* WT	NAD	0.43	0.01

TCA CYCLE

The TCA cycle oxireductases were also assayed (Table 2.8).

Table 2.8: Accumulibacter TCA cycle oxireductases specific activities (U/mg protein) in a microbial community enriched in Accumulibacter (only using CFEs from SBR-2) during both anaerobic and aerobic phases. No controls were used in these assays as the goal was to only assess the cofactor specificity, *i.e.* NAD(H) or NADP(H). Standard deviations are calculated based on, at least, two technical replicates. The clear cofactor preferences are marked in bold.

Assayed enzyme	Substrate	Type CFE	Cofactor	Specific activity NAD(P)(H) 340nm (U/mg prot)	SD
ICDH	Isocitrate	Anaerobic	NAD	0.011	0.002
		Aerobic	NAD	0.01	0.01
		Anaerobic	**NADP**	**0.80**	**0.02**
		Aerobic	**NADP**	**1.02**	**0.05**
aKGDH	aKetoglutarate	Anaerobic	NAD	0.006	0.002
		Aerobic	NAD	0.0034	0.0003
		Anaerobic	NADP	0.0007	0.0007
		Aerobic	NADP	0.006	0.002
MDH	Oxaloacetate	**Anaerobic**	**NADH**	**8.6**	**0.6**
		Aerobic	**NADH**	**1.1**	**0.2**
		Anaerobic	NADPH	0.13	0.04
		Aerobic	NADPH	0.02	0.04
ME2	Malate	Anaerobic	NADP	0.096	0.008
		Aerobic	NADP	0.0249	0.0006
FR	Fumarate	Anaerobic	NADH	0.0012	0.0004
		Aerobic	NADH	0.006	0.004

The goal was mainly to determine their cofactor specificity, nevertheless, samples were taken from both anaerobic and aerobic phases to also inspect if there would be any difference in the activity of these enzymes between both phases. From the oxidative branch of the TCA cycle, isocitrate dehydrogenase (ICDH) and α-ketoglutarate dehydrogenase (αKGDH) were investigated. From the reductive (reverse) branch of the TCA, malate dehydrogenase (MDH) and fumarate reductase (FR) were tested. Finally, from the anaplerotic routes that connect the lower glycolysis to the TCA, the malic enzyme NADP-dependent (ME 2) was tested. The NAD-dependent ME could not be tested as it is not trivial to separate the contribution of NAD-dependent MDH during the assay.

GLYOXYLATE SHUNT

Also, the presence of the glyoxylate shunt was checked by assessing the enzyme that catalyses its first step: isocitrate lyase. Here we used a coupled assay with lactate dehydrogenase which oxidizes the glyoxylate produced by the isocitrate lyase. The results can be found below in Table 2.9.

Table 2.9: Isocitrate lyase (ICL) specific activity (U/mg protein), measurement with an indirect method with rabbit lactate dehydrogenase (LDH), in a microbial community enriched in Accumulibacter during anaerobic and aerobic phases. Using as biological controls the ICL of *E. coli* WT MG1655 (strain K12) grown on MM with acetate and of *E.coli* JW3975 grown on MM with glucose. Standard deviations are calculated based on, at least, 2 technical replicates.

Type CFE	CFE	Specific activity NAD(P)H 340nm (U/mg prot)	SD
Anaerobic	SBR-2	0.07	0.01
Aerobic	SBR-2	0.02	0.01
Pos. control	*E.coli* WT (MM+Acetate)	0.28	0.02
Neg. control	*E.coli* JW3975 (MM+Glucose)	0.01	0.01

ANAEROBIC *VS.* AEROBIC CFES

In addition to assaying the cofactor preference of the aforementioned oxidoreductases, the differences in enzymatic activity between the anaerobic and aerobic phases were also evaluated. Similar activities for CFEs harvested from either condition were found. These results are in line with the previous observation from proteomics studies where relative protein abundances did not change notably across an anaerobic/aerobic cycle (Skennerton et al., 2015; Wilmes et al., 2008).

2

S5. FLUX BALANCE ANALYSIS – ADDITIONAL FIGURES AND ANALYSES

PR-COA* AND CO_2

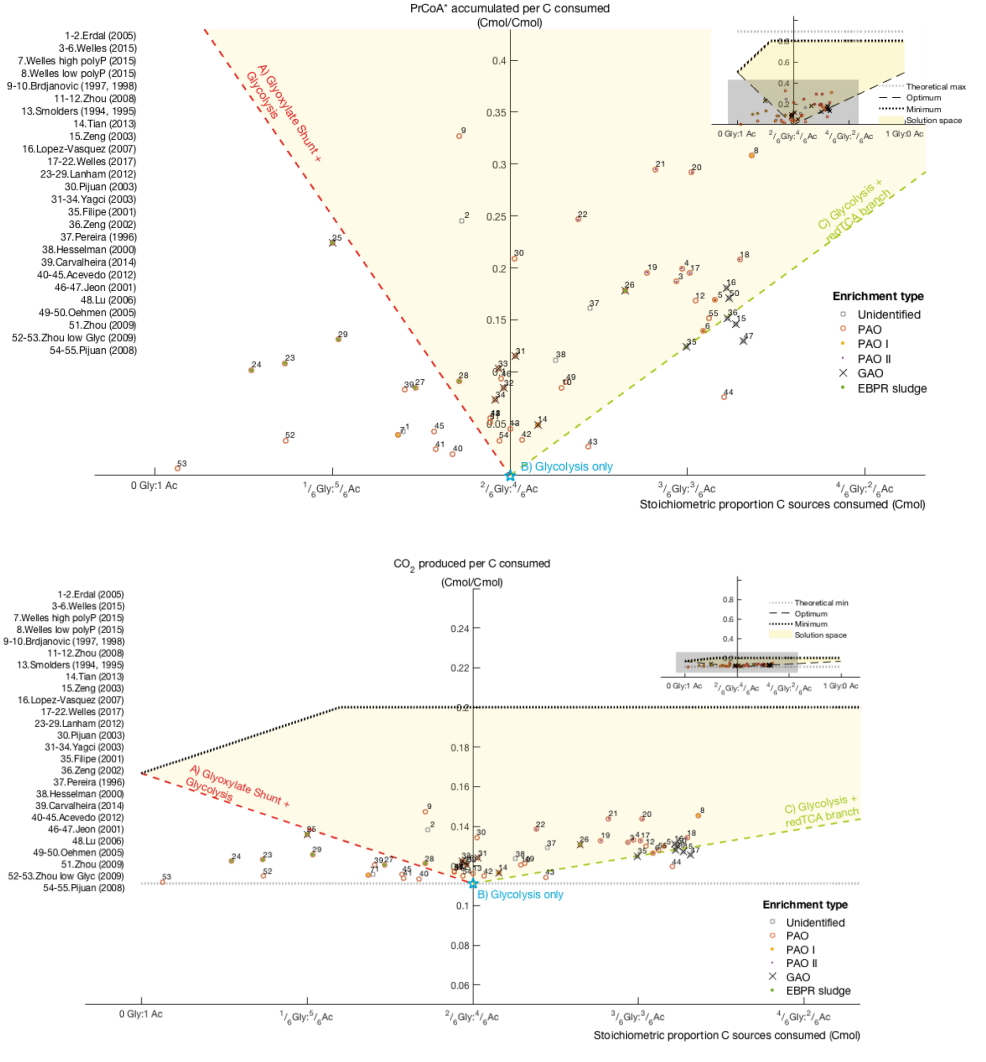


Figure 2.13: The amounts of Pr-CoA* accumulated and CO_2 released depend on the different proportion of glycogen to acetate consumed and on the available pathways.

AC-COA*, PR-COA* AND CO_2 WITH ERRORBARS FOR EXPERIMENTAL DATAPOINTS

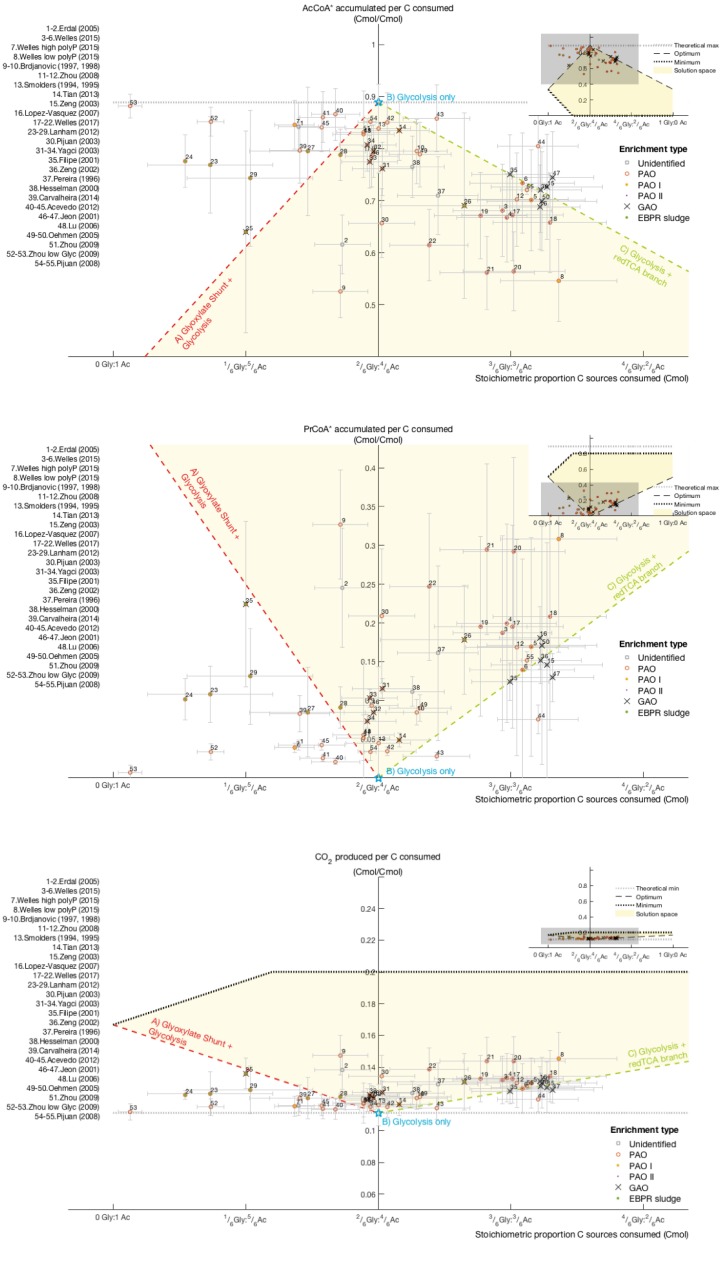
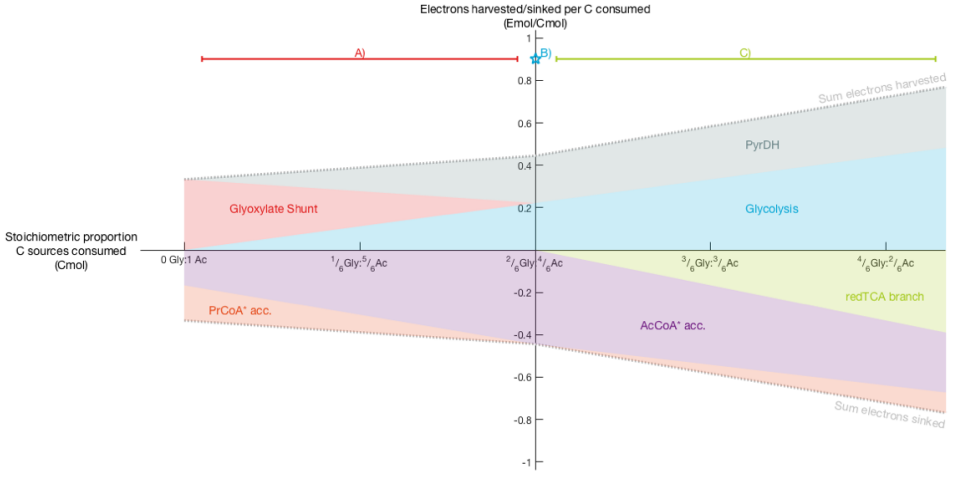


Figure 2.14: The error bars are propagated error based on published error when available or assumed (relative errors of 5% for acetate and PHB measurements and 10% for PHV, PH2MV and glycogen measurements).

ELECTRON FLUX DISTRIBUTIONS

Optimum scenario:



Minimum scenario:

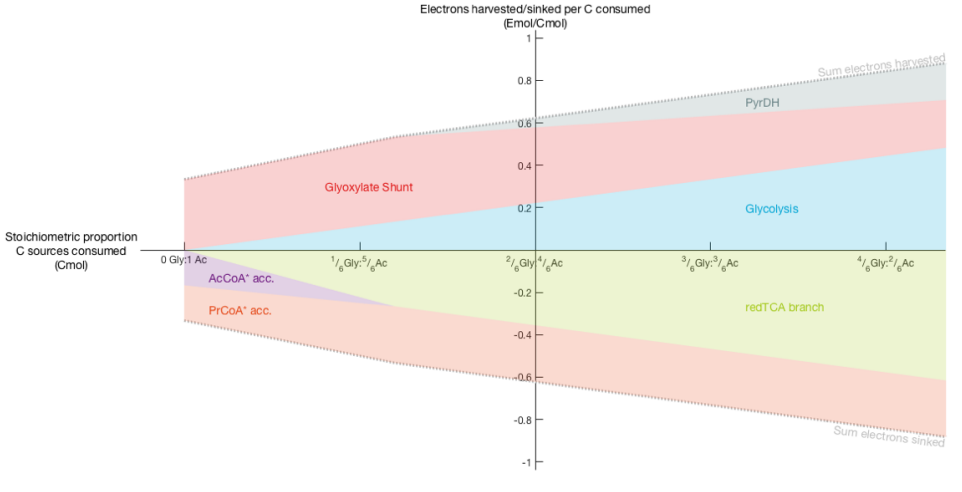


Figure 2.15: Electron fluxes through the different sources and sinks depending on the proportion of glycogen to acetate consumed. Values are normalized to C consumed. ***Optimum* scenario** – highest carbon conservation; ***Minimum* scenario** – lowest carbon conservation.

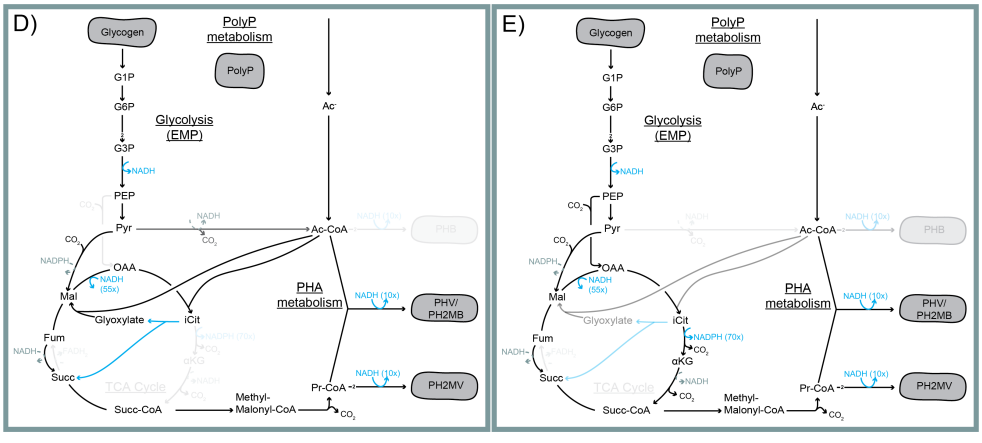


Figure 2.16: Lowest carbon conservation (*minimum*) redox balancing strategy. From another viewpoint, this is the strategy to harvest and sink the biggest amount of electrons; it makes use of both possible sources simultaneously: glyoxylate shunt and glycolysis. **D)** The simultaneous operation of these pathways allows for the biggest release of CO_2 followed closely by **E)** the strategy using the "horseshoe" TCA operation.

COMPARISON TO YAGCI'S PAO AND GAO MODELS

PAO model (Yagci et al., 2003):

$$(6+3f_{GLX})Acetate + Glycogen \rightarrow 4PHB + f_{GLX}PHV + (2+f_{GLX})CO_2$$
$$with \quad f_{GLX} = 0:1000$$

GAO model (Yagci et al., 2003):

$$Acetate + (\tfrac{1}{3} + \tfrac{2}{3}\alpha_{GAO})Glycogen \rightarrow \tfrac{2}{3}PHB + (\tfrac{1}{6} + \tfrac{2}{3}\alpha_{GAO})PHV + (\tfrac{1}{2} + \tfrac{2}{3}\alpha GAO)CO_2$$
$$with \quad \alpha_{GAO} = 0:1000$$

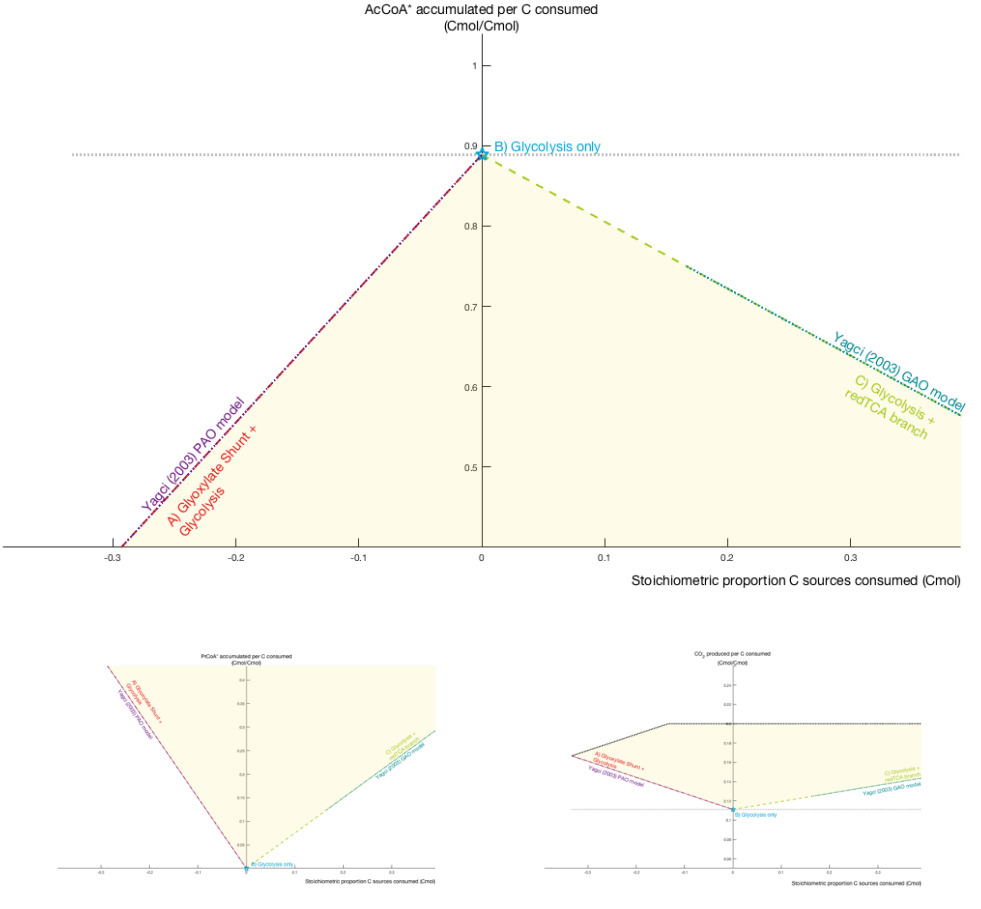


Figure 2.17: Our simulations and the (Yagci et al., 2003) models of PAO and GAO overlap. According to our observation, the PAO model should be extended to include the C) option until Yagci's GAO model and be renamed to PAM. The segment of C) that overlaps with Yagci's GAO model should be renamed to GAM.

ALTERNATIVE SCENARIOS: H_2 PRODUCTION?

The production of hydrogen gas has been observed in the studies of Oyserman and co-workers (Oyserman et al., 2016b). This is a possible way of solving excess reducing equivalents. To simulate this, we added an electron sink (H_2Prod) to the stoichiometric matrix, as follows:

$$H_2\text{Prod: } 2 \text{ electrons} \rightarrow$$

The optimization for *maximum* carbon conservation yielded the same as before, however now the *minimum* is shifted to allow more CO_2 release. As this would not explain any of the experimental datapoints outside the solution space, we tested the *maximum* Ac-CoA* (equivalent to PHB) accumulation as optimization goal. This could be the case when there is, for example, a kinetic limitation on the redTCA branch preventing excess electrons of being absorbed into Pr-CoA*. The results are shown in Figure 2.18. The electron flux distributions can be found below.

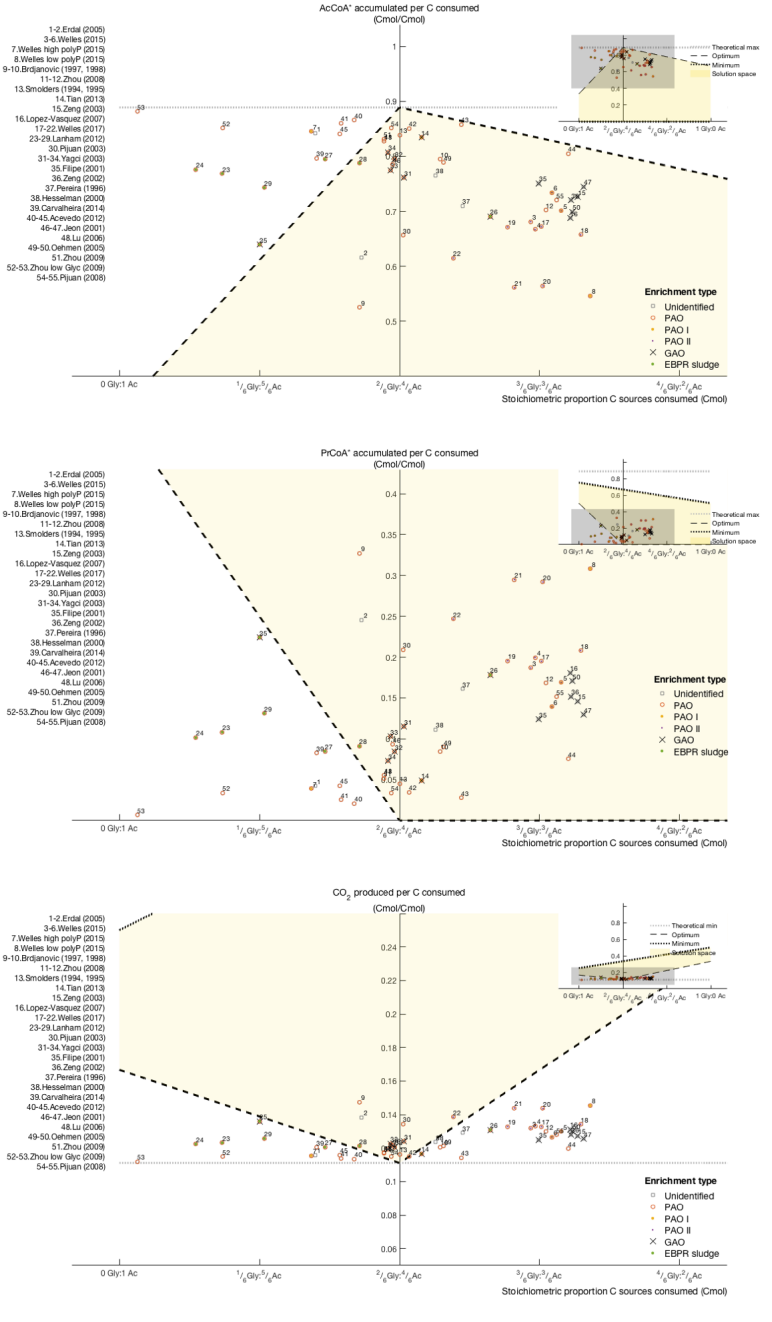
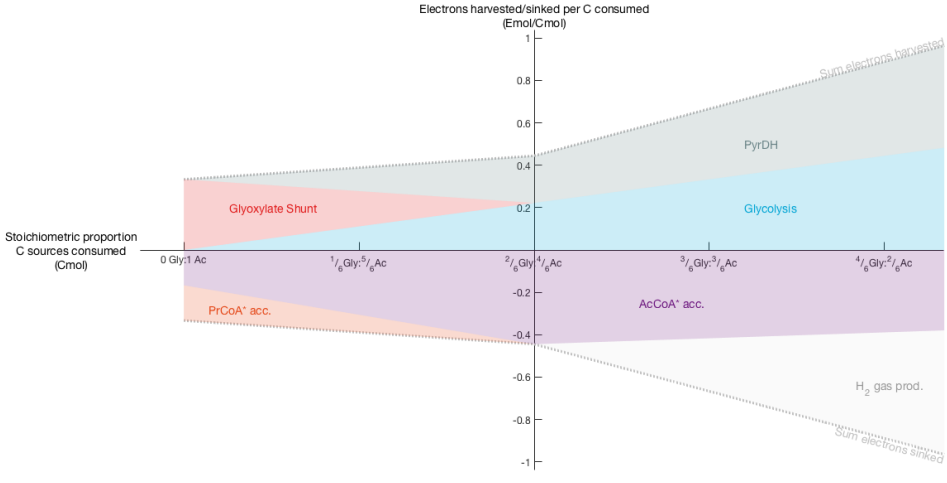


Figure 2.18: The H_2 production allows to explain the amounts of Ac-CoA* and Pr-CoA* found experimentally under the conditions of excess glycogen over acetate consumed, however it leads to higher CO_2 production which does not match experimental data.

Optimum scenario:



Minimum scenario:

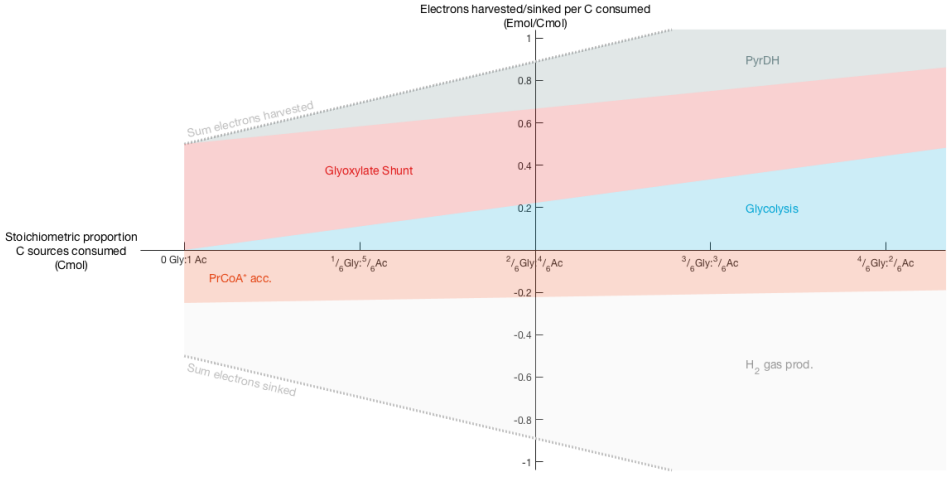


Figure 2.19: Electron fluxes through the different sources and sinks depending on the proportion of glycogen to acetate consumed. Values are normalized to C consumed. ***Optimum* scenario** – highest Ac-CoA* accumulation; ***Minimum* scenario** – lowest carbon conservation.

ALTERNATIVE SCENARIOS: FULL TCA OPERATION?

The full operation of TCA cycle anaerobically in Accumulibacter has been proposed several times, but never convincingly proven. To simulate this, we added the TCA cycle reaction to the stoichiometric matrix, as follows:

$$\text{TCA: Ac-CoA} \rightarrow 2CO_2 + 8 \text{ electrons}$$

The *optimum* scenario was obtained with the goal of *maximum* carbon conservation and the *minimum* scenario with the goal of lowest carbon conservation. The results are shown below. The electron flux distributions can be found below.

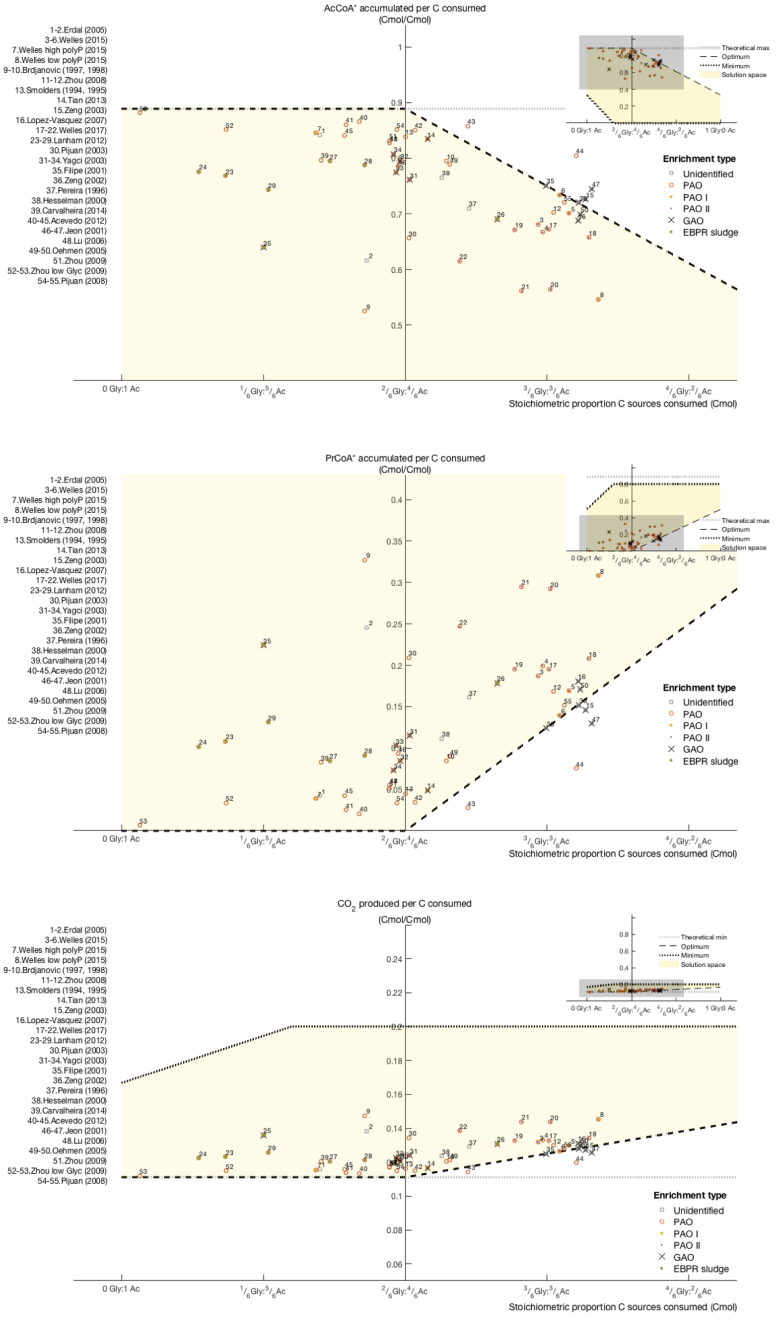
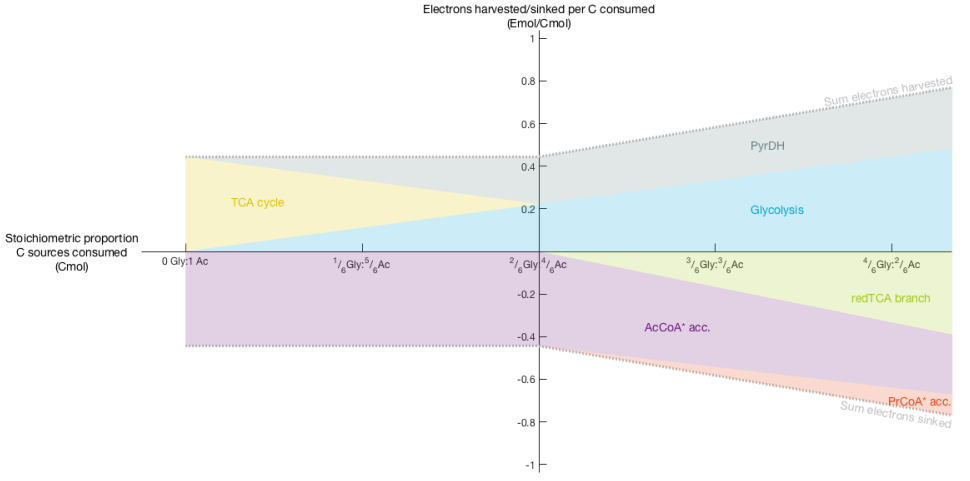


Figure 2.20: The full TCA operation allows to explain the amounts of Ac-CoA* and Pr-CoA* found experimentally under the conditions of limitation of glycogen over acetate consumed. Remarkably, this option allows to achieve the *maximum* theoretical yield of PHB (and PHA) production in "glycogen-limiting" conditions.

Optimum scenario:



Minimum scenario:

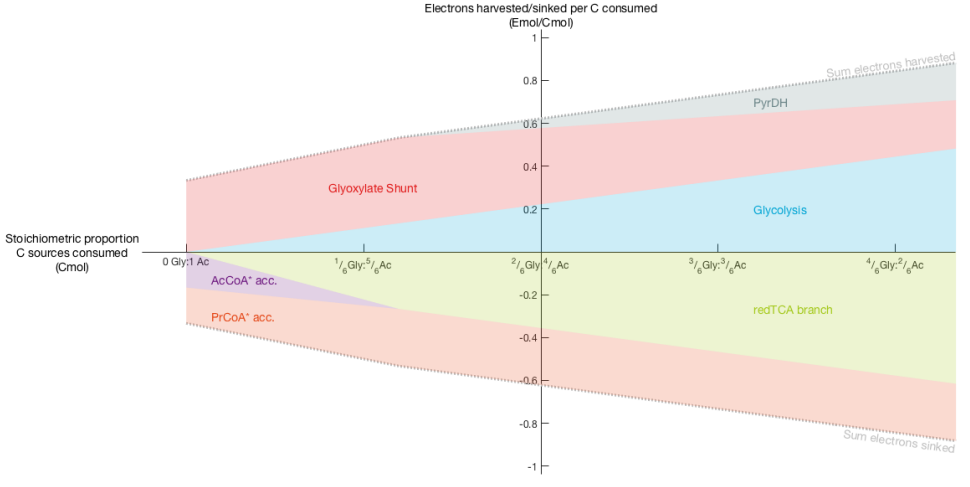


Figure 2.21: Electron fluxes through the different sources and sinks depending on the proportion of glycogen to acetate consumed. Values are normalized to C consumed. ***Optimum* scenario** – highest carbon conservation; ***Minimum* scenario** – lowest carbon conservation.

ALL SCENARIOS: BASE CASE, WITH H_2 PRODUCTION AND WITH FULL TCA

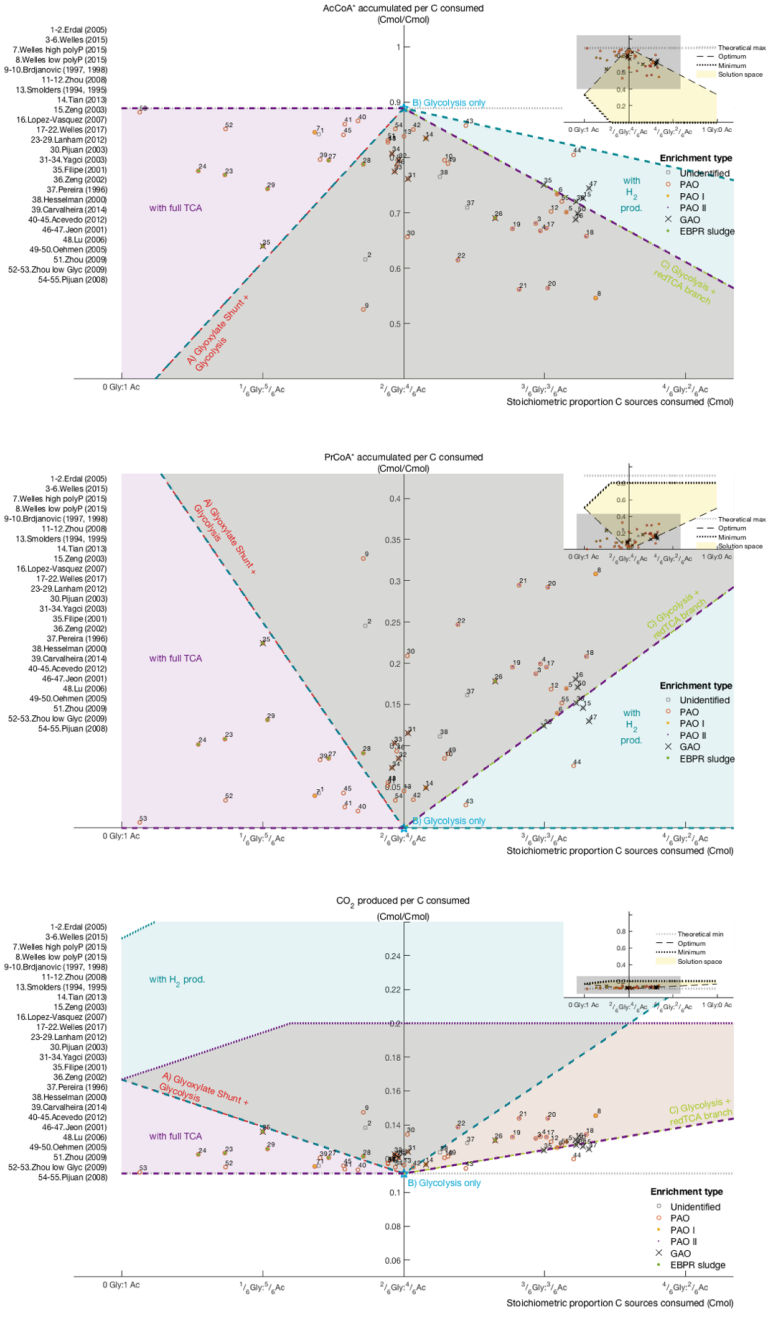
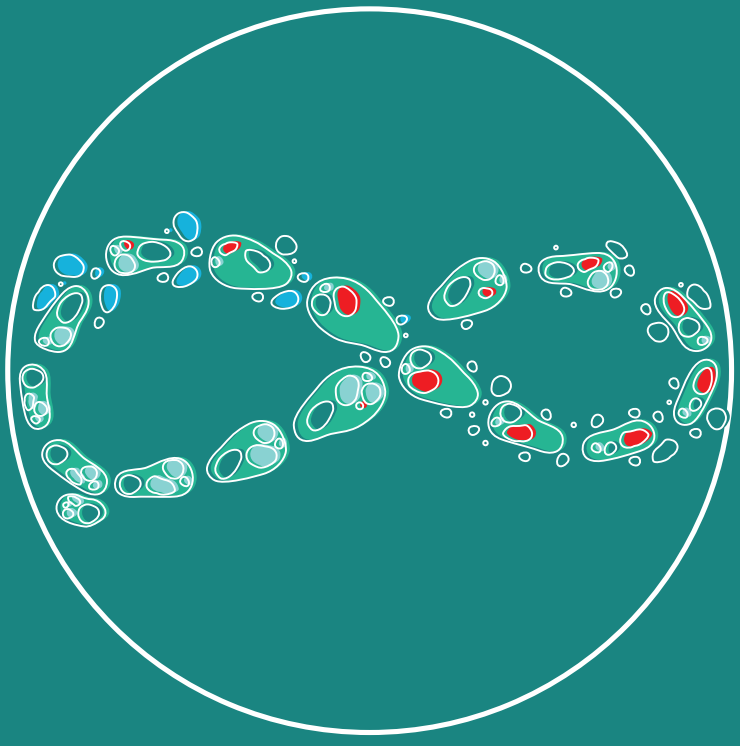


Figure 2.22: Overlapped scenarios: **Yellow** – base case; **green** – H_2 production; **purple** – full TCA.

3

CHAPTER

Energy allocation in dynamic environments



THE ENVIRONMENT SELECTS: MODELING ENERGY ALLOCATION IN MICROBIAL COMMUNITIES UNDER DYNAMIC ENVIRONMENTS

Leonor Guedes da Silva*, Sergio Tomás-Martínez*, Mark C.M. van Loosdrecht, S. Aljoscha Wahl

What will be the best metabolic strategy in a competitive environment where oxygen is periodically unavailable?

A few decades ago, an accidental, man-made cyclic anaerobic/aerobic environment selected for Polyphosphate Accumulating Organisms (PAOs) and this strategy is now widely used to allow for Enhanced Biological Phosphorus Removal (EBPR) of wastewater.

But could it have been predicted?

Here, a dynamic resource allocation modeling formalism was used to analyze the impact of selection pressures on metabolic function. With the same meta-network but modified selective pressures, different successful strategies can be predicted: Polyphosphate-AOs, Glycogen-AOs, Polyhydroxyalkanoate-AOs, and regular aerobic heterotrophs.

The results demonstrate how storage metabolism allows for different trade-offs between growth yield, robustness, and competitiveness, and highlight how each metabolic function is an important determining factor for a selective advantage in a given environment. This can be seen as an example of when *"Unity in biochemistry"* by A. Kluyver meets *"Everything is everywhere, but the environment selects"* by B. Becking and how microbial ecosystems may be described by the energy allocation phenotype instead of a detailed description of each organism.

*** equal contribution**

ACKNOWLEDGEMENTS: The authors would like to thank Professor J. J. (Sef) Heijnen for his invaluable advice on this manuscript.

INTRODUCTION

About 85 years ago, Baas Becking hypothesized that all microbial life is distributed worldwide, but the environment (*e.g.* gut, skin, plants, wastewater treatment systems) selects for and enhances a specific phenotypic function, which then becomes observable (*i.e.* above detection limit) (Baas-Becking, 1934). To date, different functions in microbial ecosystems have been described using computational models (reviewed in (Succurro and Ebenhöh, 2018)), however predictive power is still limited. For example, current kinetic models use pre-defined optimal metabolic strategies and require extensive re-calibration to simulate different environments. Consequently, these models cannot predict the outcome of a new environmental condition.

Natural environments are dynamic and changing conditions are inevitable. To cope with this, evolution has selected for a certain degree of metabolic flexibility that leads to the recently discussed rate/yield trade-offs (Abudukelimu et al., 2017; Frank, 2010; Peyraud et al., 2016; Pfeiffer, Schuster, and Bonhoeffer, 2001). Depending on the environmental pressure, different cellular characteristics arise as selective advantage, *e.g.* higher substrate uptake or growth rates *versus* higher energetic efficiency (*i.e.* higher biomass yield) *versus* membrane space (*i.e.* higher transporter capacity) *versus* storage metabolism (*i.e.* metabolic buffer capacity).

Proposed solutions to tackle some of today's grand societal challenges like wastewater treatment as well as production of chemicals from renewable resources or waste streams rely on microbial communities. In the design of these processes, engineers can exploit the selective advantage of a given community (*i.e.* intracellular storage) to, for example, remove phosphate or produce polyhydroxyalkanoates (PHA) from wastewater streams (Barnard, 1976; Kleerebezem and Loosdrecht, 2007). To select for a relevant microbial community, one of the main technological questions arising is *how to design and control the selective environment that stabilizes the open microbial community for the desired function?*

One of the best characterized and modeled microbial communities can be found in biological wastewater treatment plants. The selected microbial community was tuned over the years to promote removal of organic carbon, nitrogen and phosphorus from sewage. In its history, there have been several discoveries which led to the design of new generations of this bioprocess. An important condition for enhanced biological phosphorus removal (EBPR) was accidentally discovered in treatment plants that contained an anaerobic zone at the entrance in otherwise fully aerobic activated sludge systems (Srinath, Sastry, and Pillai, 1959). As a result, microorganisms experienced time-varying presence and absence of external electron acceptors (O_2). In the absence of O_2, ordinary aerobic heterotrophs cannot allocate energy for substrate uptake, whereas Polyphosphate Accumulating Organisms (PAOs) can thanks to their polyphosphate and glycogen storage. PAOs remove phosphate from the environment by accumulating it intracellularly as polyphosphate (Barnard, 1976; Seviour, Mino, and Onuki, 2003).

When no external electron acceptor is present (usually defined as anaerobic condition in EBPR literature), PAOs use their polyphosphate and glycogen storage as competitive advantage (Van Loosdrecht, Pot, and Heijnen, 1997). These storage compounds are used to generate ATP and NADH allowing to rapidly sequester extracellular organic carbon sources such as volatile fatty acids to store them intracellularly as polyhydroxyalkanoates (PHAs). The incorporation of these sources into PHAs is faster than into biomass synthesis (growth), representing another competitive advantage: rapid sequestration of the extracellular organic carbon sources making these inaccessible for the regular heterotrophs. In the presence of an external electron acceptor (aerobic), the accumulated PHAs are used for both growth and regeneration of the polyphosphate and glycogen storage pools. While the single metabolic traits of PAO are commonly found among microorganisms - *genotype* - the combined use of them to sequester substrates in the absence of external electron acceptors is what defines the PAO *phenotype*. For example, in periodic, dynamic environments such as activated sludge systems, other microbial functional groups can be found such as Glycogen Accumulating Organisms (GAOs), Polyhydroxyalkanoate Accumulating Organisms (PHA-AOs) and regular aerobic heterotrophs (Figure 3.1); these microorganisms share the single metabolic traits of PAOs, however each uses them to different extents - *phenotype* - which result in different levels of fitness (*i.e.* reproductive success in a given environment). An additional investment of resources in these storage phenotypes confers microorganisms robustness to cope with unexpected events during the cyclic environment (Maurer et al., 1997). Such additional storage activity requires resources (*i.e.* metabolic energy, but also additional enzymes/proteins) leading to a reduction of the maximal growth rate and yield compared to a growth-only strategy and generates a trade-off between the different metabolic processes (Figure 3.1).

	PAO	GAO	PHA-AO	Heterotroph
Metabolic trait(s)	PHA Glycogen Polyphosphate	PHA Glycogen	PHA	-
Competitive advantage	Fast acetate uptake without ext. e^- acceptor, Robustness	Fast acetate uptake without ext. e^- acceptor, Robustness	Fast acetate uptake with ext. e^- acceptor	High growth rate and yield but only with ext. e^- acceptor

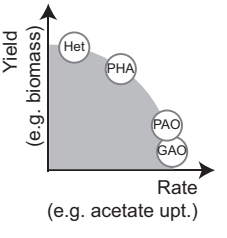


Figure 3.1: Main phenotypes found in activated sludge systems. **Left:** Metabolic traits and their respective competitive advantages. **Right:** Expected rate/yield trade-off curve. "Rate" can be seen as the competitive advantage of exclusive substrate uptake or substrate hoarding.

Recently, a modeling approach for dynamic resource allocation has been proposed that integrates stoichiometry and dynamic conditions and allows for the calculation of optimal phenotypes. This new modeling framework (Rügen, Bockmayr, and Steuer, 2015) known as Conditional Flux Balance Analysis (cFBA) is a variant of dynamic FBA and it has been developed as a dynamic resource allocation formalism to understand growth in a periodic environment. This formalism has been used previously for the analysis of axenic cyanobacterial growth in day/night cycles (Faizi et al., 2018; Reimers et al., 2017; Rügen, Bockmayr, and Steuer, 2015).

Conditional FBA (cFBA) uses as optimization objective the *"maximization of biomass synthesis over a full cycle, constrained by the conditional interdependences of cellular metabolism"* (Rügen, Bockmayr, and Steuer, 2015). This allows to model the whole anaerobic/aerobic cycle without having to explicitly set the different cellular objectives in the different phases of the cycle as it would be needed in conventional FBA (used previously in Chapter 2). The output of this approach is a prediction of the *dynamic* phenotype that has the highest fitness for the set cyclic environment.

In the present study we aim to better understand why each different metabolic strategy – PAOs, GAOs, PHA-AOs and heterotrophs – despite their common genetic traits, can all be found in a competitive environment where oxygen is periodically unavailable. To do so, we constructed a model using the novel cFBA approach (Figure 3.2) to:

a. Demonstrate that investment in (polyphosphate and/or glycogen) storage leads to improved fitness, generating a competitive advantage as well as robustness under periodic anaerobic/aerobic conditions;

b. Compare the investments on the two alternative polyphosphate or glycogen-based metabolic (PAM or GAM) strategies and show how PAOs can still adapt their metabolic strategy when polyphosphate storage is limiting;

c. Explore how the environment selects for the phenotype (metabolic trait) with highest fitness and how that leads to different rate/yield trade-offs.

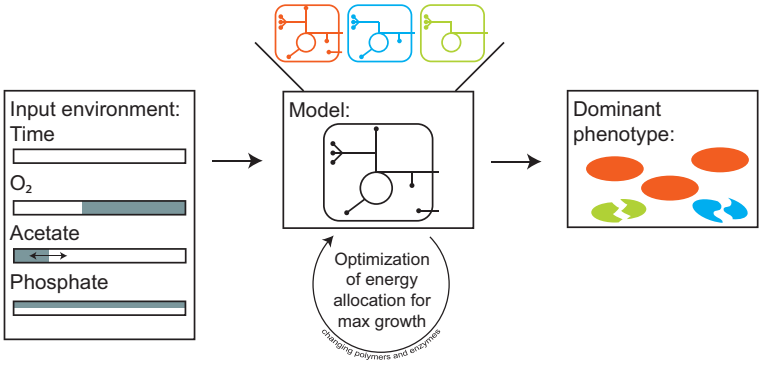


Figure 3.2: Overview of the approach used in this study. Cycle time and anaerobic/aerobic phases are fixed, while acetate uptake period and external phosphate availability are varied to create different environments.

Model Construction

In the framework of cFBA the cell is described as an autocatalytic system with different functions that contribute to growth and share common resources. Maximal reaction rates are defined by the amount of available enzyme and its respective catalytic capacity. Consequently, enzyme synthesis is also limited by the quantity of ribosomes, which in turn are synthesized by enzymes - *auto-catalysis*.

Here, the amounts of macromolecules such as biomass precursors, proteins, enzymes, ribosomes and storage polymers are explicitly modeled and are considered time-dependent (*i.e.* dynamic). Moreover, alike in conventional FBA, it is assumed that changes in metabolic intermediates occur much faster than in macromolecules, thus all intermediates are in *quasi*-steady state. To enforce the synthesis of inert compounds like biomass precursors and non-catalytic proteins, a so-called quota (*i.e.* minimum amount required) is introduced (Rügen, Bockmayr, and Steuer, 2015). Quotas for the storage polymers are also set to represent the extra investment in robustness.
In this framework, dynamic environmental transitions are described as stable, periodic cycles. Consequentially, biomass composition is the same at the end and start of a cycle. Furthermore, when such repetitive system is stable, the biomass synthesized during one cycle equals the biomass removed from the system (*i.e.* in equals out). Based on that, the net growth at end of the cycle is expressed as a multiplication of the defined individual cellular components at the beginning of the cycle.
All simulations were based on a simplified metabolic network of one of the most well studied PAOs, *Candidatus* Accumulibacter phosphatis, derived from (Oyserman et al., 2016b). This model was constructed following the steps proposed by (Reimers, Lindhorst, and Waldherr, 2017).

METABOLIC NETWORK

The model comprises the main metabolic reactions to describe PAOs growth during anaerobic-aerobic cycles (Figure 3.3).

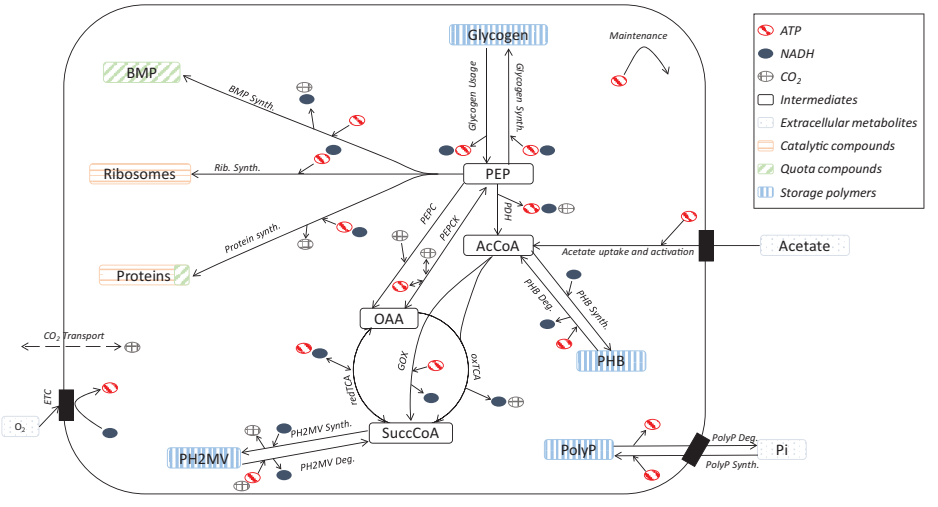


Figure 3.3: Simplified representation of the metabolic network used in this study. The complete reaction list and respective stoichiometries are shown in Table 3.1.

This works as a meta-network since the phenotypes of GAOs, PHA-AOs and heterotrophs are subsets of the metabolic network of PAOs. Traditionally metabolic models for these

organisms have been separated for the different redox conditions, the here used model approach does not need predefined modelling of anaerobic or aerobic conditions. Reaction names and stoichiometry are shown in Table 3.1. ATP requirements for acetate and phosphate uptake, together with the ATP:NADH stoichiometry of the oxidative respiration (*i.e.* P/O ratio in ETC reaction) were based on (Smolders et al., 1994b; Smolders et al., 1994a; Smolders et al., 1995). Phosphoenolpyruvate (PEP) is introduced as a link between catabolism and biosynthesis. As simplification, the different biomass precursors (BMP) are modeled as one quota component. This metabolite represents components of biomass such as DNA, RNA and phospholipids. The stoichiometry for the anabolic reactions was based on (Oehmen et al., 2007b) for BMP, and (Rügen, Bockmayr, and Steuer, 2015) for ribosomes and proteins. The different types of PHA polymers (PHB, PHV, PH2MV, PH2MB) originating from different combinations of the precursors Acetyl-CoA and Succinyl-CoA were simplified as PHB and PH2MV. With this simplification, 1 monomer of PHV or PH2MB is described by half a molecule of PHB and half a molecule of PH2MV. A maintenance reaction is introduced to represent energy requirements of the cellular processes like protein turnover among others, which currently can only be estimated from experimental data, here taken from (Smolders et al., 1995). Lastly, all redox cofactors are expressed as NADH, and all energy cofactors as ATP.

Biomass composition, quotas and enzyme capacities

In this model, biomass is composed of storage polymers and lean biomass (sum of all proteins, ribosomes, and BMP). In order to ensure the synthesis of BMP and non-catalytic proteins, minimum amounts (quotas) for those compounds are defined for all time points. On the other hand, for storage polymers (glycogen, polyphosphate and PHA), only an initial quota is needed. These values were derived from literature data: **a)** for glycogen, polyphosphate and PHAs, experimental measurements from Acevedo *et al.*(Acevedo et al., 2012) were used, **b)** total protein content was based on the work of (Yücesoy et al., 2012), **c)** composition of this protein pool was based on the metaproteomic study by Barr *et al.*(Barr et al., 2016), and **d)** for the ribosome content, no PAO data was available – here the value used in (Rügen, Bockmayr, and Steuer, 2015) for cyanobacteria was taken. An overview of quota values for these compounds can be found in Table 3.1.

Except for CO_2 diffusion across the membrane, all reactions in the system are catalyzed by enzymes or ribosomes. Constraints for the (maximal) biomass specific reaction rates are defined by the catalyst amounts and their specific activities (represented by the turnover rates, k_{cat}). Rügen and co-workers estimated k_{cat} values for each modeled reaction (often representative of whole pathways) and assuming all enzymes have the same production cost (Rügen, Bockmayr, and Steuer, 2015). For reactions missing in their model, the turnover rates were derived from the work of Davidi and colleagues (Davidi et al., 2016) by scaling them based on common reactions presented in both studies. Since these values were estimated for cyanobacteria and *E. coli*, respectively, it is reasonable to also use them to describe the bacterial ecosystems understudy (see Table 3.1). Furthermore, a sensitivity analysis was performed to study the influence of these values, *i.e.* each k_{cat} parameter was changed by one order of magnitude up and down (see Figure 3.8 in Supplementary Info).

Table 3.1: Complete reaction network, quotas and turnover rates used in this model.

Reaction ID	Stoichiometry	Enzyme ID	k_{cat} (s^{-1})	Compound quota?
Ac_upt [a, f]	Ac_{ext} + 1 ATP → AcCoA	E_Ac_upt	2.22	-
PHB_S	2 AcCoA + NADH → PHB	E_PHB	2.22	Only at t=0,
PHB_D	PHB + 2 ATP → 2 AcCoA + NADH			PHB ≥ 1.88 %(w/w)
PH2MV_S	2 SuccCoA + NADH → PH2MV + 2 CO_2	E_PH2MV	2.22	Only at t=0,
PH2MV_D	PH2MV + 2 CO_2 + 2 ATP → 2 SuccCoA + NADH			PH2MV ≥ 0.21 %(w/w)
Glyc_S	2 PEP + 2 ATP + 2 NADH → Glyc	E_Glyc_S	0.17	Only at t=0,
Glyc_D	Glyc → 2 PEP + ATP + 2 NADH	E_Glyc_D	21.87	Glyc ≥ 6.83 %(w/w)
PP_S [b, f]	1.26 ATP → PP	E_PP	0.81	Only at t=0,
PP_D [f]	PP → ATP			PP ≥ 23.85 %(w/w)
PDH	PEP → AcCoA + CO_2 + ATP + NADH	E_PDH	0.97	-
redTCA	OAA + 2 NADH + ATP ⇌ SuccCoA	E_TCA	7.59	-
oxTCA	AcCoA + OAA → SuccCoA + 2 CO_2 + 2 NADH			-
TCAGOX	2 AcCoA + ATP → SuccCoA + NADH			-
PEPC	PEP + CO_2 → OAA	E_PEPC	39.90	-
PEPCK	OAA + ATP ⇌ PEP + CO_2	E_PEPCK	3.69	-
ETC	NADH → 1.85 ATP	E_ETC	36.95	-
Maintenance [c]	ATP →	E_Maintenance	5.20	-
CO2_Export	CO2 ⇌	-	-	-
E_xxx_S [d]	15.48 PEP + 124.45 ATP + 65.768 NADH → E_xxx + 1.483 CO_2	Ribosome	1.26×10^{-3}	E_other = 18.3 %(w/w)
Ribosome_S	11.604 PEP + 44.038 ATP + 51.657 NADH → Ribosome	E_Ribosome	1.26×10^{-3}	-
BMP_S	0.635 PEP + 1.065 ATP → BMP + 1.2075 NADH + 0.905 CO_2	E_BMP	4.79×10^{-5}	BMP = 22.10 %(w/w)
Ac_feed [e]	→ Ac_{ext}	-	-	-

[a] For simplification, all acetate is considered to be passively transported into cells.
[b] This reaction was blocked in some simulations to simulate a phosphate-limiting environment.
[c] Reaction flux fixed at 0.398 mmol ATP/(g_{DW0}.h). Adapted from the estimated 0.019 mol_{ATP}/(Cmol.h) in (Smolders et al., 1995).
[d] This includes the synthesis of all the enzymes, including the group of non-catalytic proteins. It is thus assumed that all enzymes require the same resources (type and amount).
[e] Fake spontaneous reaction for acetate replenishment in the environment (feeding).
[f] The corresponding transport process is lumped in these reactions.

RESULTS & DISCUSSION

A competitive environment where oxygen is periodically unavailable was simulated using the cFBA model as previously described. As reference simulation, the experimental conditions (*i.e.* initial amounts of extracellular acetate, phosphate and intracellular polymers, and the duration of the phases) presented by Acevedo et al., 2012 were chosen. The authors reported a high enrichment of PAOs in their reactors based on high phosphate release upon acetate uptake and visual confirmation by FISH.

The reference simulation reproduced known features of PAOs' metabolism (Figure 3.4):

- Polyphosphate utilization as energy source for anaerobic acetate uptake;
- Glycogen degradation provides reducing equivalents anaerobically and extra ATP;
- Anaerobic PHA accumulation and aerobic PHA degradation for biomass, glycogen and polyphosphate synthesis;

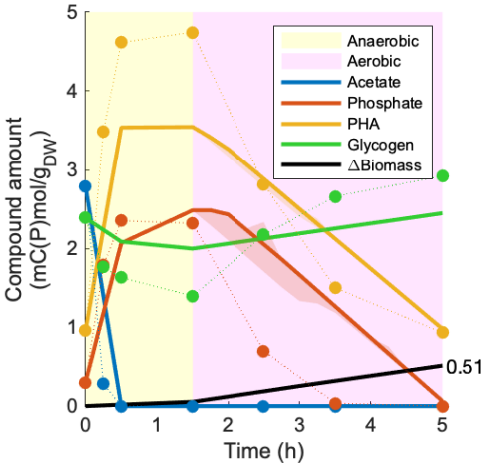


Figure 3.4: Simulated and experimental amounts of extracellular acetate and phosphate, intracellular polymers (PHA, glycogen) and lean biomass. The objective function set in this simulation was the maximization of biomass synthesis over a full cycle. Amounts are normalized to the initial biomass amount. The phosphate profile is calculated from the polyphosphate dynamics and initial phosphate supplied in the medium (experimental). The simulated PHA line is calculated from the modeled PHB and PH2MV pools. Solid lines represent the simulation and bullets represent experimental data retrieved from Fig. 3 of Acevedo et al., 2012.

Apart from the minimal quotas and turnover rates as mentioned earlier, only three (3) additional constraints were needed to simulate this PAO phenotype: **1)** amount of intracellular storage polymers at the beginning of the cycle, **2)** amount of acetate available extracellularly per cycle and **3)** maximum amount of time cells have to completely consume it (feast duration). The combination of 2) and 3) sets the minimum acetate uptake rate. The type of metabolic response was not a constraint in this model, nor any of the other kinetic rates. While the amount of acetate per g_{DW0} available is an experimental design input defining the environment, the constraints on storage quotas are specific to PAO metabolism. When no initial amount of intracellular storage polymers is set, the simulation predicts that the (optimal) cell will only accumulate the minimum amount of polymers needed to consume all acetate anaerobically; polyphosphate and glycogen would optimally be zero at the anaerobic/aerobic switch, and PHA would be zero at the beginning/end of the cycle. These are specific *optima* for the defined environmental conditions, and, as consequence, these cells have no buffer capacity to cope with fluctuations in the environment. Investing in higher accumulation as observed experimentally will increase the robustness during a deviating cycle as reserves will be available. The initial intracellular storage polymer quotas thus represent a growth trade-off towards robustness.
Furthermore, the acetate uptake duration was constrained to obtain the PAO phenotype. In a simulation without such constraint, acetate is consumed only during the aerobic phase (as seen later in Figure 3.5). This constraint thus selects for specific phenotypes with respect to a rate/yield tradeoff. Substrate uptake rate is an essential competitive advantage, as the fastest consuming organism will thrive. Anaerobic acetate consumption represents a significant rate advantage (*i.e.* earlier sequestration) compared to waiting for aerobic conditions. However, and as later seen in Figure 3.6, this represents a trade-off against growth as a significant allocation of energy is required for formation and consumption of the stored PHA. In this case, the allocated energy is mainly used for polymer cycling instead of the traditional protein resource allocation used to describe *e.g.* Crabtree effect in yeast (Nilsson and Nielsen, 2016).

POLYPHOSPHATE *versus* GLYCOGEN

The reference simulation representing the optimal operation for the given condition (Figure 3.4) deviates from the experimental observations for the glycogen usage (Acevedo et al., 2012). In their experiment as well as in the work of Welles and colleagues (Welles et al., 2015), a metabolic shift of PAOs from polyphosphate-based metabolism (PAM) to glycogen-based (GAM) was associated with different experimental conditions. This metabolic flexibility was also discussed extensively from a redox balancing viewpoint where different sources of reducing equivalents were considered: partial TCA cycle with/without glyoxylate shunt, and glycolysis (see Chapter 2). One described environmental condition that potentially promotes GAM is low phosphate availability. To simulate this limitation, the current model was used, but this time with polyphosphate synthesis impeded. As a result of this simulation, a GAM phenotype came up as the optimal strategy (Table 3.2). Note that GAM is also the strategy used by GAOs.

Table 3.2: Simulated and experimental anaerobic-feast yields [C(P)mol/Cmol]. Feast corresponds to the period in which acetate is present. Results are shown for PAM (PAOs performing polyphosphate-based metabolism), GAM (PAOs performing glycogen-based metabolism) and GAOs. To simulate GAM, polyphosphate production and consumption reactions were set to zero (*i.e.* blocked). Experimental values were converted so that 1 monomer of PHV (or PH2MB) is described by half a molecule of PHB and half a molecule of PH2MV.

	Anaerobic-Feast Yields [C(P)mol/Cmol]				
	P/Ac	Gly/Ac	PHB/Ac	PH2MV/Ac	X/Ac (overall)
PAM					
This simulation	0.67	0.12	0.51	0.41	0.18
(Acevedo et al., 2012)	0.7	0.38	1.33	0.03	-
(Welles et al., 2015)	0.64	0.29	1.31	0.05	-
GAM					
This simulation	0	1.98	0.48	1.95	0.15
(Acevedo et al., 2012)	0.08	1.08	1.85	0.17	-
(Welles et al., 2015)	0.02	1.28	1.24	0.22	-
GAO					
(Zeng, Yuan, and Keller, 2003)	n/a	1.2	1.60	0.31	-

The purpose of utilizing glycogen or polyphosphate anaerobically is the same: to provide ATP for anaerobic acetate uptake and activation for polymerization into PHAs. The choice of one ATP source over the other can be determined by the environment, *e.g.* ATP costs of transport processes across the membrane. Using the model presented in Table 3.1, PAM leads to a higher growth yield than GAM (Table 3.2). This is because polyphosphate production and consumption are energetically less costly than the glycogen cycling (Figure 3.6). Furthermore, glycogen cycling comes along with balancing reducing equivalents whereas polyphosphate not. As demonstrated in **Chapter 2**, polyphosphate leads to higher metabolic flexibility thus allowing for higher fitness to cope with changes in the environment. However, when phosphate is limited in the environment and polyphosphate accumulation is impeded, GAM is the alternative. In both PAM and GAM simulations, the acetate uptake rate and the ATP costs for both acetate and phosphate transport were set to

the same value. However, these three parameters change differently for PAOs and GAOs under different environmental conditions such as pH and temperature (Lopez-Vazquez et al., 2009). Consequentially, each parameter combination may select for either PAM or GAM, as a lower biomass yield can be compensated with a higher acetate uptake rate.

"...but the environment selects"

There are critical environmental characteristics that dictate the most favorable metabolic strategy (trait), *e.g.* rate- versus yield-strategy (Maharjan et al., 2013). Here, different rate/yield trade-offs are further analyzed by constraining the maximal time for acetate uptake during the cyclic feeding regime, *i.e.* rate-selective pressure. Based on the predicted optimal metabolic function, the dominance of PAOs/GAOs/PHA-AOs/heterotroph's strategy is discussed.

While keeping the same core model, the maximal time for acetate uptake was varied from 30 minutes (reference condition described by Acevedo et al., 2012) up to 5 hours (the whole cycle time). The simulated scenarios show distinct metabolic strategies (Figure 3.5).

When acetate is allowed to be consumed throughout the whole cycle (Figure 3.5d), the optimal solution found is to only do it once oxygen is available and only regular aerobic heterotrophic growth occurs. On the other hand, when acetate is forced to be taken up within 30 minutes into the anaerobic phase (Figure 3.5a), the optimal solution is to invest in the storage of polyphosphate and glycogen aerobically and to make use of them anaerobically. As it can be seen from the amount of lean biomass synthesized in each case, storing polyphosphate and glycogen is indeed an investment as it leads to a lower biomass yield (Figure 3.5a, 0.18 Cmol biomass per Cmol acetate) as opposed to the case where no storage metabolism is needed (Figure 3.5d, 0.21 Cmol biomass per Cmol acetate). These biomass yields on acetate obtained in the simulations are comparable with the 0.24 Cmol biomass per Cmol acetate reported by Acevedo et al., 2012 for the experimental conditions here simulated.

The amount of biomass produced is not as sensitive to the anaerobic acetate uptake rate (0.5 and 1.5h scenarios), as opposed to when acetate is consumed fully aerobically (2 and 5h). Furthermore, the simulated anaerobic biomass yields are the same for 0.5 and 1.5h scenarios. Thus, in these cases, the fastest consumer will thrive. This raises the question: *what is then the bottleneck setting the maximal biological rates in these systems?* Here it is already seen that PHA production alleviates the growth bottleneck allowing for faster acetate uptake. Thus, the question remains whether PHA production has enough processing capacity to keep up with acetate transport, or if this transport is the actual bottleneck. Other possible bottlenecks may be related with the ATP generation capacity or any other related process such as limited membrane space for transporters. To shed light on these questions, we recommend a comprehensive analysis of biomass composition, proteome (incl. transporters), membrane structure and processes.

DIFFERENT INTRACELLULAR ENERGY ALLOCATION AND RATE/YIELD TRADE-OFF STRATEGIES

To further explore the investment on each cellular process, their net ATP gain/losses are shown in Figure 3.6. At first glance, the differences in ATP generation in the ETC reaction point towards the efficiency of the strategy employed: Less ATP invested means less need for its production, and thus leading to a higher biomass yield as verified earlier.

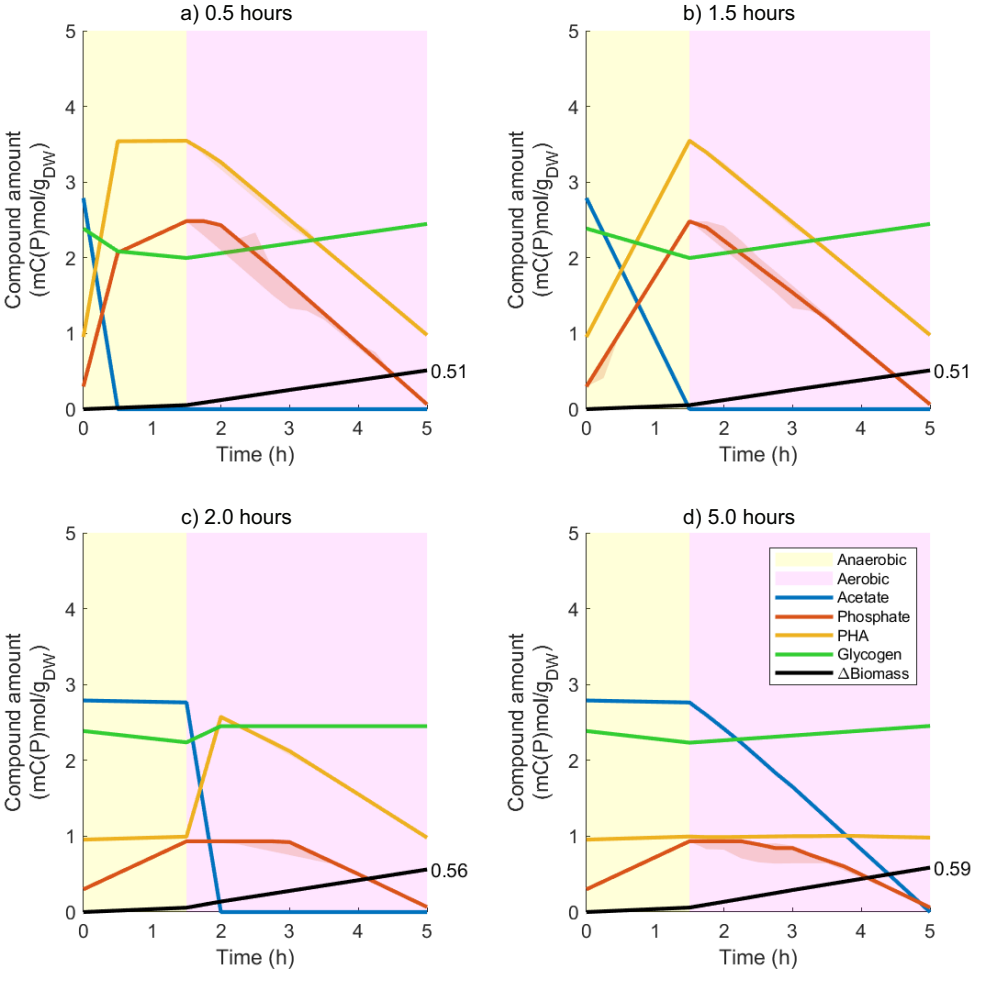


Figure 3.5: Simulated amounts of extracellular acetate and phosphate, intracellular polymers (PHA, glycogen) and lean biomass. The objective function set in this simulation was the maximization of biomass synthesis over a full cycle. The shaded areas around the simulated lines represent the output of the flux variability analysis. Amounts are normalized to the initial biomass amount. The normalized amount of new biomass at the end of the cycle is explicitly shown. The phosphate profile is calculated from the polyphosphate dynamics and initial phosphate supplied in the medium (experimental). The simulated PHA line is calculated from the modeled PHB and PH2MV pools. Scenarios: **a)** acetate uptake set to last only 30 min (also in Figure 3.4); **b)** 1.5h; **c)** 2h and **d)** 5h.

Commonly, in rate/yield trade-off analyses, the costs of higher rates (enzymes) are the culprit for the biomass yield loss caused by such investment. For the accumulating organisms (AOs) under study, there is indeed an extra investment on enzymes to enable the observed polymer cycling. However, this energy requirement is about hundred times lower (see zoomed section of Figure 3.6) than the ATP involved in the polymer cycling itself (*i.e.*

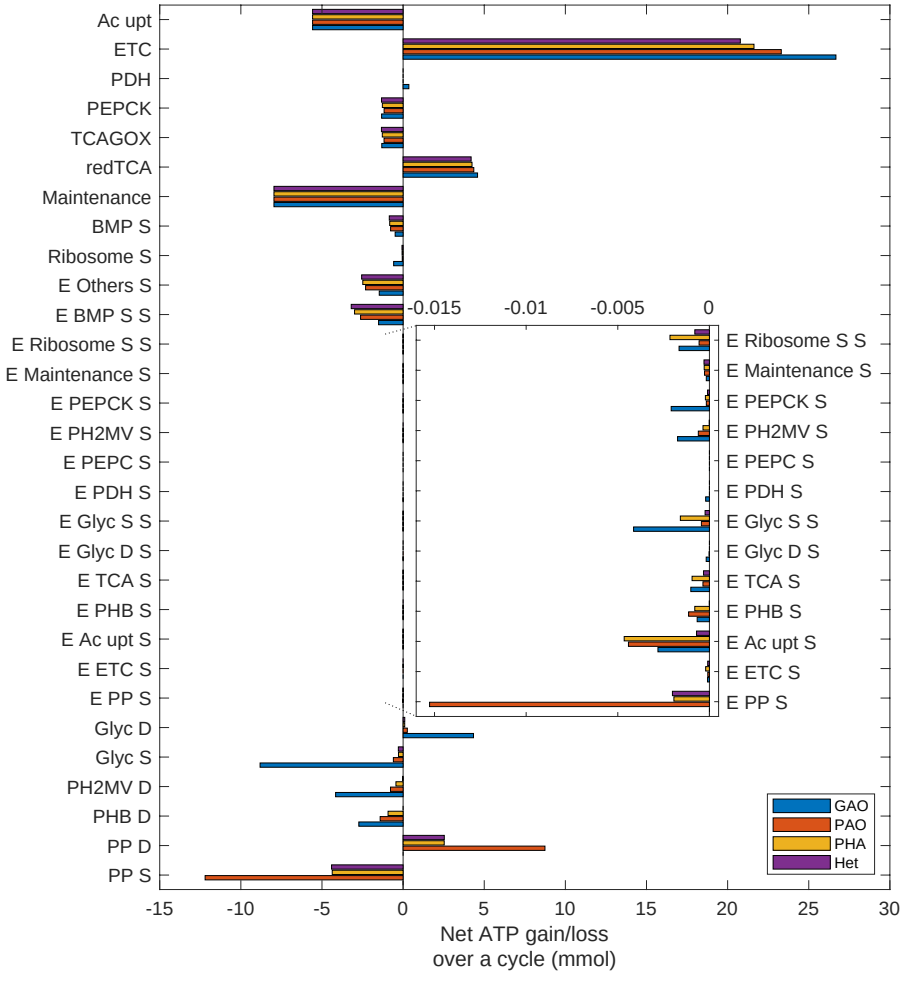


Figure 3.6: Net ATP gain/loss over a cycle for each different cellular reaction for each metabolic strategy (excluding the ATP cost of producing the enzyme catalyzing the reaction). For each storage polymer the synthesis (S) reaction is separated from the degradation (D) reaction. The zoomed inset shows the ATP costs of synthesizing each enzyme (E).

ATP requirements for transport and activation reactions). One could also argue on the investment in different enzymes, however, this model is too coarse in that respect. For a more detailed analysis on protein allocation, this model requires further improvements as it currently assumes **a)** there is no protein turnover (*i.e.* degradation is not included), **b)** all enzymes have the same production cost, and **c)** global catalytic capacities (k_{cat}) are estimated for each linear pathway, which are currently based on *in vitro* measurements but should become predictive using thermodynamics and molecular dynamics modeling (Pekař, 2015). Nonetheless, a sensitivity analysis was made by varying each assumed k_{cat} one order of magnitude higher/lower (see Figure 3.8 in Supplementary Info). The results show that the simulated output is only very sensitive to the ten times decrease of k_{cat} for BMP synthesis. However, the simulated biomass yield in that case is one order of magnitude lower than the experimentally observed, which is further from reality than the one obtained in our reference simulation. For all the other simulations, all conclusions were not sensitive to changes in (k_{cat}) across three orders of magnitude (see Figure 3.9).

From Figure 3.6 it becomes clear that the rate advantage that accumulating organisms (-AOs) have does not only come from fast acetate sequestration, but also from having the enzymes and resources that provide ATP anaerobically. To compare the different phenotypes (Figure 3.5) in a pareto-like relation between rate and yield (Figure 3.7), average acetate uptake rates were calculated taking into account the whole time in which acetate is available until it is fully consumed. For example, PHA-AOs only take 30 minutes to consume acetate but they only do so after the 1.5 hours of anaerobic phase, thus the average rate is calculated over the 2 hours of acetate availability.

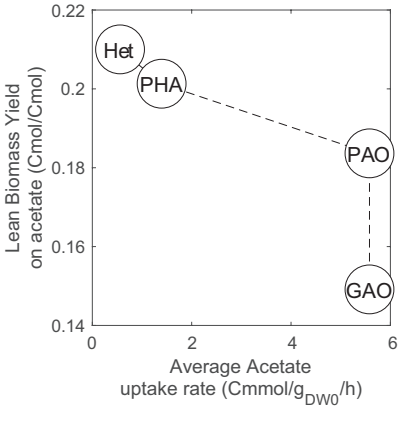


Figure 3.7: Rate/Yield trade-off curve. The curve was obtained based on the simulated results of the different metabolic strategies shown in Figure 3.5 and Table 3.2.

In systems where carbon sources are provided all-at-once and are in excess (*i.e.* in batch cultivation), a rate-strategy is likely winning. With the current information provided to the model, GAOs and PAOs are equally capable of quickly taking up acetate anaerobically and to store it in the form of PHA. The competition between PAO and GAO has been extensively studied and there are known environmental factors which balance the competition to either the PAO or GAO side; **1)** Changes in pH affect the ATP stoichiometry of transport processes, **2)** changes in temperature have a direct impact on the kinetic capacities of

pathways and, as demonstrated earlier, **3)** reduced phosphate availability can have a detrimental effect on polyphosphate-based metabolism (Acevedo et al., 2012; Lopez-Vazquez et al., 2009; Smolders et al., 1994b; Welles et al., 2015). Here, we propose taking into account proton (H^+) balancing between the intra- and extracellular space and proton translocation energetic costs in future simulations as a differentiator between PAOs and GAOs.
Another possible metabolic strategy to take up acetate anaerobically is employed by methanogens. While the acetate uptake rate is comparable, (about 5-7 $Cmmol/g_{DW}/h$ as reported for *Methanothrix* spp. by J.W.H. et al., 1994), the biomass yield is not comparable as methanogens excrete the majority of the energy present in acetate in the form of methane, thus only harvesting a small fraction for their biomass.
In the scenario where no acetate is taken up anaerobically or when it is pulse-fed into an aerobic system, PHA-AOs are known to thrive and dominate in such conditions (Beun et al., 2000; Van Loosdrecht, Pot, and Heijnen, 1997). Here, and again, organisms capable of sequestering substrate quickly will have a competitive advantage over yield-strategists. Finally, when PAOs, GAOs, and PHA-AOs fail at quickly sequestering substrate because of **a)** limitation of another nutrient, or **b)** inhibitors/predators, or **c)** when substrates are not readily available and need to be transformed first, or **d)** when substrates are continuously fed and limiting (*i.e.* in a chemostat), only then aerobic heterotrophs thrive. It is also important to note that simulations predicted that the most efficient strategy for PHA-AOs and heterotrophs would be to make use of glycogen and polyphosphate storage to cover for maintenance costs during the anaerobic starvation period. However, they may trade-off this (simulated) robustness to have a higher biomass yield. This will result in higher cell death during the anaerobic starvation period compensated by having higher amounts of offspring aerobically. In order to evaluate the investments that such strategy requires, enzyme and biomass decay should also be considered in future simulations.

A TOOL TO PREDICT PHENOTYPES BASED ON DEFINED ENVIRONMENT

The new modeling framework developed by Rügen and colleagues was used in this study as it allows for understanding growth in a periodic environment (Rügen, Bockmayr, and Steuer, 2015). Here we show how it can be applied to microbial communities by using a meta-network of the main functional groups present in such community and how it can predict which metabolic trait (phenotype) has the highest fitness for the set environment (*i.e.* combination of pathways that lead to the optimal energy allocation strategy).
Apart from using a meta-network instead of pre-defined strain-specific stoichiometric models, this formalism only requires a rough estimation of kinetic parameters to simulate an approximate proteome allocation. This approach contrasts with current kinetic models for PAOs/GAOs/PHA-AOs that use pre-defined optimal metabolic strategies, for example (Acevedo et al., 2014; Lanham et al., 2014; Marang, Loosdrecht, and Kleerebezem, 2015). These kinetic models require extensive re-calibration to simulate different environments and consequently cannot predict the outcome of a new environmental condition. Furthermore, they may be used for describing microbial competition among pre-defined metabolic strategies - *input* - however they will not be able to predict the most optimal and competitive strategy - *output*. For example, it is not trivial that to maximize biomass in a competitive system, investment in PHA cycling is what enables fast substrate sequestration that in turn results in more offspring than a biomass yield-strategy.

This set of simulations using the model presented in Table 3.1 predicted a polyphosphate-based metabolism as most adequate to cope with a competitive environment where oxygen is periodically unavailable. However, when polyphosphate is limiting, a glycogen-based metabolism emerges as an alternative. Lastly, simulations show that a faster and anaerobic acetate uptake is a strategic investment on competitiveness and represents a clear trade-off against growth. The balancing between rate *versus* yield strategies has regularly been shown in batch and chemostat studies (Bachmann et al., 2017). Here we showed that it is also the driver in microbial selection for dynamic ecosystems like wastewater treatment processes, but likely also tidal zones and systems subjected to light/dark cycles.
This is an example of when *"Unity in biochemistry"* (Kluyver and Donker, 1926) meets *"Everything is everywhere, but the environment selects"* (Baas-Becking, 1934): one metabolic network was used for all metabolic strategies (phenotypes) mentioned but it is the environment that sets which phenotype will thrive in the end.

MATERIALS & METHODS

SIMULATION AND IMPLEMENTATION

The files for the simulation were obtained from Rügen *et al.*(Rügen, Bockmayr, and Steuer, 2015) and executed in MATLAB version 9.4 (R2018a). The following changes were implemented: **1)** LINPROG was used as linear optimization solver; **2)** the simulation was adapted to this case study to model the usage of a defined external carbon source (acetate), within a specified amount of time. To this effect, acetate is modelled as an unbalanced metabolite (Ac_{ext}), however without any contribution to the composition of biomass (*i.e.* it has a weight of zero on the Biomass composition vector). A spontaneous reaction (Ac_{feed}) was added to ensure the replenishment of acetate for the next cycle, which is only active at the end of the cycle.
All simulations were performed to mimic the experimental set-up used by Acevedo *et al.* (Acevedo et al., 2012), *i.e.* cycles of 5 hours divided into 20 uniform intervals of 15 minutes, with 1.5 hours of anaerobiosis and 3.5 hours of aerobic phase. To represent these two different conditions, ETC was set to zero (*i.e.* blocked) and redTCA as irreversible during anaerobiosis, and then both were let free during the aerobic phase.
To simulate the different scenarios, the following constraints were used:

- Polyphosphate production and consumption reactions were set to zero (*i.e.* blocked) to obtain the results presented in Table 3.2;
- The maximal time for acetate uptake was constrained to 30 min, 1h30, 2h and 5h to produce Figure 3.5. For the remaining simulations, this value is set to 30 min.

DATA AVAILABILITY

- Original model retrieved from Rügen, Bockmayr, and Steuer, 2015;
- Adapted model available at `https://github.com/cell-systems-engineering-tud/energy-allocation`;
- Experimental data retrieved from (Acevedo et al., 2012).

SUPPLEMENTARY INFO

SENSITIVITY ANALYSIS - TURNOVER RATE, K_{CAT}

In order to study the effect of the value of the different turnover rates, k_{cat}, a sensitivity analysis was made by varying each assumed k_{cat} one order of magnitude higher/lower. As it can be seen in Figure 3.8, the change in these parameters does not alter the qualitative analysis performed in this study, except for the decrease of k_{cat} for BMP synthesis. However, that simulation leads to about one order of magnitude lower biomass yield as compared with experimental data (Table 3.2), which indicates the unlikelihood of this value. All these simulations, together with the sensitivity analysis for the GAM were used to generate Figure 3.9, showing that the conclusions drawn from Figure 3.7 remain the same.

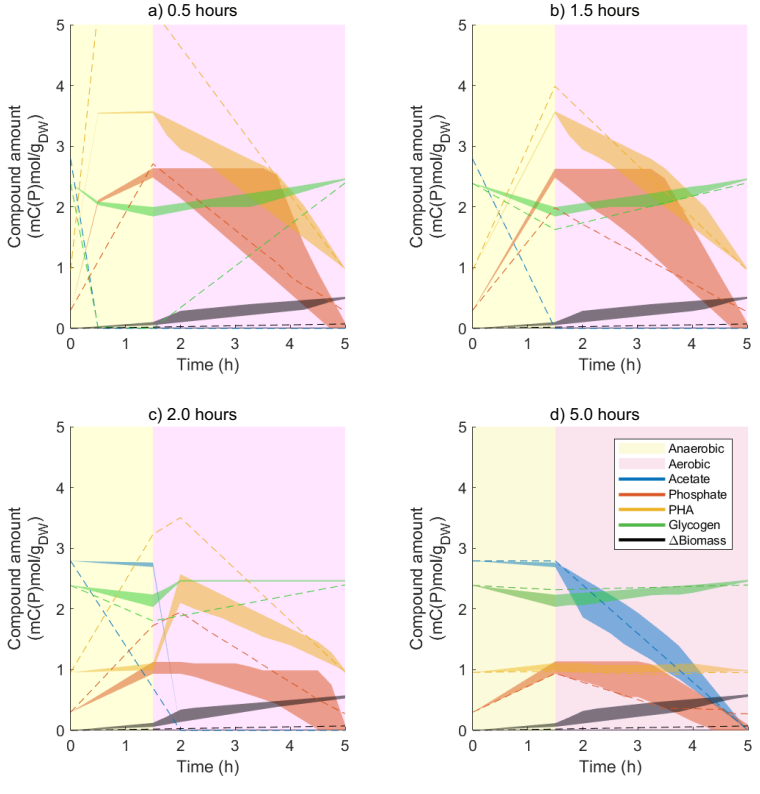


Figure 3.8: Simulated amounts of extracellular acetate and phosphate, intracellular polymers (PHA, glycogen) and lean biomass for the sensitivity analysis of the turnover rates (k_{cat}). Individual values of the different k_{cat} were changed by a factor of 0.1 and 10. Shaded areas represent the grouped output of all the simulations, except for the decrease of k_{cat} for BMP synthesis, which is represented by the dashed line. Amounts are normalized to the initial biomass amount. The phosphate profile is calculated from the polyphosphate dynamics and initial phosphate supplied in the medium (experimental). PHA is calculated from PHB and PH2MV. Scenarios: **a)** acetate uptake set to last only 30 min (same as in Figure 3.4); **b)** 1.5h; **c)** 2h and **d)** 5h.

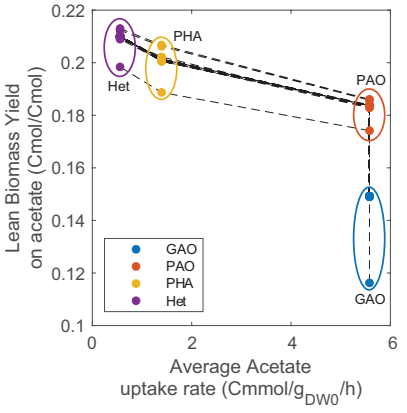


Figure 3.9: Rate/Yield trade-off curve for the sensitivity analysis of the turnover rates (k_{cat}). The curve was obtained based on the simulated results of the different metabolic strategies shown in Figure 3.8 and the sensitivity analysis for GAO. The output from the simulation of the decrease of the k_{cat} of BMP synthesis was excluded from the figure.

FULL OR PARTIAL OPERATION OF THE TCA CYCLE

The usage of a full TCA cycle anaerobically has been hypothesized many times in PAO literature (Lemos et al., 2003; Louie et al., 2000; Pereira et al., 1996; Zhou et al., 2009). In this model, the full TCA cycle can be set active in the anaerobic phase by unbounding redTCA during both anaerobic and aerobic phases. In this scenario (Figure 3.10b), less glycogen cycling is needed compared to the reference simulation (Figure 3.4 and Figure 3.10a). These simulated results agree with the simulations done earlier in **Chapter 2**, in which it is discussed that a full operation of the TCA cycle anaerobically could explain the higher PHA yields found in EBPR than predicted by models. A higher PHA yield corresponds to a higher biomass yield, thus a fully operating TCA cycle in the absence of an external electron acceptor represents an improved polyphosphate-based metabolism (PAM).

When the TCA cycle is blocked at the level of the succinate dehydrogenase (Figure 3.10a), then a combination of glycogen metabolism with the oxidative TCA branch (isocitrate to succinyl-CoA) and the glyoxylate shunt are used. This latter strategy yields 73% PHB/PHA in a Cmol basis, while when the TCA is fully active, 90% of all PHA is PHB.

This change in strategy also impacts the biomass yield in each case being the best strategy when the TCA cycle is fully operational anaerobically (0.19 instead of 0.18 Cmol biomass per Cmol acetate). One of the simplifications in this model is to consider all reducing equivalents are in the form of NADH however, $FADH_2$ is produced in the succinate dehydrogenase reaction step. The mechanisms for re-oxidizing this $FADH_2$, even if feasible without an external electron acceptor, may come with additional ATP costs, which should be considered in a future simulation.

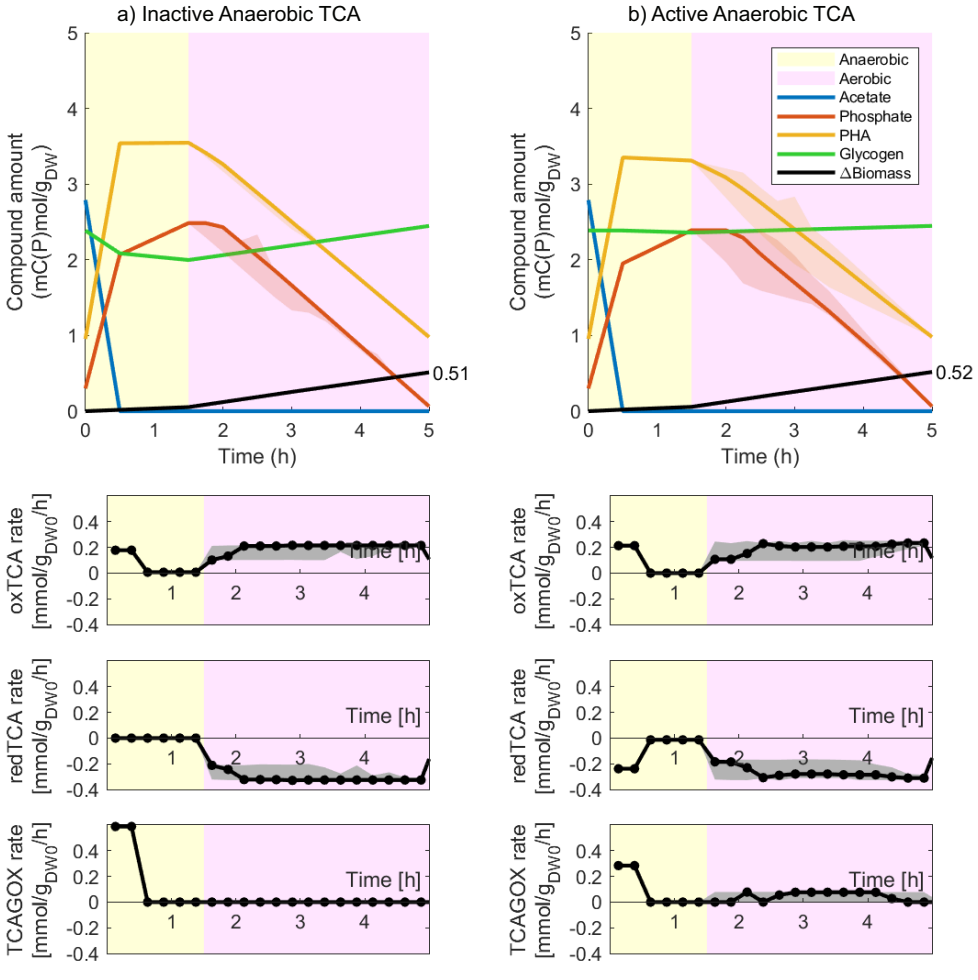
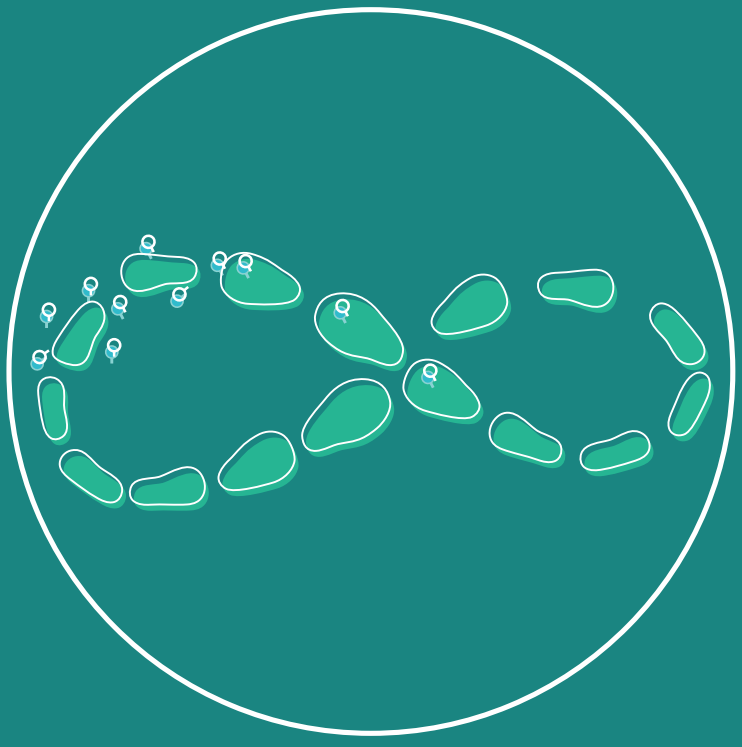


Figure 3.10: Simulations comparing the impact of an (in)active TCA cycle in the absence of an external electron acceptor (anaerobic): **a)** Reference simulation with an inactive TCA cycle anaerobically and **b)** with an active anaerobic TCA cycle.

4 CHAPTER

^{13}C tracing of Accumulibacter's metabolism



CAPTURING THE DYNAMICS OF CENTRAL CARBON METABOLISM OF *Candidatus* ACCUMULIBACTER PHOSPHATIS DURING AEROBIC/ANAEROBIC CYCLES USING ^{13}C TRACING

Leonor Guedes da Silva, Mark C.M. van Loosdrecht, S. Aljoscha Wahl

In natural and industrial, engineered environments, organisms are exposed to dynamic conditions with non-continuous supply of carbon and energy sources. Under such conditions, storage metabolism can increase competitiveness and robustness, however storage and mobilization can also lead to significant energy losses. *Candidatus* Accumulibacter phosphatis (hereafter referred to as Accumulibacter) are highly enriched in enhanced biological phosphorus removal (EBPR) processes – these organisms cycle different storage polymers such as polyhydroxybutyrate (PHB), glycogen and polyphosphate to master the alternating anaerobic-feast and aerobic-famine conditions.

To monitor intracellular switches of carbon flows, uniformly ^{13}C labelled acetate was fed to an enrichment culture of Accumulibacter. The metabolome was captured upon **1)** anaerobic feeding of acetate after a period of famine, and **2)** oxygen sparging after the anaerobic period when acetate was no longer available.

The ^{13}C-enrichment patterns of intracellular metabolites reveal that anaerobic acetate uptake and PHB formation occurred instantaneously upon acetate addition. Release of $^{13}CO_2$ to the offgas points at the oxidation of labelled acetate anaerobically, which was consistent with an initial ^{13}C-enrichment of TCA cycle intermediates. However, this enrichment decreases again, suggesting a metabolic transition during the anaerobic-feast phase. Distinct anaerobic/aerobic ^{13}C-patterns in TCA intermediates hint to a changed glyoxylate shunt operation, suggesting a regulatory mechanism impeding this shunt's anaerobic activity. As expected, ^{13}C enrichment of upper glycolytic intermediates was only observed upon the anaerobic-to-aerobic switch, indicating a tight regulatory checkpoint of gluconeogenesis.

This study shows how ^{13}C tracing can generate insight on metabolic switches of microbial communities cultured in dynamic systems. It provides a base for hypothesis-driven investigation on the regulatory mechanisms governing the lifestyle of Accumulibacter, consequently enabling the design of novel control strategies to improve EBPR processes.

ACKNOWLEDGEMENTS: Marissa Janssens and Sergio Tomás Martínez provided essential help in the execution of this ^{13}C batch experiment. Mass isotopomers were carefully measured and analysed by Cor Ras and Patricia van Dam from the CSE analytical team. Several EBT members also played an important role: Aerobic granular sludge used to inoculate the bioreactor used for this study was kindly supplied by Danny de Graaff; Bioreactor control systems were provided and kept smoothly running thanks to Gerben Stouten. FISH images for microbial community characterization were kindly prepared by Roel van de Wijgaart; The 16S rRNA gene amplicon analysis was kindly executed by Ben Abbas.

INTRODUCTION

In natural and industrial environments, organisms are exposed to dynamic conditions with non-continuous supply of carbon and energy sources. Under such conditions, storage metabolism can increase competitiveness leading to enrichment of organisms with this function (Van Loosdrecht, Pot, and Heijnen, 1997). In enhanced biological phosphorus removal (EBPR) systems, microorganisms are exposed to alternating anaerobic-feast and aerobic-famine conditions (Mino, Van Loosdrecht, and Heijnen, 1998; Seviour, Mino, and Onuki, 2003). Here, anaerobic-feast refers to the absence of an external electron acceptor while organic carbon sources are available such as acetate. Aerobic-famine refers to the opposite condition, *i.e.* presence of an external electron acceptor (oxygen) while no extracellular organic carbon source is available. Under these conditions, polyphosphate accumulating organisms (PAOs) such as *e.g.* *Candidatus* Accumulibacter phosphatis (hereafter referred to as Accumulibacter) have a selective advantage thanks to a metabolic strategy based on switching of synthesis and degradation of polyphosphate, glycogen and polyhydroxyalkanoates (Figure 4.1).

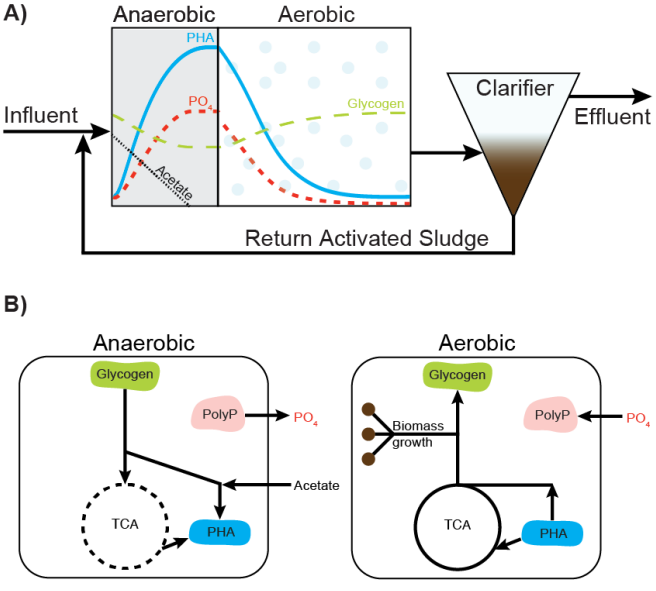


Figure 4.1: Schematic diagram of an EBPR process with nutrient/polymer profiles **(A)** and corresponding metabolic strategy **(B)**. Recirculated activated sludge containing PAOs is mixed with influent wastewater in an anaerobic reactor. To compensate for the absence of an external electron acceptor, Accumulibacter use intracellular polyphosphate and glycogen reserves to generate ATP to take up organic carbon sources (e.g. acetate) and synthesize polyhydroxyalkanoates (PHA). Phosphate is released at this stage. The TCA cycle is represented by a dashed line as it can have different operating modes. When oxygen becomes available, Accumulibacter makes use of the previously stored PHA for biomass synthesis and regeneration of glycogen and polyphosphate pools. The accumulation of polyphosphate in growing biomass and subsequent purge of this biomass leads to the net removal of phosphate from wastewater. Adapted from (McMahon and Read, 2013; Oyserman et al., 2016a). **PHA:** polyhydroxyalkanoates, **PolyP:** polyphosphate, **PO_4:** phosphate, **TCA:** tricarboxylic acid cycle.

The stoichiometry during the anaerobic phase is not yet fully elucidated as the discussion stands on the possibility of an anaerobic TCA cycle operation or glyoxylate shunt to provide for reducing equivalents for PHA accumulation (see **Chapter 2**). The main question is how FAD can be regenerated without an external electron acceptor (Mino, Van Loosdrecht, and Heijnen, 1998).

The switch between environmental conditions, *i.e.* with respect to substrate or oxygen availability, can be very rapid (*i.e.* less than one (1) minute). Thus, it is expected that Accumulibacter metabolism adapts, at least in the order of minutes. This is also the sampling frequency usually used for extracellular substrates and storage polymer profiles available in literature (Acevedo et al., 2012; Carvalheira et al., 2014; Filipe, Daigger, and Grady, 2001b; Oehmen et al., 2005; Welles et al., 2015). The environmental changes thus trigger fast metabolic pathway switches (Figure 4.1b), *e.g.* glycolysis to gluconeogenesis (and *vice-versa*), which entails a very interesting regulatory challenge as parallel pathway activity could lead to significant energy losses by futile cycling (Berg, Tymoczko, and Stryer, 2002).

Earlier carbon tracer experiments (also referred to as stable isotope probing) indicated the regular cycling of storage polymers under the anaerobic/aerobic regime (Hesselmann et al., 2000; Maurer et al., 1997; Pereira et al., 1996) but the regulatory mechanisms involved are still unknown. In these studies, only the positional labelling pattern imprinted in the storage polymers (glycogen, PHB and PHV) was measured, allowing to estimate carbon flows over the whole anaerobic or aerobic periods, but not the (short-term) dynamics in-between.

The publication of the first genome of Accumulibacter (García Martín et al., 2006) has enabled proteome (Wexler, Richardson, and Bond, 2009; Wilmes and Bond, 2006; Wilmes et al., 2008) and transcriptome (Oyserman et al., 2016a) studies with the aim to find the regulatory mechanisms that govern the metabolism of Accumulibacter under EBPR's changing conditions. However, these have not been able to explain the markedly different metabolism observed between the anaerobic and aerobic conditions. This suggests that the regulation of the lifestyle of Accumulibacter is mainly occurring at the metabolome level by post-translational, allosteric or thermodynamic control.

In this study, we aim to monitor the ^{13}C enrichment of intermediates of the central carbon metabolism of Accumulibacter and observe intracellular switches of carbon flows upon changes in oxygen or external organic carbon availability. To this end, U-^{13}C-acetate was used and the metabolome's ^{13}C enrichment was monitored every minute during two imposed transition periods: **1)** anaerobic feeding of acetate after a period of famine, and **2)** oxygen sparging after the anaerobic period when acetate is no longer available.

Materials & Methods

Medium and cultivation

An Accumulibacter enriched culture was obtained using 2 L (1.5 L working volume) laboratory bioreactor of glass with a stainless-steel bottom containing rapid sampling ports (Applikon Biotechnology B.V., The Netherlands), inoculated with aerobic granular sludge kindly supplied by D. de Graaff (TU Delft, The Netherlands). The bioreactor control systems are described in (Stouten et al., 2019). The sequencing batch reactor (SBR) was operated in cycles of 6 hours with alternated anaerobic and aerobic phases (120 min anaerobic, 185 min aerobic and 30 min settling phase).

Before feeding substrate, the broth was sparged with nitrogen gas to strip any dissolved

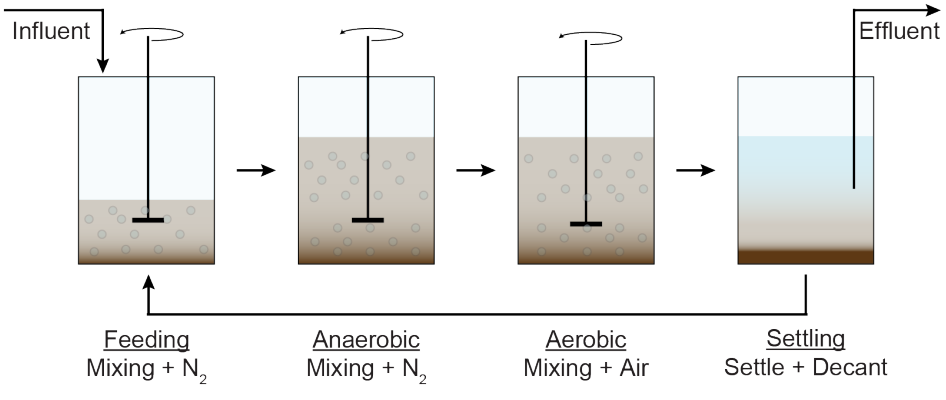


Figure 4.2: Schematic diagram of the anaerobic-aerobic SBR used to enrich for Accumulibacter in this study.

carbon dioxide and oxygen. During the anaerobic phase, the N_2 sparging rate was set at 0.383 L/min (approx. 0.26 vvm) and the stirring speed was 200 rpm. Later, in the aerobic phase, compressed air was sparged instead of N_2 at the same rate and keeping the same stirring speed. The pH was controlled at 7.6 ± 0.1 by titration with 0.2 M NaOH and 0.2 M HCl. The temperature was controlled at 20 ± 0.5 °C. The hydraulic retention time (HRT) was 12 hours (750 mL/cycle, 95 mL of broth before settling and 655 mL of supernatant after settling). The total solids retention time (SRT) was approximately 4 days (aerobic SRT of 2.1 days, equivalent to a dilution rate of approx. 0.01 h^{-1}) and was controlled by removing about 95 mL broth every cycle before settling.

The SBR was fed with two media containing **A)** 40.6 mM sodium acetate trihydrate, 12.5 mM sodium propionate, 0.02 g/L yeast extract and **B)** 40 mM NH_4Cl, 7.3 mM $MgSO_4.7H_2O$, 1.9 mM $CaCl_2.2H_2O$, 9.7 mM KCl, 12.5 mM $NaH_2PO_4.H_2O$, 6 mL/L of trace element solution prepared as in (Smolders et al., 1994b), and 0.04 g/L of allylthiourea (ATU) to inhibit nitrification. Of each medium, 75 g was added per cycle, together with 600 g of demiwater. The exact amounts of medium added were determined gravimetrically. The final feed (influent) contained 400 mg COD/L of acetate and propionate in a 65:35 COD-based ratio.

MICROBIAL COMMUNITY CHARACTERIZATION

The microbial community present in the enrichment culture under study has been characterized by three orthogonal approaches, Fluorescence *in situ* hybridization, *ppk1* gene analysis and 16S rRNA gene amplicon sequencing as earlier described in **Chapter 2**. The *ppk1* gene sequence obtained in this study have been deposited in the GenBank database under accession numbers MT079116. The 16S rRNA gene amplicon data have been deposited in GenBank under Bioproject PRJNA490689.

U-^{13}C ACETATE LABELLING EXPERIMENT (BATCH TEST)

For this experiment, medium **A)** was replaced by an uniformly labelled acetate-only solution with 0.4721 g sodium U-^{13}C-acetate (99 atom % ^{13}C, CortecNet, France) dissolved in 30 mg demi-water, which was dosed manually using a syringe at minute zero (0) of the experiment. To compensate the lower amount of medium added (30 g instead of 75 g), the amount of demi-water was adjusted (645 g instead of 600 g). The estimated concentration in the reactor upon the pulse is about 4.2 mM, which corresponds to 267 mg_{COD}/L in the reactor (534 mg_{COD}/L in the influent). This is 67 mg_{COD}/L higher than the total COD load in the reactor after addition of the standard carbon medium (medium **A)**), however this is not expected to influence the speed or nature of the metabolic shift since substrate is present in non-rate limiting concentrations. Furthermore, the cycle time during the ^{13}C switch was adjusted given the focus on the metabolic transitions **1)** anaerobic famine-to-feast, and **2)** anaerobic-to-aerobic: The anaerobic phase was ended after all acetate was consumed (*i.e.* after 105 min instead of 120 min, as the famine period was not relevant for this experiment). Additionally, due to the lower broth volume caused by the intense sampling, pH control had to be turned off at minute 194 of the experiment (90 minutes after the anaerobic-to-aerobic transition).

EXTRACELLULAR ANALYSIS

Around 4 mL of broth was taken from the reactor and filtered through a 0.22 μm PVDF Millex®-GV Syringe Filter Unit (Merck) into two 2 mL tubes (Eppendorf). Samples were stored at -80 °C until further analysis. Prior to injection, the samples were diluted based on the expected concentration range. The phosphate, ammonium and acetate concentration were then measured with a ThermoFisher Scientific® Gallery Analyzer. Acetate was additionally measured by HPLC using a Bio-Rad HPX-87H 300 column (7.8 mm). The column was eluted with phosphoric acid (1.5 mM in Milli-Q water) at a flow rate of 0.60 mL/min. This method was less sensitive to the sample's matrix interference.

GAS PHASE ANALYSIS

Both in- and offgas compositions were analysed by mass spectrometry (PRIMA BT Benchtop, Thermo Scientific, UK). The gas inflow was controlled, and the gas outflow was estimated based on the mass balance of N_2 gas, which is assumed to not be consumed nor produced by the microorganisms present in the bioreactor. The CO_2 and O_2 production and consumption rates, respectively, were calculated assuming there was no accumulation or depletion of these gases in the liquid phase. However, this is clearly not valid at the start of the anaerobic phase, and during the switch from anaerobic to aerobic.

RAPID SAMPLING AND QUENCHING FOR INTRACELLULAR METABOLITES

About 4 g of broth were rapidly withdrawn (approx. 4 s) from the reactor with a sampling device (Lange et al., 2001) and injected into a 50 mL Falcon tube containing 24 mL of 60 %(v/v) aqueous methanol quenching solution pre-cooled to -40 °C (mixture temperature: -21 °C). The content of each tube was homogenized by vortexing (5 s) and immediately poured onto a filter precooled with 20 mL of the same -40 °C cold quenching solution. Filtration was performed using 1 μm glass fibre filter disks (PALL glass fibre type E). The quenched biomass retained on the filter was washed with 20 mL of quenching solution. This cold filtration method has been previously used and described for filamentous fungi (Douma et al., 2010; Lameiras, Heijnen, and Gulik, 2015).

EXTRACTION AND CONCENTRATION OF INTRACELLULAR METABOLITE

Boiling ethanol extraction was performed to ensure complete cell disruption and inactivation of enzyme activity, according to a method modified from (Lameiras, Heijnen, and Gulik, 2015; Lange et al., 2001). Each sample was extracted immediately after sampling and quenching. For intracellular samples, the cooled filter paper with quenched biomass was placed in a falcon tube containing 25 mL of 75 % ethanol (pre-heated at 75 °C). Each tube was immediately vortexed and placed in a water bath at 95 °C. After 3 min each tube was placed on ice, and later stored at -80 °C. Polymer samples were also submitted to the same process, by withdrawing about 4 g of broth from the reactor directly into 25 mL of 75 % ethanol (pre-heated at 75 °C) and boiled for 3 min at 95 °C. All extracts were evaporated at 30 °C to dryness in a Rapid-Vap (Labconco, Kansas City, MO) under vacuum. After evaporation, the residues were resuspended in 600 μL of MilliQ water, transferred into a 1.5 mL tube (Eppendorf), and centrifuged (Heraeus Biofuge Pico microcentrifuge) at room temperature for 10 min at 10,400 × *g* to remove cell debris. The supernatants were transferred to a new tube, centrifuged again, transferred to a screw-cap sample vial tube, and finally stored at -80 °C until further analysis.

MASS ISOTOPOMERS MEASUREMENTS

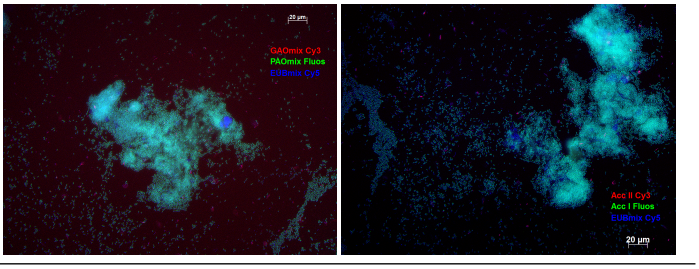
The ^{13}C enrichment in intracellular metabolites was obtained from the measurement of mass isotopomers using coupled chromatography-MS systems depending on the nature of the metabolites analysed. The mass isotopomers of glycolytic sugar phosphates and di- and tricarboxylic acids were determined by anion- exchange LC-MS/MS (Alliance HT pump 2795 LC system and, Quattro LC™ triple quadrupole, Waters, UK) (Canelas et al., 2009). For GC-MSMS analysis of glycolytic, pentose phosphate pathway, TCA, and storage metabolism intermediates 150 μL of sample was freeze-dried together with 20 μL of AAL signal boosting mix (Niedenführ et al., 2016; Pierick et al., 2014) and subsequently derivatized as described in (Cipollina et al., 2009). Measurement was carried out using a GC-MSMS (7890A GC coupled to 5975C MSD, Agilent, USA) as described in (Niedenführ et al., 2016). For GC-MS analysis of aminoacids 150 μL of sample were transferred to a glass vial, 30 μL of 100 mg/mL NaCl were added and the mixture was freeze-dried. Derivatization and measurement were then carried as described in (Jonge et al., 2011) using a GC-MS (7890A GC coupled to 5975C MSD, Agilent, USA). Carbon skeleton ^{13}C enrichments measured by GC-MS and GC-MSMS were corrected for natural isotopes of non-carbon skeleton atoms (Niedenführ et al., 2016; Wahl, Dauner, and Wiechert, 2004).

RESULTS & DISCUSSION

CHARACTERIZATION OF ACCUMULIBACTER ENRICHMENT

This study was carried out using an Accumulibacter enrichment culture continuously cultivated in a sequencing batch reactor (SBR-3). A summary of the main process parameters and key performance indicators are found in Table 4.1.

Table 4.1: Process parameters and key performance indicators of the Accumulibacter enrichment used in this study (SBR-3). A FISH analysis allowed for a visual assessment of the proportion of general bacteria, GAO, PAO and the two different types of PAO, I and II. The 16S rRNA gene analysis provided information on the *genus* of the most abundant sub-populations next to Accumulibacter and the *ppk1* gene analysis further specified which Accumulibacter I clade was dominant.

KPIs	**SBR-3**
Anaerobic P-release per C-consumed	0.65 Pmol/Cmol (SBR cultivation) 0.67 Pmol/Cmol (U-^{13}C-acetate batch)
Anaerobic-feast length	~43 min (SBR cultivation, 400 mg_{COD}/L influent, 65:35 Ac:Pr) ~70 min (U-^{13}C-acetate batch, 534 mg_{COD}/L influent, 100:0 Ac:Pr) Total anaerobic period was 105 minutes.
FISH	A) B) 
16S rRNA gene amplicon sequencing (dominant OTUs)	Dominant: Ca_Accumulibacter Sub-populations (<5% of rRNA count): *genus* from f__Hyphomonadaceae, *Plasticicumulans acidivorans*
ppk1 analysis	Dominant: *Ca.* Accumulibacter phosphatis clade IA

A) blue: EUB mix (Cy5); purple (superposition of blue and red): GAO mix (Cy3); and cyan green (superposition of blue and green): PAO mix (Fluos).
B) blue: EUB mix (Cy5), purple (superposition of blue and red): PAO type II (Cy3), and cyan green (superposition of blue and green): PAO type I (Fluos).

The high anaerobic P-release per C-consumed indicates that the PAO metabolism is the dominant mechanism for acetate uptake and that glycogen accumulating organisms (GAO) are likely not present (Welles et al., 2017). The anaerobic phosphate release to acetate uptake ratio measured in this study matches the predicted values for PAO metabolism of 0.59-0.66 Pmol/Cmol at pH 7.6 (Filipe, Daigger, and Grady, 2001b; Smolders et al., 1994b). This is corroborated through the microbial characterization by FISH that confirmed Accumulibacter type I formed the vast majority of biovolume of biomass present as it can

be seen by the high overlap between the Accumulibacter-specific probe and the general Bacteria probe (see Table 4.1). The *ppk1* gene analysis further specified Accumulibacter clade IA was dominant and the 16S rRNA gene analysis provided information on the *genus* of the most abundant sub-populations next to Accumulibacter (see Table 4.1). The detailed results can be found in section 4.5.

U-^{13}C-ACETATE BATCH EXPERIMENT

The Accumulibacter enrichment characterized above was used for a ^{13}C tracer experiment. In this batch experiment only U-^{13}C-acetate was fed (*i.e.* no propionate nor yeast extract). Typical acetate, orthophosphate, ammonium, and produced CO_2 profiles for an Accumulibacter enrichment are shown in Figure 4.3 together with an overview of the intracellular samples taken in this experiment.

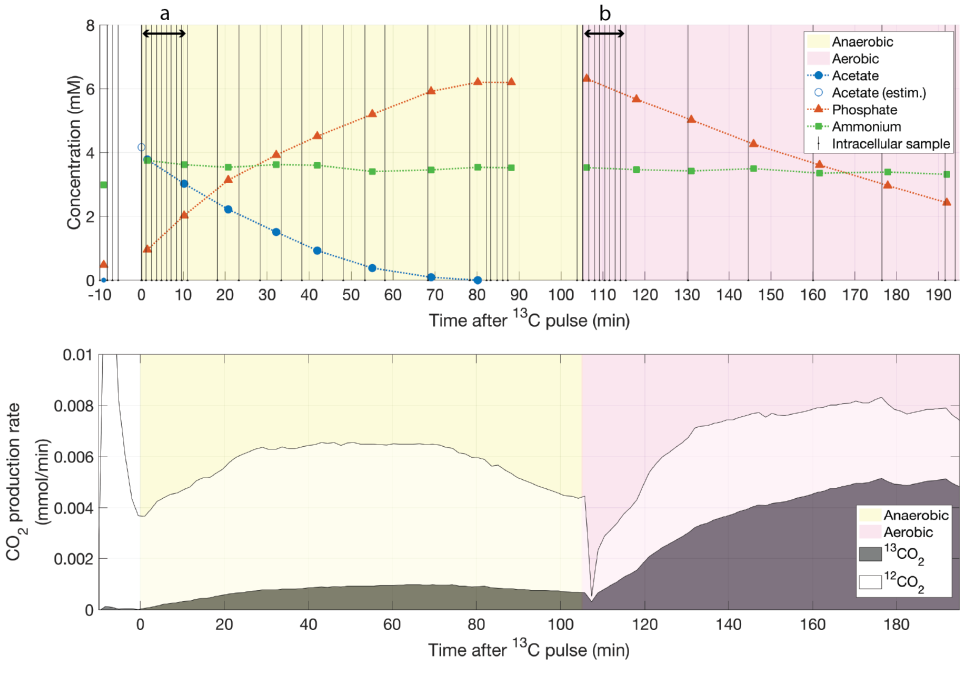


Figure 4.3: Acetate, phosphate, ammonium concentrations (top) and CO_2 profiles (bottom) during the U-^{13}C-acetate batch experiment. **Top:** The intracellular state of Accumulibacter was measured with a higher frequency (sampling indicated by black vertical lines, about 1 sample per minute) during the two transition periods: **a)** anaerobic famine-to-feast, *i.e.* when acetate is fed (starting at 0 min), and **b)** anaerobic-to-aerobic, *i.e.* when oxygen becomes available (starting at 105 min). **Bottom:** Calculated CO_2 production rate for $^{12}CO_2$ and $^{13}CO_2$. The line on top of the white shaded area corresponds to total CO_2 production rate.

ANAEROBIC FAMINE TO FEAST TRANSITION

EBPR microorganisms undergo a transition from famine to feast when they encounter influent wastewater rich in organic carbon sources after a period when these were unavailable (Figure 4.1). The first extracellular sample was taken 1.5 min after U-^{13}C-acetate was added to the bioreactor and the consumption of acetate together with phosphate release was already observable (Figure 4.3). A higher sampling frequency would be needed to determine the exact time it takes for these processes to become active. Therefore, we cannot discriminate between active regulation (allosteric or post-translational modifications) or thermodynamic control of the acetate uptake and phosphate release. Nevertheless, based on transcriptional evidence acetate can be activated to acetyl-CoA by both high and low affinity pathways (Oyserman et al., 2016b) and phosphate efflux can be carried *via* a H^+ symporter (Pit), which has been postulated several times in literature (Burow et al., 2008; Saunders et al., 2007; Veen, 1997).

The detection of $^{13}CO_2$ in the off-gas indicates that a fraction of the fed U-^{13}C-acetate is immediately oxidized to CO_2 in the anaerobic phase, likely originating from the decarboxylation reactions of the oxidative TCA cycle branch. This hypothesis is also supported by the observed enrichment of ^{13}C in isocitrate to α-ketoglutarate and α-ketoglutarate to succinyl-CoA (Figure 4.4).

Acetate is taken up by Accumulibacter and activated to acetyl-CoA. In regular aerobic acetate-consumers, this acetyl-CoA would be channelled to biosynthetic pathways like gluconeogenesis. However, in Accumulibacter no ^{13}C reaches glucose-6-phosphate (G6P) indicating gluconeogenesis is inactive during the anaerobic phase. This was expected since Accumulibacter is known to use glycogen *via* glycolysis under anaerobic acetate uptake conditions to provide the reducing equivalents for PHB accumulation from acetate (Mino, Van Loosdrecht, and Heijnen, 1998).

In Accumulibacter a large fraction of acetyl-CoA is used for PHB accumulation. The mass isotopomers of 3HB (PHB precursor) were measured (Figure 4.4). Despite the noise (mostly from the +4 mass isotopomer measurement), it is observed that about 40% of the 3HB is labelled 1 min after the acetate addition and reaches a stable enrichment of about 60% after 10 minutes. The regulation of the pathway leading to PHB accumulation is postulated to be related with free CoA metabolite concentration, e.g. as described for *Methylobacterium rhodesianum* (Mothes, Rivera, and Babel, 1996) and *Azotobacter beijerinckii* (Senior and Dawes, 1973): Once acetate enters the cell and is activated to acetyl-CoA, the free CoA concentration drops and the competitive inhibition on β-ketothiolase is released (*i.e.* kinetic control). It has also been proposed that the acetoacetyl-CoA reductase also plays a regulatory role in PHB synthesis in response to the cell's NADPH/$NADP^+$ ratio (*i.e.* thermodynamic control), but with a smaller influence than the CoA/acetyl-CoA ratio has on β-ketothiolase (Leaf and Srienc, 1998).

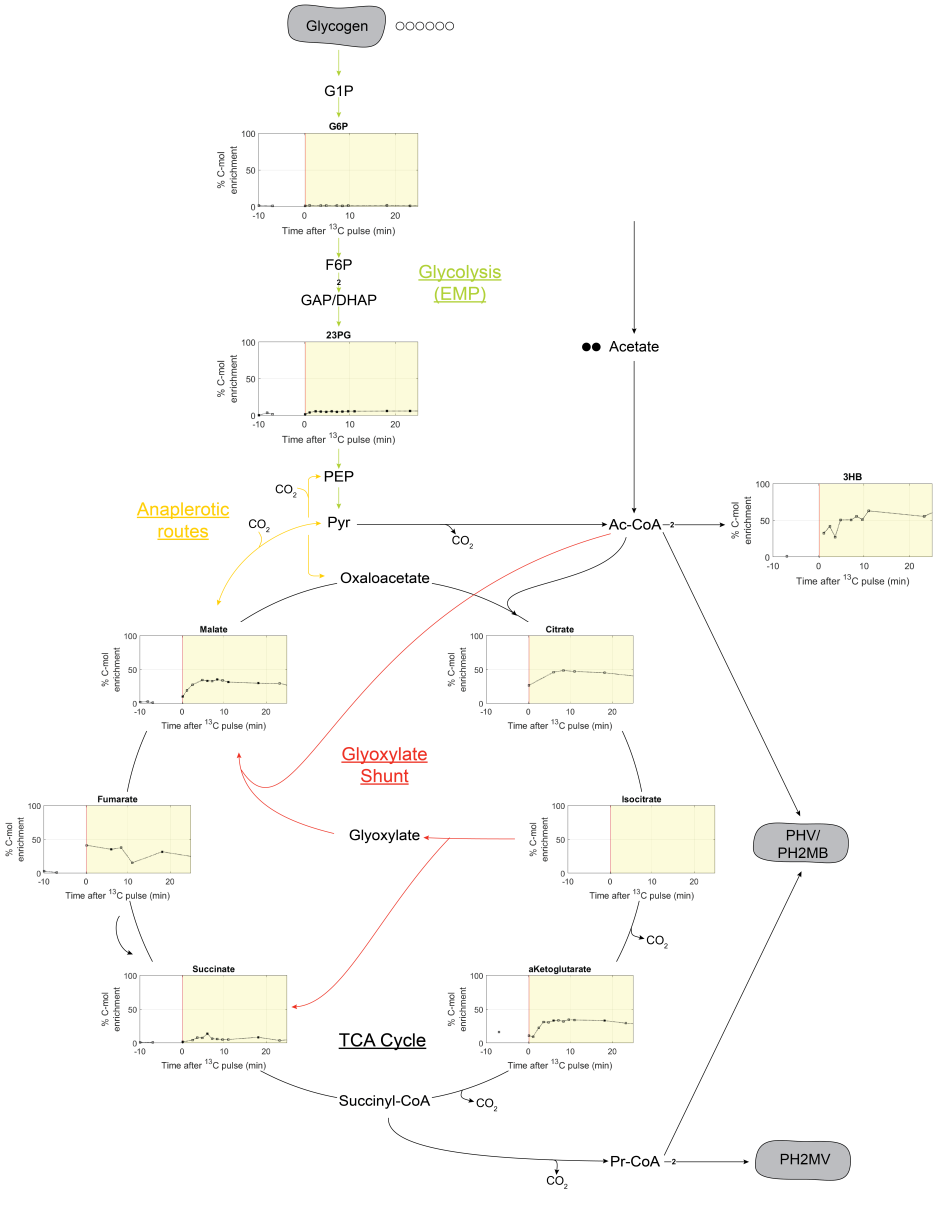


Figure 4.4: Measured ^{13}C enrichments during the anaerobic famine-to-feast transition. Bold squares are an average of both GC-MS/MS and LC-MS/MS methods. ATP and redox cofactors were omitted for simplicity. Isocitrate signal was below the detection limit throughout the whole anaerobic phase.

The synthesis of PHB from acetate requires reducing equivalents (e.g. NADH) and these can either be provided by glycolysis, full/partial TCA cycle or glyoxylate shunt (see **Chapter 2**). If all 3HB originates from fully labelled acetate and unlabelled glycogen, then after 10 minutes the C-mol ratio of 40% U-^{12}C-glycogen to 60% U-^{13}C-acetate consumed corresponds to an optimum flux distribution for PHB synthesis – *i.e.* glycogen degradation to acetyl-CoA provides reducing equivalents and only a small surplus of NAD(P)H is regenerated in the reductive branch of the TCA cycle (see Figures 2.3 and 2.4 from **Chapter 2**). However, this interpretation does not correspond with the ^{13}C found in the analysed tri- and dicarboxylic acids (*i.e.* TCA cycle intermediates) in the anaerobic phase (Figure 4.4). After 5 minutes, the amount of ^{13}C in tri- and dicarboxylic acids reaches about 40% suggesting incorporation of U-^{13}C-acetate *via* the glyoxylate shunt and/or a full oxidative TCA together with the generation of reducing equivalents. The different metabolic modes possible are discussed later and shown in Figure 4.7. Unexpectedly, instead of stabilizing after about 10 minutes as for 3HB, the amount of ^{13}C in tri- and dicarboxylic acids decreases slowly until reaching about 15% by the end of the anaerobic phase (about twenty-five times (25x) slower than the initial ^{13}C-enrichment rate). This ^{13}C-de-enrichment is likely due to a change in the acetate to glycogen consumption ratio. The unlabelled tri- and dicarboxylic acids are then produced from the anaplerotic conversion of unlabelled glycogen-derived metabolites whereas ^{13}C-enriched acids originate from U-^{13}C-acetate incorporation. Solely based on this observation, it can be concluded that the carbon for tri- and dicarboxylic acids originating from glycogen and acetate changed in time. A high acetate fraction in the beginning, while there is a higher glycogen fraction after about 5 to 10 minutes into the anaerobic-feast phase. For Accumulibacter, this represents a transition from acetate- to glycogen-sourced reducing equivalents during the anaerobic-feast phase.

ACETATE- TO GLYCOGEN-SOURCED REDUCING EQUIVALENTS (UNEXPECTED) TRANSITION

While the two main transitions occurring are imposed by external conditions and are expected, the internal metabolic transition at 5-10 min was unexpected. Without quantitative metabolite concentration measurements, it is difficult to pinpoint what triggered the metabolic response. In literature, some putative mechanisms were discussed: A build-up of reducing equivalents, NADH or NADPH, could inhibit the citrate synthase (Swissa and Benziman, 1976; Weitzman and Jones, 1968) and prevent acetyl-CoA from being further incorporated in the TCA cycle. Further, phosphoenolpyruvate carboxylase is often allosterically activated by acetyl-CoA (Morikawa et al., 1980), which would increase the flow of glycogen-derived pyruvate into oxaloacetate and consequentially result in the ^{13}C-de-enrichment of tri- and dicarboxylic acids. The study of this and other anaplerotic routes is recommended to better understand their role in Accumulibacter's metabolism.

ANAEROBIC TO AEROBIC TRANSITION

The metabolism of EBPR microorganisms switches again upon the transition from an anaerobic to an aerated zone (Figure 4.5).

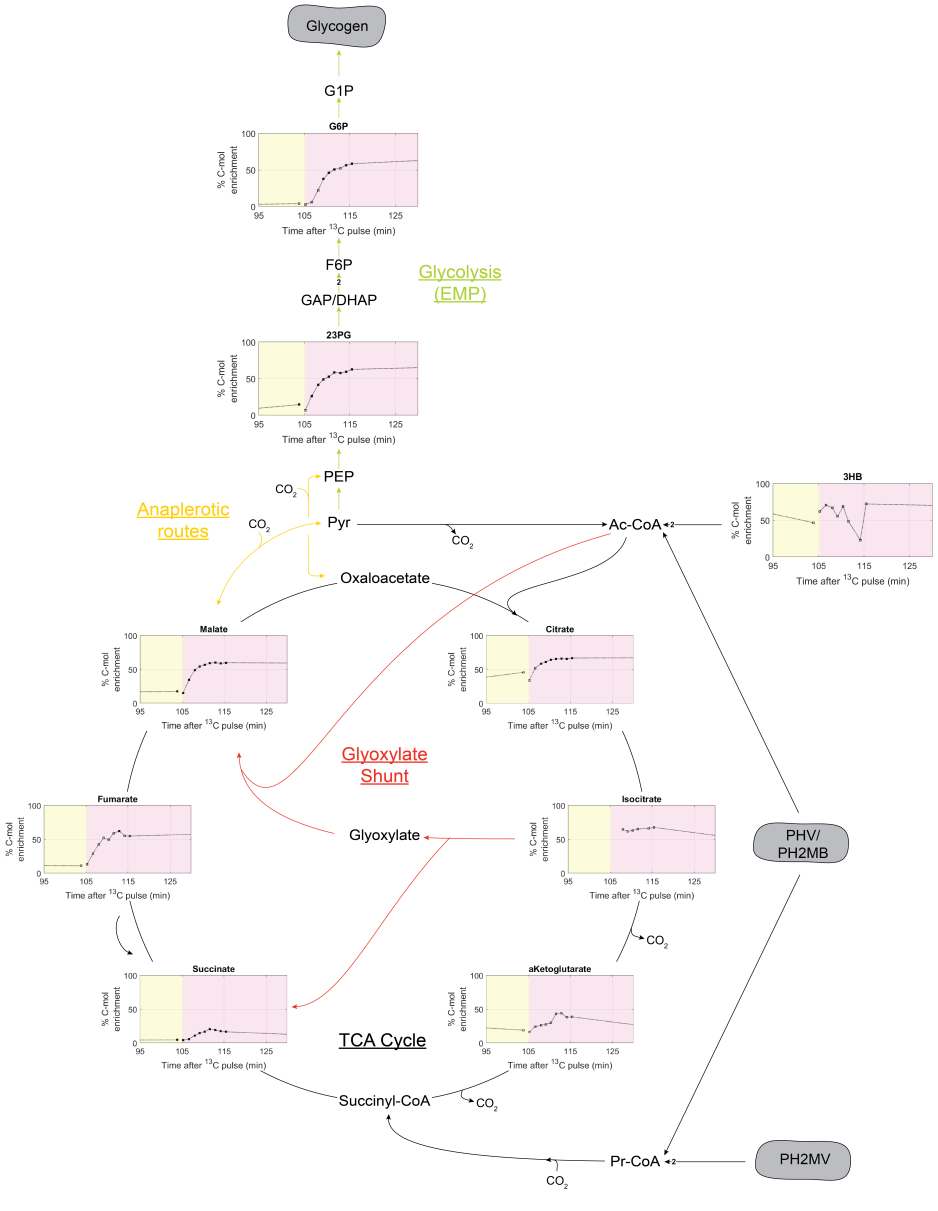


Figure 4.5: Measured ^{13}C enrichments following the anaerobic-to-aerobic transition (t > 105 min). Bold squares are an average of both GC-MS/MS and LC-MS/MS methods. ATP and redox cofactors were omitted for simplicity.

Once oxygen becomes available, the ^{13}C enrichment in CO_2 and the tri-/di-carboxylic acids and in the glycolytic intermediates rises to about 60% as expected since that was the ^{13}C enrichment found in 3HB (now substrate). Succinate exhibits a lower ^{13}C enrichment, which again does not match that of its flanking metabolites, fumarate and α-ketoglutarate, which suggests additional, not yet described sources of succinate (including degradation during sample processing, or other biases). Isocitrate levels were below the detection limit during the anaerobic phase but are well detectable during the aerobic phase. For most metabolites, the ^{13}C enrichment stabilizes about 7 minutes after oxygen becoming available. The exception is the ^{13}C enrichment of G6P, which is likely dependent on the activation of gluconeogenesis. In Figure 4.6, G6P and 2-/3PG are compared during the anaerobic-to-aerobic transition.

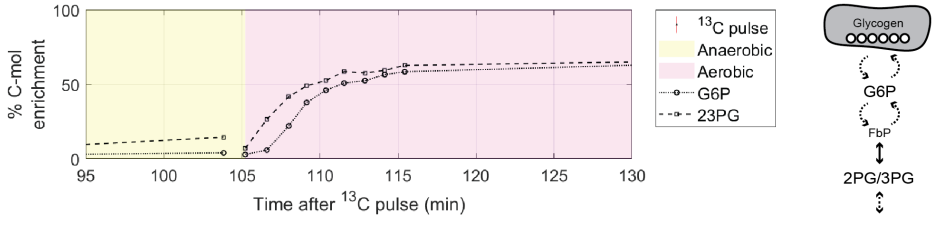


Figure 4.6: Comparison between G6P and 2-/3PG around the anaerobic-to-aerobic transition. Note the lag between 105 and 106 minutes for G6P while 2-/3PG are already getting ^{13}C-enriched.

The influx of ^{13}C into G6P seems to be delayed by about 1 minute with respect to that in 2-/3PG (lag between 105 and 106 minutes), which hints for the expected regulatory mechanism in-between these metabolites; the reactions of fructose-6-phosphate (F6P) into fructose-1,6-phosphate (FbP) and *vice versa*, are commonly regulated steps in glycolysis and gluconeogenesis. In the glycolysis direction the reaction is catalysed by phosphofructokinase (PFK), which consumes ATP. In the gluconeogenic direction, fructose bisphosphatase (FBPase) catalyses the reaction, which only releases phosphate instead of producing ATP. To avoid futile cycling of ATP, FBPase in *E. coli* (same FBPase type 1 as in Accumulibacter) is regulated by allosteric effectors like AMP and G6P (inhibitors) or PEP (activator) (Hines et al., 2007). From transcriptomic data, the transcripts coding for both enzymes are present throughout the EBPR cycle (Oyserman et al., 2016b) and do not hint for genetic regulation leading to changes in enzyme synthesis. Thus, allosteric regulation is the expected mechanism to explain the observed ^{13}C enrichment pattern, *i.e.* no gluconeogenesis anaerobically and delayed gluconeogenesis start aerobically. We recommend performing an enzymatic activity study on this step between F6P and FbP on cell free extracts from Accumulibacter harvested in different phases of the EBPR cycle to confirm this is indeed the regulatory mechanism controlling glycolysis and gluconeogenesis. Additionally, the activity of a pyrophosphate—fructose-6-phosphate-1-phosphotransferase, which uses inorganic phosphate (PPi) as phosphoryl donor instead of ATP, should also be assessed as this enzyme was found present in a metaproteomics study (Barr et al., 2016).

SWITCHES IN ^{13}C PATTERNS IMPLY DIFFERENT METABOLIC MODES

Every few hours, Accumulibacter switches cyclically between anaerobic and aerobic conditions. When oxygen is available, the TCA cycle, the glyoxylate shunt and the anaplerotic routes leading to gluconeogenesis are undoubtedly active in Accumulibacter. In the subsequent anaerobic phase, when oxygen is no longer available, these enzymes remain present (Wexler, Richardson, and Bond, 2009; Wilmes and Bond, 2006; Wilmes et al., 2008) and different biochemical models supported by experimental observations have proposed different metabolic modes to describe flux through the abovementioned pathways (Hesselmann et al., 2000; Pereira et al., 1996; Yagci et al., 2003). All three metabolic transitions showed earlier are marked by a shift in the ^{13}C enrichment pattern in the tri- and dicarboxylic acids. In Figure 4.7, malate is examined in detail as it is a key intermediate to assess the trajectory of U-^{13}C-acetate. Each ^{13}C enrichment pattern of malate, *i.e.* relative proportion of mass isotopomers, is then likely stemming from the different metabolic modes proposed. Further analysis of different ^{13}C labelling patterns in malate depending on metabolic modes can be found later in Supplementary Table 4.2.

The appearance of ^{13}C in tri- and dicarboxylic acids to such an extent (40% in 5 minutes into the anaerobic-feast period) can only be possible *via* the glyoxylate shunt, or a full TCA cycle operation. To distinguish between metabolic modes, one can compare the three different ^{13}C patterns (Figure 4.7) generated in each of the identified transitions:

A. **Anaerobic famine to feast (upon U-^{13}C-acetate feeding):** A glyoxylate operation will quickly generate both +2 and +4, whereas a full TCA cycle will first generate mainly +2 and then sequentially +3, and +4. Given that mostly +2 is found and followed sequentially by heavier isotopomers, likely the full TCA is the active operating mode and the glyoxylate shunt seems to be inactive.

B. **Acetate- to glycogen-sourced reducing equivalents transition:** After about 5 minutes, the ^{13}C enrichment pattern changes its course; Normally, the incorporation of ^{13}C into the TCA cycle would continue onwards to generate the higher isotopomers. Here, it not only stabilizes as it goes in the opposite direction – at this point, ^{13}C-acetyl-CoA is no longer being incorporated by citrate synthase and only ^{12}C-glycogen-derived precursors are entering the malate pool *via* pyr and/or PEP and triggering a reversed operation of the TCA – reductive TCA branch.

C. **Anaerobic to aerobic (O_2 sparging start):** When oxygen becomes available, +2 is fastest, followed by +4 and both reach about the same enrichment within about 10 minutes, as expected for a glyoxylate operation. Upon this observation, it becomes very clear that the glyoxylate shunt cannot be the one contributing to the labelling pattern of malate under anaerobic conditions. The +3 comes slower, likely from the TCA cycle, and, to a smaller extent, from the exchange between Mal/OAA and Pyr/PEP.

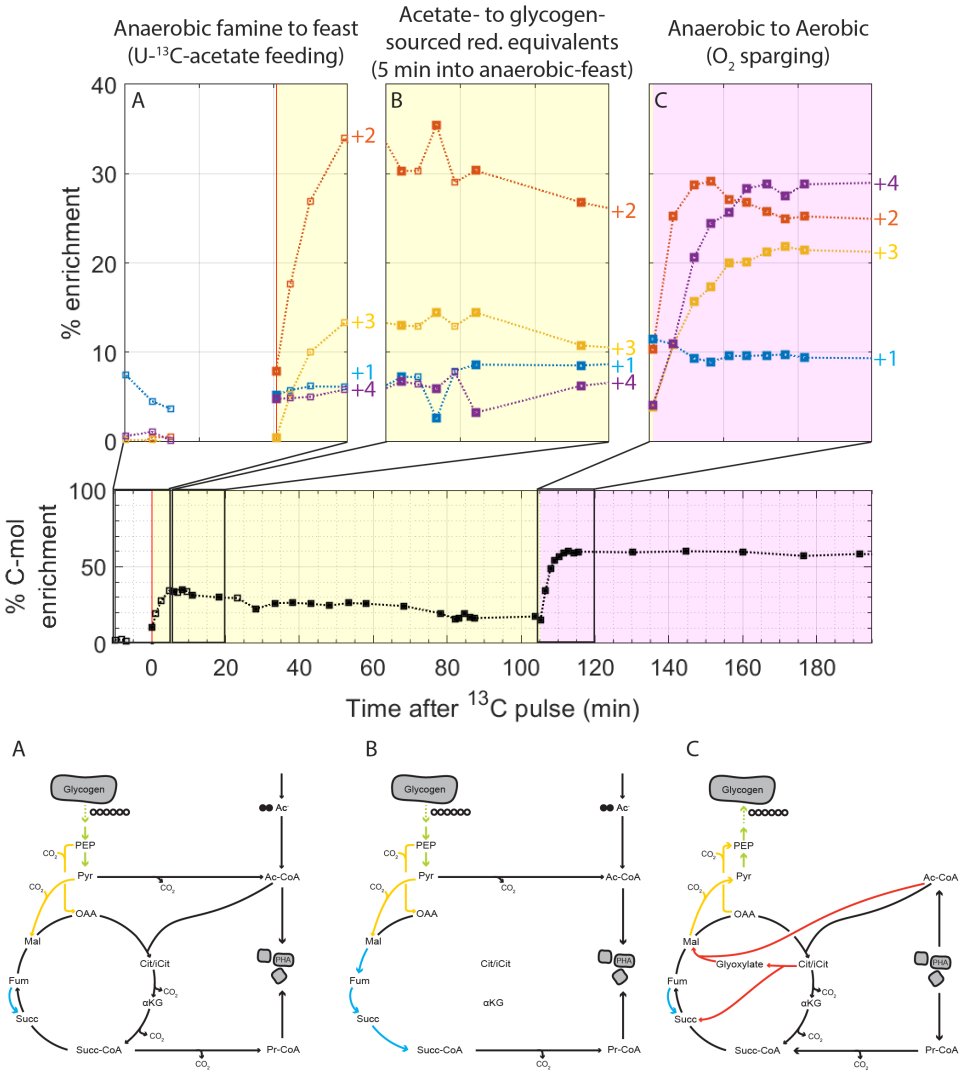


Figure 4.7: Comparison of the ^{13}C enrichment pattern of malate, *i.e.* relative proportion of mass isotopomers, stemming from different metabolic modes. **A)** acetate is added to the medium under anaerobic conditions and activated to acetyl-CoA that then flows through the TCA cycle, **B)** change in the ^{13}C enrichment trajectory likely because ^{13}C-acetyl-CoA is no longer being incorporated by citrate synthase and only ^{12}C-glycogen-derived precursors are entering the malate pool, **C)** oxygen becomes available and acetyl-CoA originates intracellularly from 60% ^{13}C-PHB and flows through both the TCA cycle and the glyoxylate shunt metabolic modes. Further analysis of different ^{13}C labelling patterns in malate depending on metabolic modes can be found later in Supplementary Table 4.2.

The activity of the glyoxylate shunt has been observed several times for Accumulibacter in **Chapter 2** but also in past studies (Burow, Mabbett, and Blackall, 2008; He and McMahon,

2011a; Hesselmann et al., 2000; Oyserman et al., 2016b; Skennerton et al., 2015; Wexler, Richardson, and Bond, 2009; Wilmes et al., 2008; Yagci et al., 2003). Its most logic operation lies during the aerobic period when cells use acetyl-CoA from PHA to grow and replenish glycogen (Figure 4.7C). This shunt seems to be inactive during the anaerobic phase (Figure 4.7A and B). The low glyoxylate shunt activity may be related with the observation that isocitrate was below the limit of detection throughout the whole anaerobic period and only became measurable once oxygen was available. A kinetic characterization of the isocitrate dehydrogenase (ICDH) and isocitrate lyase (ICL) of Accumulibacter is recommended to assess the sensitivity of these enzymes to a low isocitrate concentration as their different affinity to isocitrate may play a role in how flux is split at this branching point (Wendisch et al., 2000). Furthermore, in *E. coli* growing on acetate aerobically, this shunt is inhibited if glucose becomes available (LaPorte, Walsh, and Koshland, 1984) by an ICDH-kinase/phosphatase. The gene coding for this enzyme is present in the genome of Accumulibacter and is adjacent to that of ICDH (García Martín et al., 2006). In this case, phosphoproteomics would be an interesting method to track the phosphorylation state of ICDH throughout an EBPR cycle and relate it to its maximum velocity. Lastly, the *in vivo* estimation of intracellular fluxes through these pathways and branch point would be ideal to solve this question, however quantitative metabolomics in Accumulibacter remains a challenge due to the broth heterogeneity of these granular enrichment cultures.

The ^{13}C labelling pattern of malate (Figure 4.7A) is consistent with that of a full TCA cycle operation in the first period of the anaerobic phase. This is deemed unlikely due to the absence of an external electron acceptor supposedly needed to regenerate FAD (Mino, Van Loosdrecht, and Heijnen, 1998). Typically, it is argued that fully anaerobic conditions were not yet attained due to the residual presence of oxygen, nitrate or other oxidised compounds, but that was not the case here. *Thiobacillus ferrooxidans* are capable of regenerating FAD using uphill electron transfer (Elbehti, Brasseur, and Lemesle-Meunier, 2000), and inspired by it, García-Martin *et al* suggested a novel fusion protein was present in the genome of Accumulibacter that could allow for such mechanism (García Martín et al., 2006). However, this has not yet been demonstrated experimentally, and first steps towards elucidating such mechanisms are done in **Chapter 5**.

TOWARDS A REGULATORY MODEL FOR ACCUMULIBACTER

The switches in ^{13}C enrichment patterns observed in this study allow to generate hypotheses on regulatory mechanisms governing the lifestyle of Accumulibacter upon changes in oxygen or external organic carbon availability. In Figure 4.8, an overview is presented on the presumed regulated steps based on the observed ^{13}C patterns.

The interplay of the pathway leading to PHA, Glycolysis, Gluconeogenesis, TCA cycle, Glyoxylate shunt, Succinate-Propionate Shunt, and other anaplerotic routes in Accumulibacter is presumably orchestrated by different types of regulation over multiple checkpoints. The causal chain until the root causes (drivers) remains vague and further detailed investigation on each regulatory mechanism combined with mathematical modelling will enable the construction of a regulatory model for Accumulibacter.

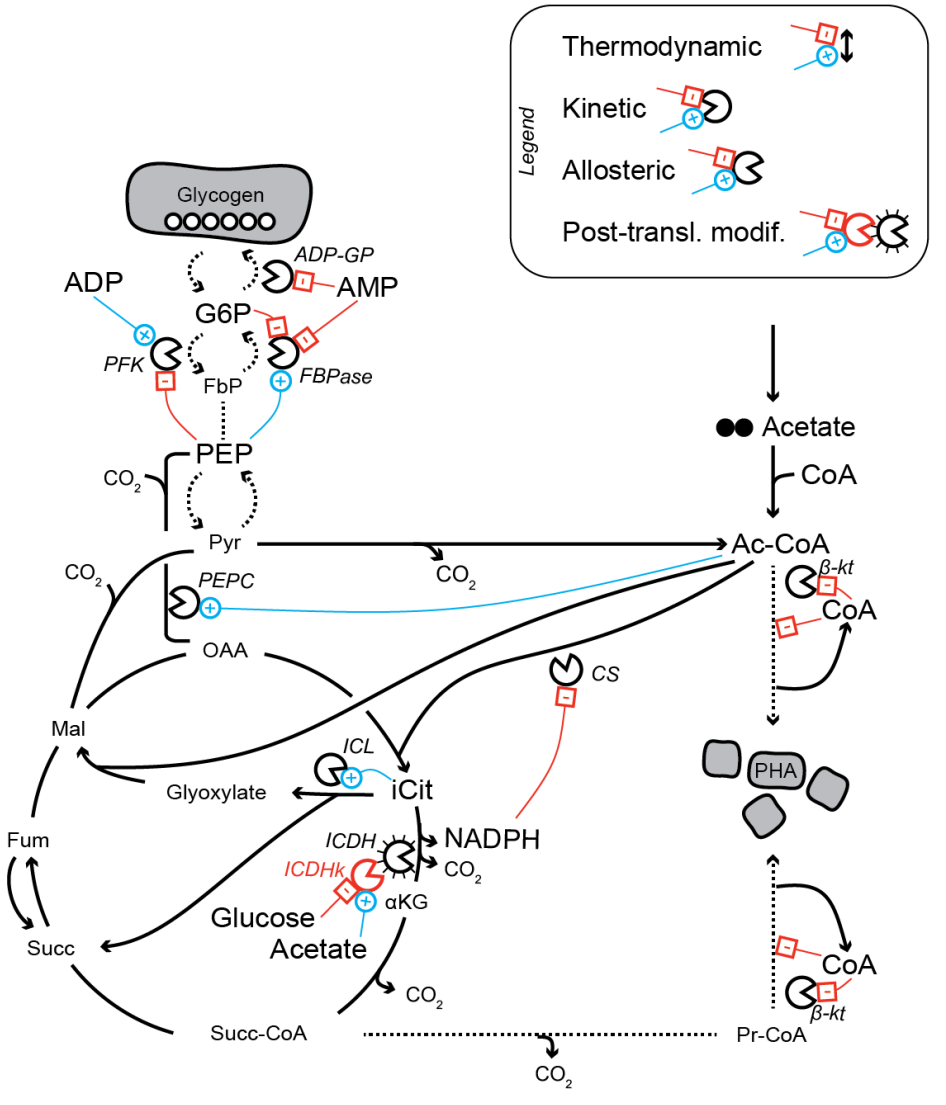


Figure 4.8: Presumed regulated checkpoints based on observed ^{13}C patterns and postulated mechanisms based on analogous bacterial systems described in literature (Gottschalk, 1986; LaPorte, Walsh, and Koshland, 1984; Hines et al., 2007; Leaf and Srienc, 1998; Morikawa et al., 1980; Mothes, Rivera, and Babel, 1996; Senior and Dawes, 1973; Wendisch et al., 2000).
ADP-GP: Glucose-1-phosphate adenylyltransferase, **PFK:** Phosphofructokinase, **FBPase:** Fructose 1,6-biphosphatase, **PEPC:** Phosphoenolpyruvate carboxylase, **CS:** Citrate synthase, **ICL:** Isocitrate lyase, **ICDH:** Isocitrate dehydrogenase, **ICDHk:** Isocitrate dehydrogenase kinase, β-**kt:** β-ketothiolase

Conclusion

A time series ^{13}C labelled metabolome was used to gain insight into enriched bacteria cultivated in dynamic, non-axenic conditions. As case-study, a highly enriched Accumulibacter culture was examined in different phases of a typical EBPR cycle, including the expected metabolic transitions anaerobic famine-to-feast and anaerobic-to-aerobic.

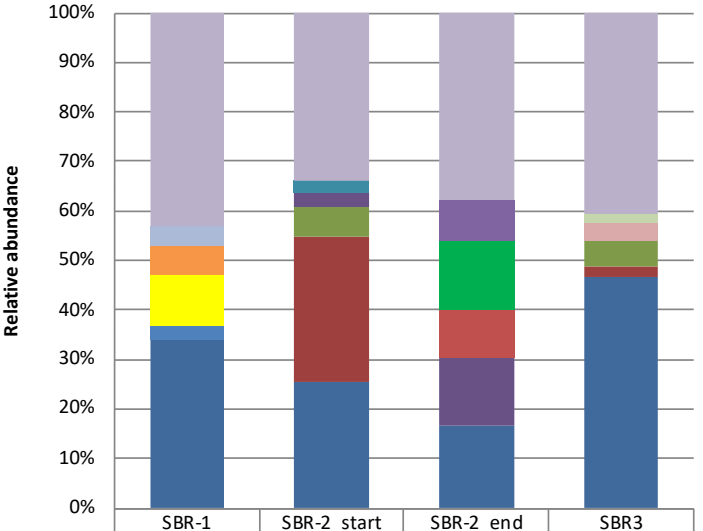
A third, unexpected metabolic transition was observed 5 minutes into the anaerobic-feast phase, providing experimental evidence for the flexibility of Accumulibacter to balance reducing equivalents anaerobically. Beyond observing metabolic shifts leading to different "steady-state" ^{13}C:^{12}C enrichments, ^{13}C labelling delays were observed, for example between gluconeogenesis intermediates that indicate the presence of regulated steps separating them.

Based on the analysis of the different proportion of mass isotopomers of a metabolic intermediate such as malate, distinctive metabolic modes were inferred that combined differently the TCA cycle, its branches and the glyoxylate shunt throughout the EBPR cycle. Another key intermediate at a branching point is succinate, which presented a strange behavior and requires further analysis given its link with the FAD regeneration problem.

Finally, regulated checkpoints and mechanisms are here postulated, providing a framework to construct a regulatory model for Accumulibacter. Such mechanistic model can enable the design of novel strategies to improve EBPR processes.

SUPPLEMENTARY INFO

MICROBIAL COMMUNITY CHARACTERIZATION



	SBR-1	SBR-2_start	SBR-2_end	SBR3
others	43.2	34.1	37.7	40.8
Acidovorax delafieldii				1.6
Plasticicumulans acidivorans				3.5
Lachnotalea	3.6			
Blastochloris	6.2			
Rhodopseudomonas	10.3			
Aquimonas			8.3	
Chryseobacterium			14.0	
Flavobacterium			9.6	
Rhodobacter	2.7			
Lactobacillus		2.4		
genus from o__Sphingobacteriales		2.7	13.7	
genus from f__Hyphomonadaceae		6.0		5.3
Dechloromonas		29.3		2.4
Candidatus_Accumulibacter	34.1	25.5	16.7	46.5

Figure 4.9: Taxonomic distribution of microbial community based on 16S rRNA gene copy numbers. The 16S rRNA gene amplicon libraries have been deposited under project PRJNA490689. SBR-3 was used in this study.

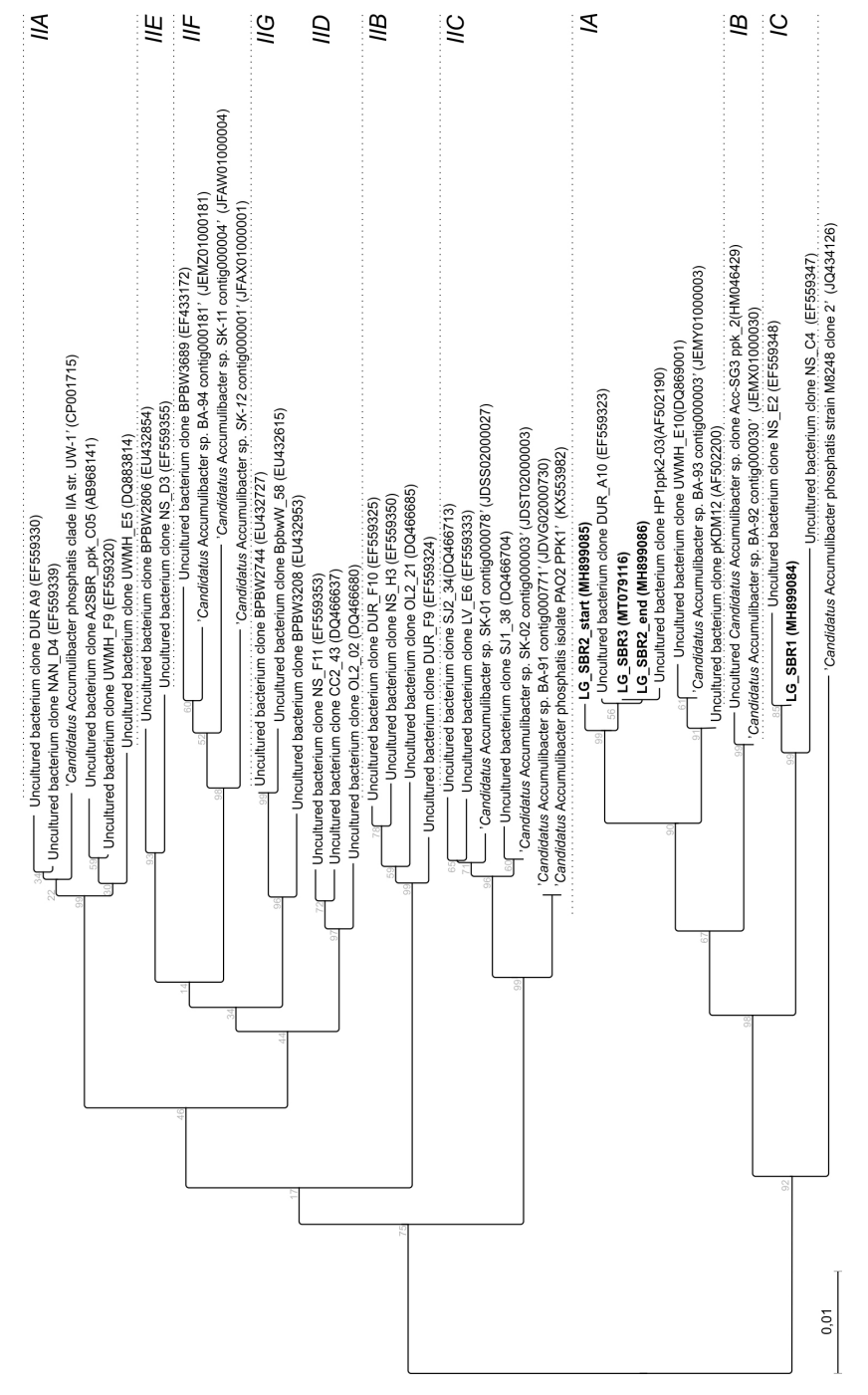


Figure 4.10: Phylogenetic tree based on Accumulibacter *ppk1* gene analysis. SBR-3 was used in this study and Accumulibacter clade IA was dominant.

OVERVIEW OF COMPLETE EBPR CYCLE

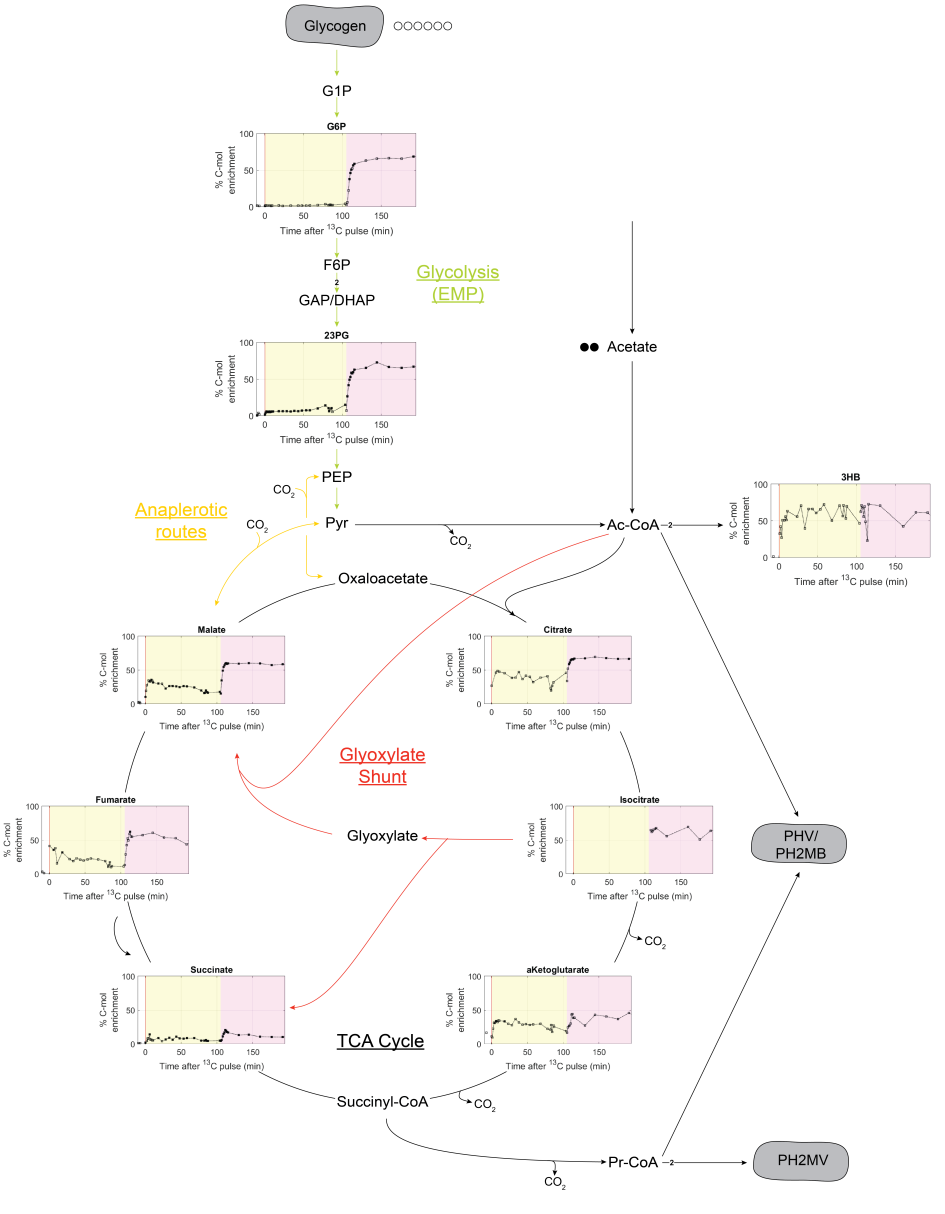
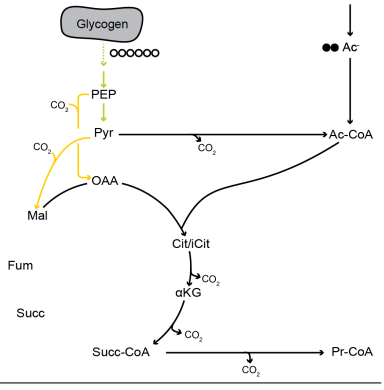


Figure 4.11: Measured ^{13}C enrichments throughout the whole experiment. Bold squares are an average of both GC-MS/MS and LC-MS/MS methods.

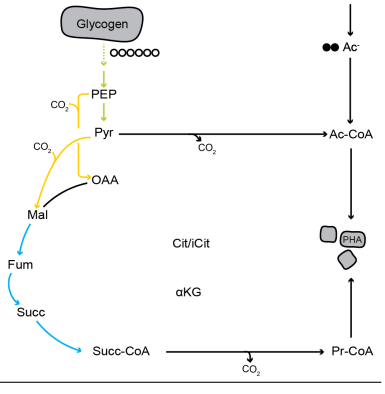
DIFFERENT ^{13}C LABELLING PATTERNS IN MALATE DEPENDING ON METABOLIC MODE

Table 4.2: Expected ^{13}C labelling patterns in malate, *i.e.* relative proportion of mass isotopomers, depending on the metabolic mode (reference available if ever proposed in PAO literature). The expected ^{13}C pattern in malate was derived from the work of Walsh and Koshland (Walsh and Koshland, 1984)

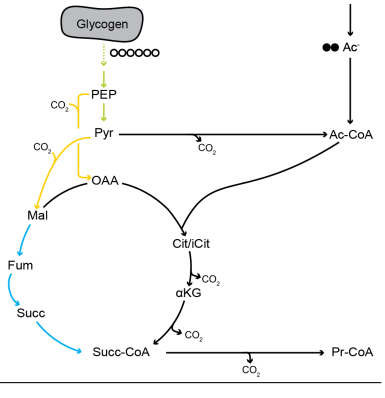
Oxidative TCA branch
No ^{13}C expected in malate since it is produced from ^{12}C-glycogen-derived precursors.



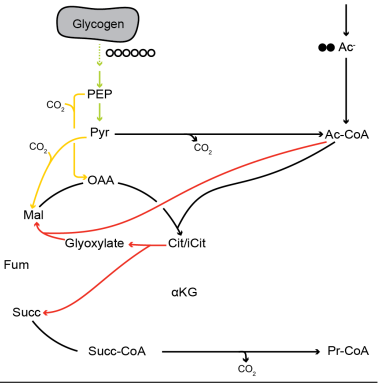
Reductive TCA branch (Pereira et al., 1996)
If any, only a minimal amount of +1 ^{13}C is expected in malate from CO_2.



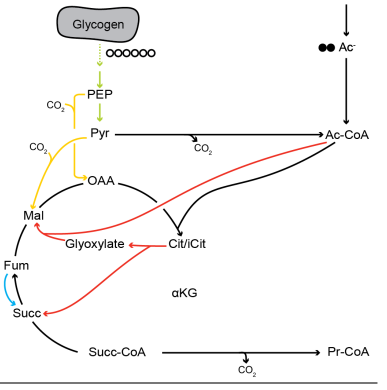
Horse-shoe (Hesselmann et al., 2000)
If any, only a minimal amount of +1 ^{13}C is expected in malate from CO_2.



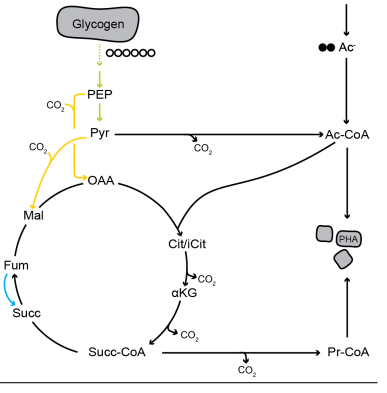
Glyoxylate shunt (without $FADH_2$ production) (Yagci et al., 2003)
The first acetyl-CoA always goes to succinate. The second acetyl-CoA goes to malate but ends in succinate in the second round. Thus: +2 ^{13}C is the majority and some +4 only if equilibration with fumarate occurs. "Odd" masses +1 and +3 can only occur from the equilibration with PEP/pyruvate and CO_2.



Glyoxylate shunt (with $FADH_2$ production)
The first acetyl-CoA always goes to succinate (+2). The second acetyl-CoA goes to malate (+2) but ends in succinate in the second round (+4). Thus: +2 and +4 ^{13}C are the majority. "Odd" masses +1 and +3 can only occur from the equilibration with PEP/pyruvate and CO_2.



Full TCA cycle (Pereira et al., 1996)
In the first cycle, no $^{13}CO_2$ is generated, thus only +2 is expected. In the second cycle, +4 and +3 are generated and in the third cycle even more +4 is generated. "Odd" mass +1 can only occur from the equilibration with PEP/pyruvate and CO_2.



OFFGAS ANALYSIS

Upon acetate feeding (0 min), the evolution of O_2 becomes negative. This is due to the injection of the acetate solution which is in equilibrium with air. Air was also injected together with the solution to guarantee nothing would remain in the syringe. The amount of O_2 dissolved that entered with the pulsed acetate solution is 0.002 mmol (solubility = 9.8 mg O_2/L at 20 °C, 21% O_2 in air, 30 mL solution). This matches with the DO readout of

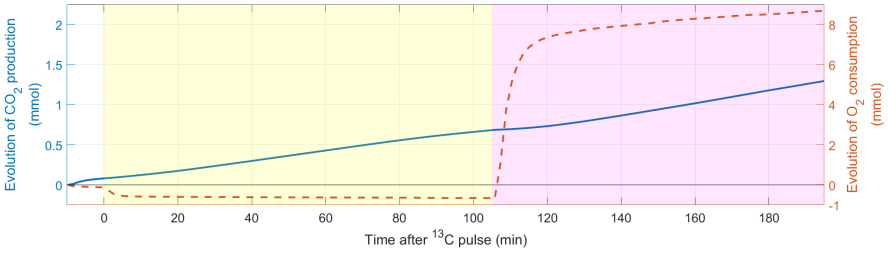


Figure 4.12: Evolution of CO_2 produced and O_2 consumed based on offgas measurements during U-^{13}C-acetate batch experiment.

1.5% in the first minute upon acetate dosing. The remaining amount likely corresponds to air that was injected into the headspace (unknown) and thus measured in the offgas but which did not go through the broth.
The amount of O_2 added to the broth with the acetate solution, 0.002 mmol, is about a thousand times (1000x) lower than that needed to fully oxidize the amount of acetate consumed in first 5 minutes (~ 0.9 mmol). On the other hand, about 0.02 mmol of CO_2 are produced (amount retained in broth unknown) in these 5 minutes which correspond to 0.01 mmol fully oxidized acetate. Assuming only $FADH_2$ would require O_2 to be oxidized then 0.01 mmol O_2 would be required, which is five times (5x) more than what is added with the acetate solution.

GLUTAMATE AND CO_2 FIXATION AS ADDITIONAL ELECTRON SINK UNDER ANAEROBIOSIS

When Accumulibacter relies on glycogen as ATP source, an excess of NADH is generated that is absorbed into the reductive branch of the TCA cycle (malate → fumarate → succinate) *via* fumarate reductase. Succinate is usually not detected as a by-product but is thought to be converted *via* the methyl-malonyl-CoA pathway into PHV. Again, mass isotopomers of metabolites containing a CoA group cannot be assessed and only the first intermediate of PHV's depolymerization could be measured, 3HV. It was detected using GC-MS/MS however there were no signs of ^{13}C enrichment throughout the whole EBPR cycle. During the second metabolic mode in the anaerobic phase, all TCA intermediates get slowly de-enriched in ^{13}C (Figure 4.4). A forward operation would lead to the incorporation of ^{13}C from acetyl-CoA, thus only a reverse could cause a decrease in ^{13}C content. Another supporting observation comes from glutamate (Figure 4.13).

The ^{13}C enrichment of glutamate follows that of α-ketoglutarate as expected from the equilibrium between these two metabolites. However, the glutamate pool size increases during the anaerobic period. In the first half of the anaerobic phase it is under detection limit, then its signal rises to about 50% in the second half of the anaerobic phase and it reaches its maximum (100%) later in the aerobic phase. It likely gets exhausted afterwards during the settling phase and that is why it would be under detection limit at the beginning of the anaerobic phase. Thus, glutamate could likely be an additional electron sink for Accumulibacter under anaerobic conditions.

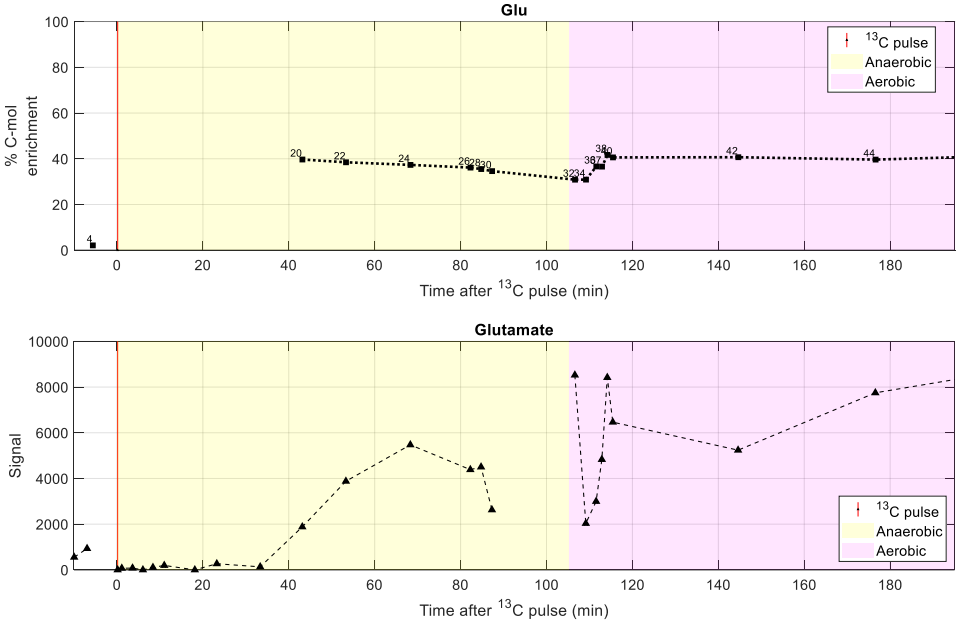
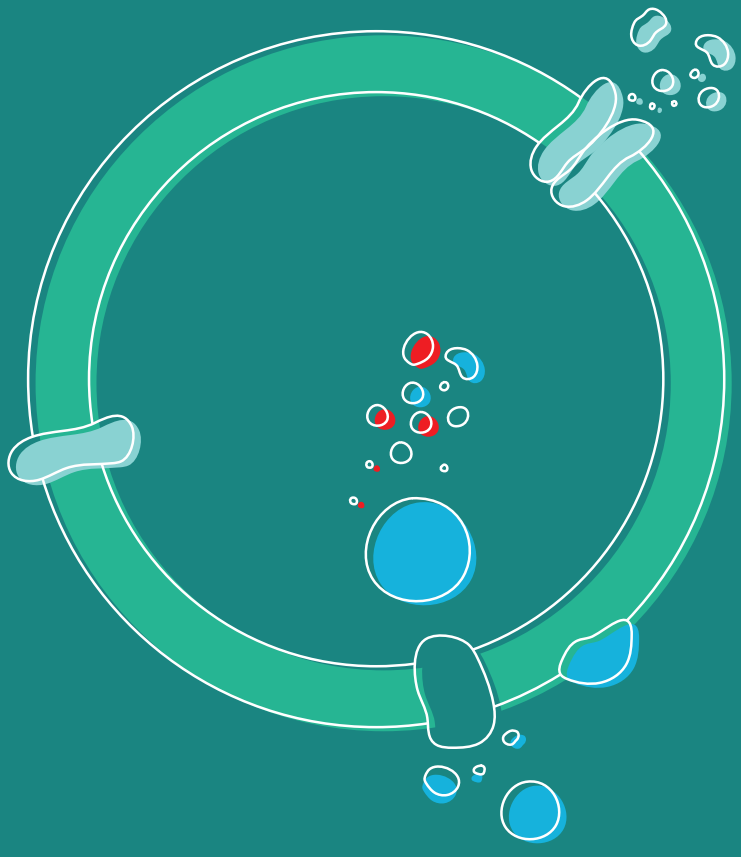


Figure 4.13: Profiles in glutamate (glu), an amino acid generated from α-ketoglutarate. **Top:** Profile of ^{13}C enrichment. **Bottom:** Profile of the total signal sum of glutamate. This metabolite was analysed using GC-MS and it has been corrected for natural isotopes of non-carbon skeleton atoms.

In the radioproteomics study by Wexler and colleagues, glutamine synthetase produced during the anaerobic phase (Wexler, Richardson, and Bond, 2009). This may be related with the increasing glutamate levels inside the cells. The levels of α-ketoglutarate and citrate also increased throughout the anaerobic phase and were much higher in the aerobic phase hinting that likely these pools get refilled after being exhausted during the famine/starvation period preceding the anaerobic-feast phase (*i.e.* settling).
Fixation of CO_2 was previously proposed by Barr and colleagues, based on proteomics measurements. Especially, pyruvate synthase (pyruvate:ferredoxin oxidoreductase) seemed to have a special role (Barr et al., 2016). This enzyme uses ferredoxin as electron carrier which could be donated to the annotated oxoglutarate:ferredoxin oxidoreductase to generate α-ketoglutarate from succinate-CoA, while fixating CO_2. We recommend a tracer experiment with labelled CO_2 to test these possibilities in parallel with a proteomic study to identify whether these ferredoxin-dependent enzymes are present.

5 CHAPTER

Membrane bioenergetics of Accumulibacter



EXPLORING MEMBRANE BIOENERGETICS OF *Candidatus* ACCUMULIBACTER PHOSPHATIS: CAN FAD BE REGENERATED WITHOUT AN EXTERNAL ELECTRON ACCEPTOR?

Leonor Guedes da Silva, Mark C.M. van Loosdrecht, S. Aljoscha Wahl, D. McMillan

Microorganisms present in Enhanced Biological Phosphorus removal (EBPR) systems experience cyclic absence and presence of external electron acceptors (here, anaerobic and aerobic conditions, respectively). It has been postulated that under anaerobic conditions a fully oxidative TCA cycle operates in these microorganisms. This sets a challenge since succinate dehydrogenation is commonly coupled to $FADH_2$ production, which needs a strong electron acceptor to be regenerated to FAD or high energy inputs to regenerate *via* reverse electron transport.

Here we evaluated the potential for *Candidatus* Accumulibacter phosphatis (hereafter referred to as Accumulibacter) to regenerate FAD when there is no external electron acceptor. Enzymatic activities of NADH dehydrogenases (NDH), Succinate dehydrogenases (SDH) and of putative NAD reductases (Nred) were evaluated in inverted membrane vesicles of Accumulibacter-enriched cultures. NDH and SDH activity was observed using oxygen as electron acceptor. Cyanide was used to inhibit cytochrome oxidases and prevent electron transfer to oxygen while assaying the putative Nred with NAD^+ as internal electron acceptor. Under the conditions tested, we could not detect any appreciable signal. We discuss the possibilities of this Nred depending on electron bifurcation or proton motive force across the membrane and propose steps to further elucidate this putative FAD regeneration mechanism.

A full oxidative TCA cycle functioning without an external electron acceptor would explain several experimental observations that the current biochemical model of Accumulibacter cannot describe. An active TCA can be a source of reducing equivalents that alleviates or even eliminates the need for cycling glycogen, as long as no ATP investment is required. This would allow Accumulibacter to outcompete other microorganisms and may explain why these PAOs dominate EBPR communities.

ACKNOWLEDGEMENTS: Several BOC members provided valuable help in the execution of these experiments: Albert Godoy Hernandez, Laura Koekkoek, Lloyd Mallée, Marc Strampraad, and Morten van Schie. Several EBT members also played an important role: Aerobic granular sludge used to inoculate the bioreactor used for this study was kindly supplied by Danny de Graaff; Bioreactor control systems were provided and kept smoothly running thanks to Gerben Stouten. FISH images for microbial community characterization were kindly prepared by Roel van de Wijgaart; The 16S rRNA gene amplicon analysis was kindly executed by Ben Abbas and Sergio Tomás Martínez.

INTRODUCTION

Three decades have already passed since it was for the first time hypothesized that Polyphosphate Accumulating Organisms (PAO) use the Tricarboxylic Acid cycle (TCA) in the absence of external electron acceptors (Comeau et al., 1986). This pathway is hypothesized to be a source of reducing power for the anaerobic accumulation of polyhydroxyalkanoates (PHA) from acetate (Figure 5.1) based on experimental approaches such as using a ^{13}C tracer to estimate intracellular carbon flows or by interpreting stoichiometric relationships between substrates/products with and without the use of inhibitors (Lemos et al., 2003; Louie et al., 2000; Pereira et al., 1996; Zhou et al., 2009; Zhou et al., 2010).

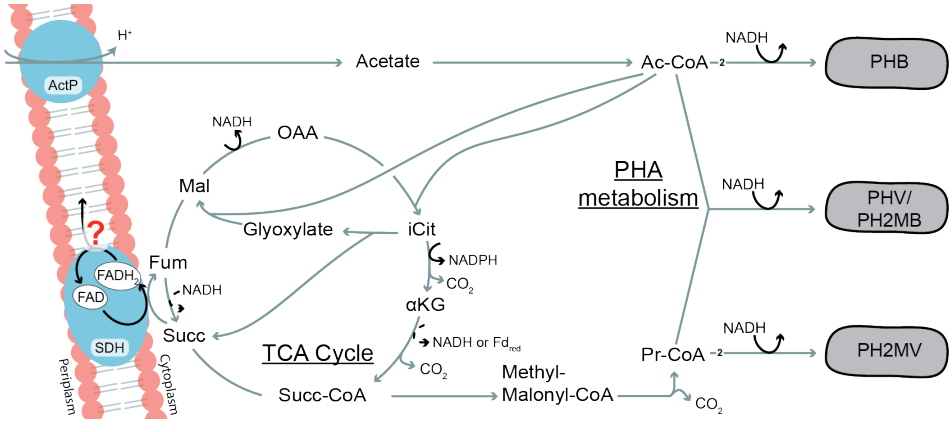


Figure 5.1: Simplified central carbon metabolic network of Accumulibacter highlighting the FAD regeneration problem (with a question mark) arising from the operation of a full TCA cycle in the absence of an external electron acceptor. Dashed black lines indicating oxidoreductase cofactor preference have been assumed by analogy with other microorganisms and full black lines identify oxidoreductases' preferences assayed previously in **Chapter 2**. This metabolic network is based on the ancestral genome reconstruction done by (Oyserman et al., 2016a). The arrows indicate the expected flux direction under anaerobic conditions, however the use of the reductive branch Mal-Fum-Succ seems to change throughout this phase (discussed earlier in **Chapter 4**). Glycogen and polyphosphate metabolism were omitted for simplicity. OAA, oxaloacetate; Ac-CoA, Acetyl-CoA; iCit, isocitrate; αKG, α-ketoglutarate; Succ-CoA, succinyl-CoA; Succ, succinate; Fum, fumarate; Mal, malate; Pr-CoA, propionyl-CoA; TCA, tricarboxylic acid cycle; ActP, acetate permease; SDH, succinate dehydrogenase.

Earlier in **Chapter 2**, an intracellular flux balance analysis (stoichiometric) was compared to several experimental datasets of the PAO *Candidatus* Accumulibacter phosphatis (hereafter referred to as Accumulibacter). It revealed how flexible Accumulibacter metabolism can be depending on its intracellular polymeric reserves. Still, only a biochemical model including a full TCA cycle operation anaerobically explains experimental observations (numbered markers in Figure 5.2) which are not explained otherwise with the accepted biochemical models: **A)** glyoxylate shunt + glycolysis, **B)** glycolysis only; **C)** glycolysis + reductive TCA branch or a mixture of all as represented by the yellow solution space in Figure 5.2.

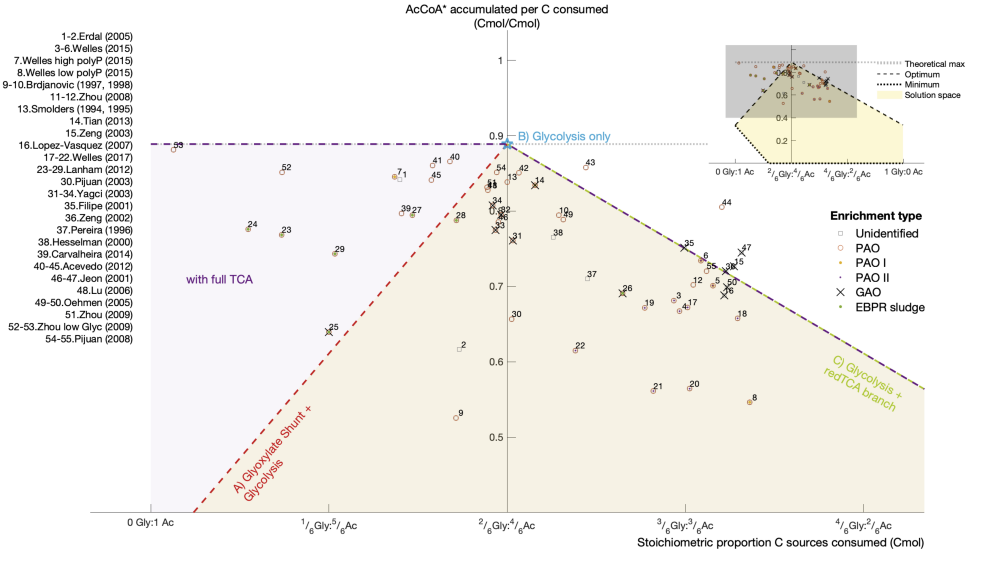


Figure 5.2: The amount of Ac-CoA* accumulated (PHB and PHV precursor) as a function of the proportion of glycogen to acetate consumed in the absence of an external electron acceptor. The yellow shaded area (below red and green dashed lines) corresponds to the possible stoichiometric combinations between acetate, glycogen and the different PHA precursors that are possible without a full TCA cycle. The purple area corresponds to the expansion of the possible combinations by assuming an active TCA cycle. Experimental datasets normalized to respect carbon and energy conservation principles (see **Chapter 2**). Adapted from Figure 2.22.

However, as depicted in Figure 5.1, the remaining question is: *how do PAOs regenerate FAD in the succinate dehydrogenase active site in the absence of an external electron acceptor?*

THE TCA CYCLE, ELECTRON TRANSPORT CHAIN (ETC) AND OXIDATIVE PHOSPHORYLATION

Most dehydrogenases that catalyze reactions of the TCA cycle produce soluble reducing equivalents like NADH and NADPH, except the succinate dehydrogenase (SDH). SDH is embedded in the cytoplasmic membrane and transfers the electrons from succinate to bound FAD, yielding $FADH_2$. Electrons are then transferred from $FADH_2$ to the membrane-bound quinone pool, and, under aerobic conditions, oxygen is the final electron acceptor. In between these electron acceptor/donor systems are proteins organized in such a way that the electrons from succinate (donor) can flow to oxygen (acceptor) *via* carriers of increasing oxidation-reduction potentials (electron transport chain, ETC). The transport of electrons results in the export of protons through the membrane creating an electrochemical gradient across the membrane. The chemical component is a proton gradient and the electric component is a charge gradient (membrane potential, $\Delta\Psi$). The free energy harvested from importing protons back across the membrane due to this resulting electrochemical gradient is known as proton motive force (PMF). This PMF is then used

to drive protons through the ATP synthase allowing for the phosphorylation of ADP. This whole process is known as oxidative phosphorylation and the involved enzymes have been annotated in the genome of Accumulibacter (Figure 5.3).

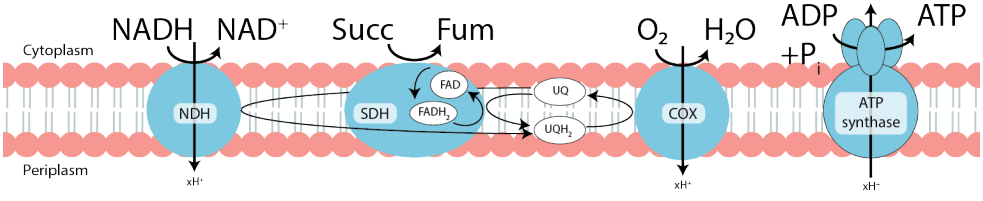


Figure 5.3: Simplified overview of the enzymes involved in the ETC and oxidative phosphorylation processes of Accumulibacter in the presence of oxygen as final electron acceptor. Interactive map of the oxidative phosphorylation pathway/enzymes can be found in `https://www.genome.jp/kegg-bin/show_pathway?app00190` based on the genome annotation of Accumulibacter IIA by García Martín *et al.*(García Martín et al., 2006).

THE FAD REGENERATION CONUNDRUM

In the absence of a high potential electron acceptor like oxygen, FAD cannot be regenerated without energy investment (Figure 5.4) and becomes a bottleneck. Traditionally this is solved by an energy demanding reversed/uphill electron transfer producing NADH (Gottschalk, 1986). More recently, electron bifurcation mechanisms have been proposed where one electron from low potential ferredoxin is coupled to one electron from a higher potential donor, yielding a "in-between-potential" electron couple that can be used to generate NADH without additional energy investment (Buckel and Thauer, 2013). Based on the genome annotation of Accumulibacter, ferredoxin can be reduced either by the α-ketoglutarate:ferredoxin oxidoreductase in the oxidative branch of the TCA cycle or by the pyruvate:ferredoxin oxidoreductase in the conversion of pyruvate to acetyl-CoA (Oyserman et al., 2016a).

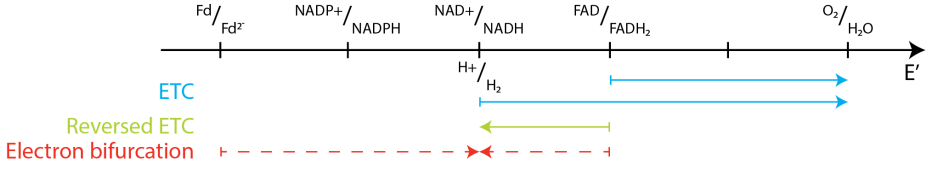


Figure 5.4: Relative scale of typical physiological oxidation-reduction potentials of different electron acceptor/donor couples based on values mentioned by Buckel and Thauer (Buckel and Thauer, 2013). The redox potential of H^+/H_2 is likely the one in this scale that varies most, but here we would like to point out that it can be very close to that of NAD^+/NADH.

In principle, if no external electron acceptor is available electrons can be transferred from $FADH_2$ to the lower potential NAD^+ (reversed/uphill electron transfer) *via* the NADH dehydrogenase (NDH). For example, *Rhodobacter capsulatus* uses NDH to reduce NAD^+ in

the presence of light (Herter, Kortlüke, and Drews, 1998). However, this reaction does not contribute for PMF generation, on the contrary it consumes it and thus why, in this case, reversed electron transfer is coupled to light.
Next to the reversible NADH dehydrogenase annotated for Accumulibacter, García Martín and colleagues (García Martín et al., 2006) have proposed a fusion protein consisting of a cytochrome *b/b6* domain with five transmembrane helices and binding domains for soluble NAD(P)$^+$ and flavin in Accumulibacter IIA (Figure 5.5). In 2006, this domain configuration was unique in public databases.

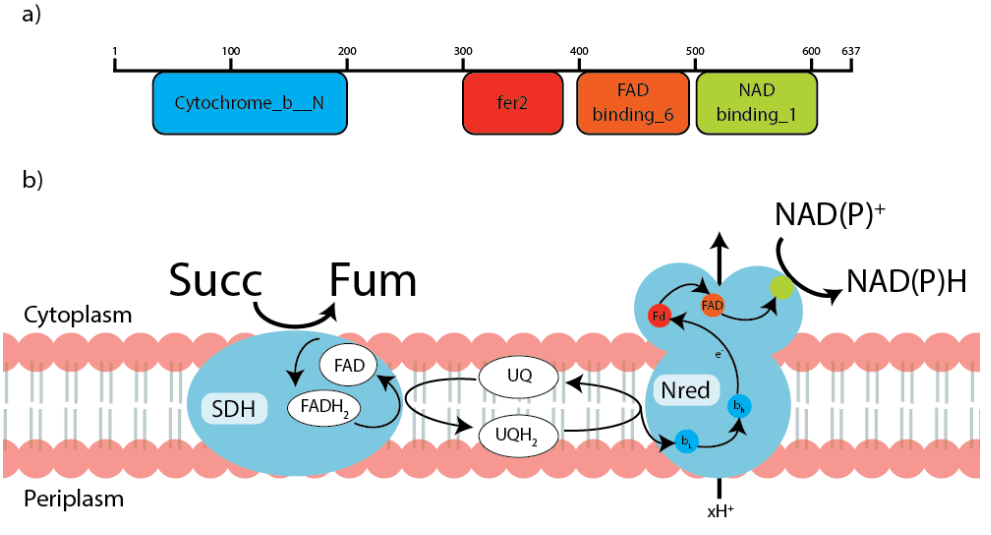


Figure 5.5: Proposed fusion protein, an ubiquinol:NAD$^+$ oxidoreductase here named NAD$^+$ reductase (Nred), consisting of a cytochrome *b/b6* domain with five transmembrane helices and a soluble NAD(P)$^+$- and flavin-binding domain. This complex would allow for Accumulibacter IIA to regenerate FAD and a full TCA cycle could then be functional in the absence of an external electron acceptor. **(a)** The presented domain structure has one gene with a cytochrome *b/b6* domain (`https://www.ncbi.nlm.nih.gov/protein/ACV33672.1`) and another gene with binding domains for soluble ferredoxin, NAD(P)$^+$ and flavin (`https://www.ncbi.nlm.nih.gov/protein/ACV34648.1`). **(b)** Hypothesized quinol reductase function that would be responsible for the uphill electron transfer from $FADH_2$ to NAD(P)$^+$, at the cost of proton motive force.
Figure adapted from (García Martín et al., 2006).

It is suggested that this fusion protein can accept electrons from the reduced quinone pool (*i.e.* from $FADH_2$) and transfer them *via* the cytochrome, ferredoxin and flavin groups to reduce NAD(P)$^+$, again at the expense of the PMF. In another study, Flowers and colleagues found a similar domain for Accumulibacter IA in their metagenomic analysis (Flowers et al., 2013). Moreover, they also found that recent studies exploring the expression of this fusion protein targeted the wrong gene (Burow, Mabbett, and Blackall, 2008; He et al., 2010) due to a wrong gene id provided in the original report (should be JGI-IMG/M gene id: 645 009 126, with the *locus* tag: CAP2UW1_1324 on finished genome: 644 736 333). In a recent metatranscriptomics study of an Accumulibacter enrichment (Oyserman et al.,

2016b), the expression pattern of this gene clustered together with others displaying the anaerobic acetate contact pattern (*i.e.* drastically increase after acetate contact and peak before oxygen contact), thus highlighting its role within the anaerobic phase (Figure 5.6). In contrast, in the proteomic study of Barr and colleagues, no peptide hits to this fusion protein were detected (Barr et al., 2016), however, special care in the preparation should have been taken to enable detection of integral membrane proteins in such proteomics analyses.

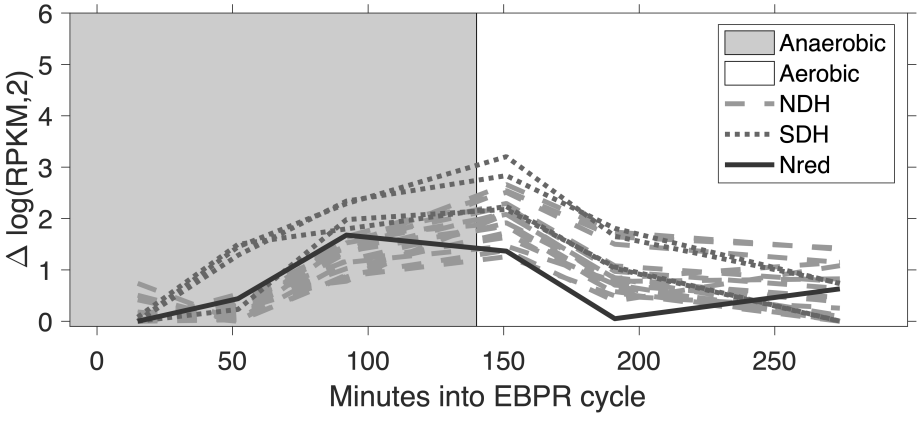


Figure 5.6: Time-series gene expression profile pattern during a single EBPR cycle retrieved from the supplementary data of (Oyserman et al., 2016b). The Nred profile corresponds to the gene encoding for the fusion protein (CAP2UW1_1324) proposed by (García Martín et al., 2006). The pattern exhibited by this gene, alike that of NDH and SDH, matches the anaerobic acetate contact pattern, which is characterized by a drastic increase after acetate contact and peaks before oxygen contact. This transcript trend clustered within Trend Category Q, which contains mainly genes from the category "Energy production and conversion".

Despite the presence of genes that could potentially code for enzymes involved in the regeneration of FAD in different Accumulibacter genomes and with an expression pattern consistent with such a role, the mechanism for regeneration of FAD in succinate dehydrogenases in the absence of external electron acceptors remains to be experimentally validated. It is the goal of the present study to experimentally assess the activity of enzymes involved in this hypothetical process. Activities will be measured in inverted membrane vesicles of Accumulibacter-enriched cultures harvested from both anaerobic and aerobic conditions. Firstly, NADH dehydrogenase (NDH) will be assayed to confirm the success of membrane vesicles preparation. Then, succinate dehydrogenase (SDH) will be assayed to confirm the transfer of electrons from succinate to the membrane vesicles. Lastly, the fusion protein proposed by (García Martín et al., 2006), an ubiquinol:NAD oxidoreductase here named NAD reductase (Nred), will be assayed. This way, we hope to give the first steps towards demonstrating the existence of a mechanism that allows for FAD regeneration and, consequentially, a full TCA cycle operation without an external electron acceptor.

MATERIALS & METHODS

BIOREACTOR OPERATION

Accumulibacter were enriched in a 2L lab-scale bioreactor (1.5L working volume) from aerobic granular sludge kindly supplied by D. de Graaff (TU Delft, The Netherlands). The bioreactor control systems are described in (Stouten et al., 2019). Temperature and pH were controlled at 20 °C and 7.6, respectively. The most important phases of the Accumulibacter enrichment cultivation are depicted in Figure 5.7. Total cycle time was 6h, with 0.5h of settling, 2h anaerobic and 3h aerobic, with a hydraulic retention time (HRT) of 12h and a solids retention time (SRT) of about 5 days (approx. 2.5 days aerobic SRT and equivalent to a growth rate of about 0.01 h^{-1}). The medium fed to the reactor contained 400 mg COD/L (65:35 $Acetate_{COD}$:$Propionate_{COD}$), and a phosphate load of 0.1 Pmol/Cmol.

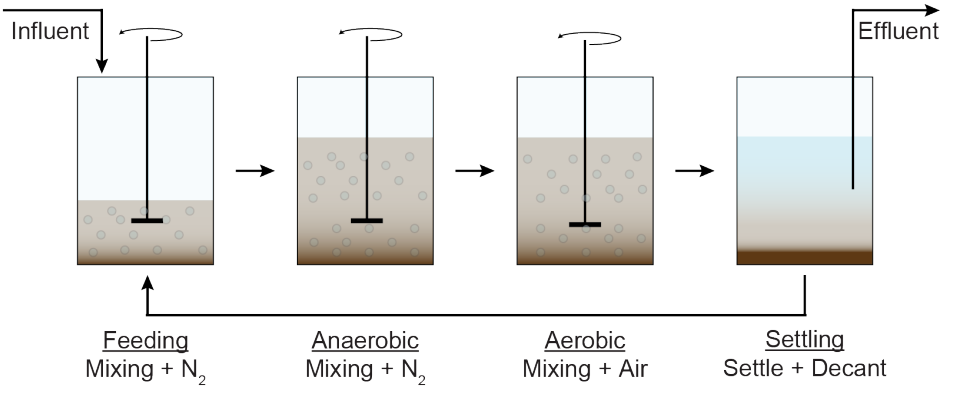


Figure 5.7: Phases of the sequencing batch reactor (SBR) used to enrich for Accumulibacter.

The enrichment culture exhibited a PAO phenotype, with 0.76 Pmol released anaerobically per Cmol substrate consumed. The microbial community present in the enrichment culture under study has been characterized by two orthogonal approaches, Fluorescence *in situ* hybridization, and 16S rRNA gene amplicon sequencing as earlier described in **Chapter 2**. FISH micrographs confirmed PAO were the dominant bacteria in this microbial community, and 16S rRNA gene amplicon confirmed Accumulibacter was the dominant PAO (see section 5.5). The 16S rRNA gene amplicon data have been deposited in GenBank under Bioproject PRJNA490689.

BIOMASS SAMPLING AND WASHING

Broth samples (200 mL at a biomass concentration of about 1 g_{DW}/L) collected from the bioreactor during both anaerobic and aerobic phases were centrifuged (6,000 × *g*, 15 min, 4 °C) and the pellet was washed using 5 mL buffer (hereafter named Buffer 1X) containing 50 mM Tris at pH 8, 5 mM $MgCl_2$, 5 mM NaCl and 5% (v/v) glycerol, and the obtained

suspension was centrifuged again (6,000 × *g*, 15 min, 4 °C). After centrifugation, pellets were suspended in 10 mL of a buffer for membrane vesicles preparation (hereafter named prep buffer) containing 20 mM Tris at pH 8, 30 mM Na_2SO_4, 2 mM $MgSO_4.7H_2O$, 5 mM NaCl and 10% (v/v) glycerol and supplemented with 2 mM (L+D) 1,4-Dithiothreitol (DTT), a spatula tip of DNAse per 40 mL buffer volume and 1 tab of cOmplete^{TM} mini protease inhibitor cocktail (Roche).

INVERTED MEMBRANE VESICLE PREPARATION AND PROTEIN QUANTIFICATION

Inverted membrane vesicles (Figure 5.8) were prepared as described in (McMillan et al., 2016) with the following modifications: Cells were vigorously homogenized resulting in a highly viscous solution before lysis using a cell disrupter (constant systems).

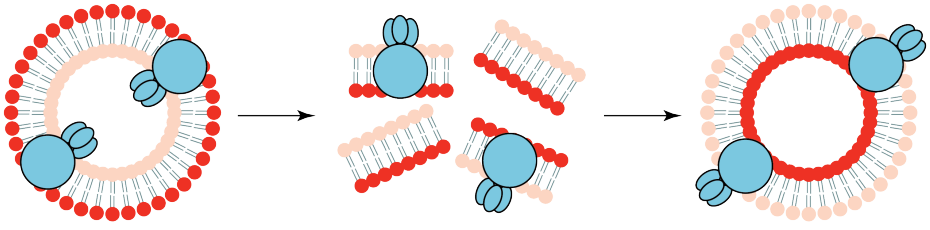


Figure 5.8: Preparation of inverted membrane vesicles. The inside-out vesicles are naturally forming after cells have been opened by a mechanical approach.

The resuspended pellets were passaged twice through the cell disrupter at a pressure of 1,500 Kpi. Disrupted cells and foam were collected and centrifuged (7,000 × *g* for 15 min at 4 °C). After this step, a floating layer was discarded, the supernatant was transferred to ultracentrifugation tubes and tubes were filled to the rim and balanced with extra prep buffer. A Beckman ultracentrifuge was used at 180,000 × *g*, for 45 min at 4 °C. The obtained supernatant was discarded, and the membrane vesicles pellet was carefully resuspended in 400 µl prep buffer. This final membrane vesicles suspension was used for the enzymatic assays. Protein concentrations were measured using a BCA protein assay kit (ThermoFischer Sci.).

ENZYMATIC ASSAYS

The reaction mixtures used for each enzymatic assay are described in Table 5.1. Reaction buffer contained 20 mM Tris (pH 8), 100 mM KCl and 30 mM Na_2SO_4. This buffer was also used to prepare the following solutions: 10 mM NADH, 9 mM sodium cyanide (NaCN), 0.3 M succinate, 10 mM NAD^+, 1 M DTT. Other solutions prepared: 20 mM Menadione (MD) in ethanol, 1 mM Dichlorophenolindophenol (DCPIP) in H_2O, 250 mM ATP pH-neutralized in H_2O, 50 µM sodium azide in H_2O. Purified electron shuttle proteins such as HiPiP (High Potential Iron Protein), cytochrome C (cytC), and ferredoxin (Fd) were used in excess in

the assays at a concentration of 20 μM.
Absorbance changes and spectra were recorded spectrophotometrically using 1-cm light path cuvettes. Temperature was controlled at 30 °C and a stirring bar was used at 800 rpm. NADH evolution was monitored at 340 nm, while DCPIP was monitored at 600 nm.

Table 5.1: Substrates (electron donor and acceptor), inhibitors, expected absorbance readout and references used for each enzymatic assay.

Tested enzyme	**Electron donor**	**Electron acceptor**	**O_2 respiration inhibitors**	**Readout (expectation)**	**Reference**
NADH dehydrogenase (NDH)	100 μM NADH	200 μM MD, dissolved O_2 from atmosphere	90 μM cyanide	NADH consumption (Abs 340 nm ↘)	(McMillan et al., 2009)
Succinate dehydrogenase (SDH)	30 mM succinate	90 μM DCPIP, dissolved O_2 from atmosphere	90 μM azide	DCPIP reduction (Abs 600 nm ↘)	(McMillan et al., 2009)
NAD reductase (Nred)	30 mM succinate	100 μM NAD^+	90 μM cyanide, 10 mM DTT	NADH production (Abs 340 nm ↗)	(this study)
NAD reductase (Nred) – Electron bifurcation	30 mM succinate, 20 μM HiPIP or cytC or Fd	100 μM NAD^+	90 μM cyanide, 10 mM DTT	NADH production (Abs 340 nm ↗)	(this study)

Results & Discussion

NADH dehydrogenase and succinate dehydrogenase activities were first tested in the presence of oxygen. The purpose of such test was two-fold: **1)** to validate the membrane vesicles preparation protocol for Accumulibacter enrichment cultures and **2)** to assess their activity in Accumulibacter cells harvested from either anaerobic or aerobic conditions. Then two different cytochrome oxidase inhibitors were tested, azide and cyanide, to simulate a situation where oxygen can no longer be the final electron acceptor.

NADH dehydrogenase (NDH)

This is the first protein complex of the respiratory chains for a wide variety of organisms and it catalyzes NADH oxidation to NAD^+. The NDH-1 type has been annotated in the genome of Accumulibacter (García Martín et al., 2006) and is thought to translocate protons. Menadione (MD) is used as final electron acceptor of this assay to have a direct readout of NDH activity without possible intermediate bottlenecks, however oxygen is also available and may be reduced as well.

At the start of the assay, all reagents but one are added to establish a baseline (gray datapoints). We noted that when membrane vesicles are added there is an inherent drift in the absorbance readout, which would not be taken into account in the baseline if these vesicles were used to trigger the reaction. Thus, the reaction was instead triggered by NADH (Figure 5.9). An additional *E. coli* membrane extract was used as positive control (see Figure 5.17). This control was important to be sure the low activity is due to the Accumulibacter enrichment and not the method used. Nevertheless, while the conditions used are suitable for *E. coli*, they might need further optimization for Accumulibacter. Hereto we suggest investigating the usage of buffers with different pH, for both preparation of the vesicles and for the assay itself.

NDH activity was detected in membrane vesicles of Accumulibacter harvested during the anaerobic and aerobic phases. Furthermore, different amounts of membrane extract were added to examine if the membrane extracts were indeed responsible for oxidation of NADH. The specific activity calculated (Figure 5.9) is an average of those assays with different amount of membrane extract, which contribute for an increased standard deviation as compared to when replicates using the exact same amount would be performed.

Additionally, cyanide was used to inhibit any cytochrome oxidase (COX) activity, preventing electrons from being donated to O_2 as final electron acceptor. Inhibiting COX is important to later simulate the anaerobic condition in which the Nred is most likely to be functional. As expected, in both anaerobic/aerobic-vesicles, cyanide had an inhibitory effect on COX activity. However, the inhibition extent cannot be evaluated while MD is present as final electron acceptor. Here, it is assumed COX would be the only way electrons can go other than MD. Besides ensuring complete inhibition of COX activity is attained to properly assay Nred, one should also be careful with other NADH-consuming membrane enzymes like hydrogenases as these are expressed in Accumulibacter (Oyserman et al., 2016b).

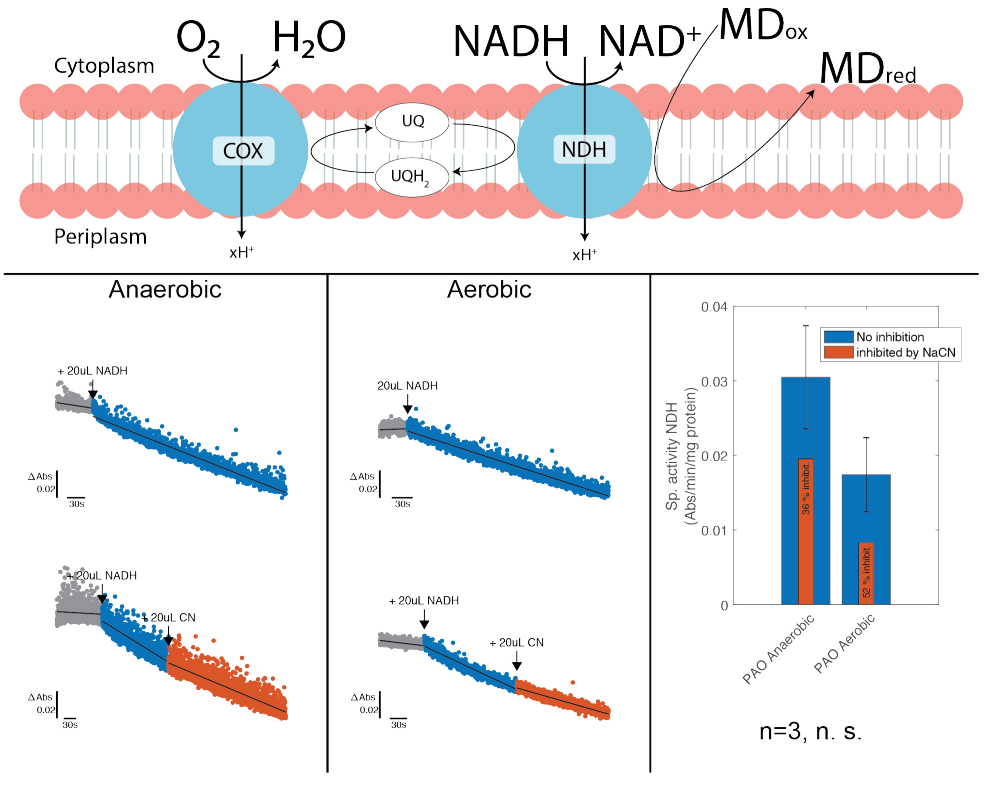


Figure 5.9: Consumption of NADH by Accumulibacter membrane vesicles monitored at 340 nm. Both oxygen and menadione (MD) were used as final electron acceptors. Cyanide (CN) was used to inhibit cytochrome oxidase activity (COX). **Top)** reaction scheme – electrons are transferred from NADH to NDH, then to the quinone pool and finally to either oxygen or MD. **Left)** NDH activity of Accumulibacter harvested during the anaerobic phase. **Middle)** NDH activity of Accumulibacter harvested during the aerobic phase. **Right)** Comparison of NDH activities of Accumulibacter harvested during the anaerobic and aerobic phases. Activities for each phase were measured in three (3) replicates but according to a *t*-test the number of samples analyzed is not enough to determine a significant difference between phases. The extent of inhibition (in %) is indicated in the orange bars for each condition.

SUCCINATE DEHYDROGENASE (SDH)

This TCA cycle enzyme catalyzes the oxidation of succinate to fumarate with reduction of FAD and subsequent electron transport to quinones (UQ). Due to its need for a high potential electron acceptor, the reaction this enzyme catalyzes is the culprit for stalling the TCA cycle operation in the absence of an external electron acceptor. In this assay, Dichlorophenolindophenol (DCPIP) is the final electron acceptor of this reaction, turning from dark blue to colorless (*i.e.* absorbance decreases when DCPIP is reduced), but oxygen is also available and may be reduced as well. The activity of this enzyme was monitored by following the reduction of DCPIP at 600 nm. The reaction was triggered by the addition of membrane vesicles, either from an anaerobic or aerobic biomass sample. Furthermore, different amounts of membrane extract were added to examine if the membrane extracts were indeed responsible for the reduction of DCPIP. The specific activity calculated is an average of those assays with different amount of membrane vesicles, which contribute

for an increased standard deviation as compared to when replicates using the exact same amount were performed.

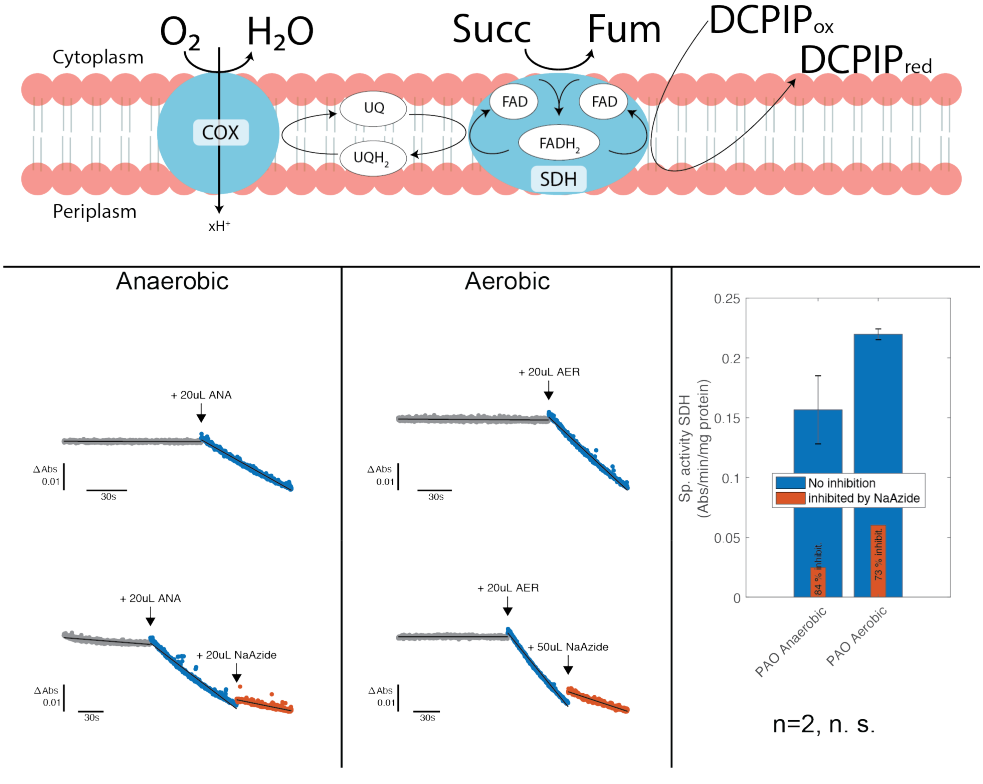


Figure 5.10: Reduction of $DCPIP_{ox}$ by Accumulibacter membrane vesicles monitored at 600 nm. Both oxygen and $DCPIP_{ox}$ were used as final electron acceptors. Azide (NaAzide) was used to inhibit cytochrome oxidase activity (COX). **Top)** reaction scheme – electrons are transferred from succinate to SDH, then to FAD, then the quinone pool and finally to either oxygen or $DCPIP_{ox}$. **Left)** SDH activity of Accumulibacter harvested during the anaerobic phase. **Middle)** SDH activity of Accumulibacter harvested during the aerobic phase. **Right)** Comparison of SDH activities of Accumulibacter harvested during the anaerobic and aerobic phases. Activities for each phase were measured in two (2) replicates but according to a *t*-test the number of samples analyzed is not enough to determine a significant difference between phases. The extent of inhibition (in %) is indicated in the orange bars for each condition.

SDH activity was detected in membrane vesicles of Accumulibacter harvested during the anaerobic and aerobic phases. In a recent metatranscriptomics study of an Accumulibacter enrichment (Oyserman et al., 2016b), the transcript levels for SDH fluctuated depending on the cycle phase, exhibiting an increase after anaerobic acetate contact and peaking only after oxygen contact (aerobic), thus highlighting its increasing importance throughout the cycle (Figure 5.10). Azide was used to stop COX activity (donation of electrons to O_2), but it also inhibited the reduction of DCPIP, indicating that SDH may also be sensitive to azide. Later for the Nred assay cyanide is used instead, however it should still be tested whether SDH is also sensitive to this inhibitor even if unlikely.

NAD^+ REDUCTASE (NRED)

When the first genome of Accumulibacter became available in 2006, its authors proposed a fusion protein that allows for uphill electron transfer, and, consequently allows for anaerobic TCA cycle function (García Martín et al., 2006). This enzyme catalyzes the reverse reaction of that catalyzed by NADH dehydrogenase – it reduces NAD^+ to NADH with electrons originating from the electron transport chain. In the case of Accumulibacter, these electrons are thought of coming from the dehydrogenation of succinate to fumarate by the succinate dehydrogenase, allowing for a full TCA cycle operation when no external electron acceptor is available.

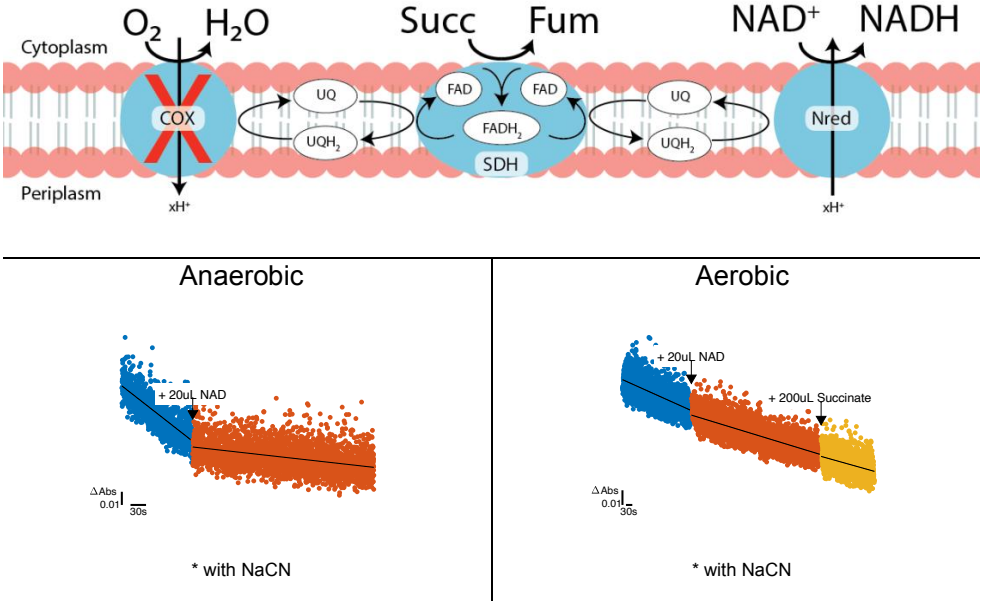


Figure 5.11: Reduction of NAD^+ by succinate in Accumulibacter membrane vesicles monitored at 340 nm. Cyanide (NaCN) was used to inhibit cytochrome oxidase activity (COX). **Top)** reaction scheme – electrons are transferred from succinate to SDH, then to FAD, then the quinone pool and finally to NAD^+. **Left)** Nred activity of Accumulibacter harvested during the anaerobic phase. Reaction was triggered by the addition of NAD^+. **Right)** Nred activity of Accumulibacter harvested during the aerobic phase. Here the reaction was triggered by succinate, which was only added after NAD^+. The signal-to-noise ratio was too low to allow for accurate slope calculations and hence why activities could not be calculated and compared.

To test the presence of the NAD^+ reductase, we designed the following assay: succinate was used as electron donor to the previously assayed succinate dehydrogenase, and NAD^+ was used as final electron acceptor. Cyanide was used to inhibit the terminal cytochrome oxidase (COX) and prevent electrons from using O_2 as final electron acceptor, thus circumventing electron escape to oxygen and effectively funneling them back to NAD^+.

The activity of this enzyme was monitored by following NADH at 340 nm. If active, an increase in absorbance (*i.e.* NADH production) is expected. While some signal was indeed observed, conclusive evidence was not obtained due to the low signal-to-noise ratio. An

attempt to use a higher amount of membranes to increase the signal did not result in improved signals, but rather created optical interference. Therefore, another system such as bioelectrochemistry (Li et al., 2015; McMillan et al., 2013) would need to be employed to supply electrons directly to the quinone pool without the need for soluble mediators which may complicate output measurements. Strict anaerobic conditions might also be needed in case this enzyme is highly inhibited by oxygen. Furthermore, while being highly unlikely, one cannot discard the interference of other NADH consuming activities since *Ca.* Accumulibacter also expresses a membrane-bound hydrogenase (Oyserman et al., 2016b) that can function inversely and either consume NADH or it could take the electrons directly from the quinol pool to produce H_2.

ELECTRON BIFURCATION

In the putative ubiquinol:NAD oxidoreductase proposed by Garcia-Martin and colleagues, a binding domain for a soluble ferredoxin is predicted (García Martín et al., 2006). It could be that this ferredoxin is not constantly attached to the protein as suggested in their scheme (see Figure 5.5), but that it is more of a pocket where soluble ferredoxin can attach and donate electrons, in an electron bifurcation type mechanism (Buckel and Thauer, 2013). Here we hypothesize a mechanism combining the Q-cycle described in mitochondrial membranes (Osyczka, Moser, and Dutton, 2005) and the bacterial Rnf complexes, which couple ferredoxin oxidation by NAD^+ with proton/sodium ion translocation (Biegel et al., 2011). Thus, we propose that the two (2) electrons donated by ubiquinol (UQH_2) are separated to different energy levels like the tines of a fork (from Latin, *furca*=fork): one electron is combined with another electron donated from ferredoxin and both electrons are then donated to NAD^+; the second electron is donated back to the ubiquinone pool (UQ). The electron movements generate potential across the membrane ($\Delta\Psi$) leading to proton/sodium translocation. Thus, the ubiquinol:NAD oxidoreductase understudy might have a similar mechanism in which electrons donated by the high-potential fumarate/succinate couple to the UQ/UQH_2 are combined with electrons originating from the lower-potential ferredoxin to be donated to the "in-between-potential" NAD^+/NADH couple.

Again here, if Nred is active, an increase in absorbance (*i.e.* NADH production) is expected. Ferredoxin (Fd) was added to the mixture, however the signal-to-noise ratio is too low. The assay with anaerobic vesicles should be repeated with DTT to draw any conclusion since DTT is crucial to reduce ferredoxin so that it can function as an electron donor. Other higher potential electron carriers were used, like HiPiP (High Potential Iron Protein) and cytochrome C (cytC), but again the signal-to-noise ratio is too low to draw any conclusion. Instead of indirectly donating electrons to Nred *via* succinate, DTT could also act as electron donor in the presence of MD as electron carrier, however this combination alone (*i.e.* without any membrane extract present) contributes for the absorbance at 340nm, thus making it difficult to solely assess the production of NADH.

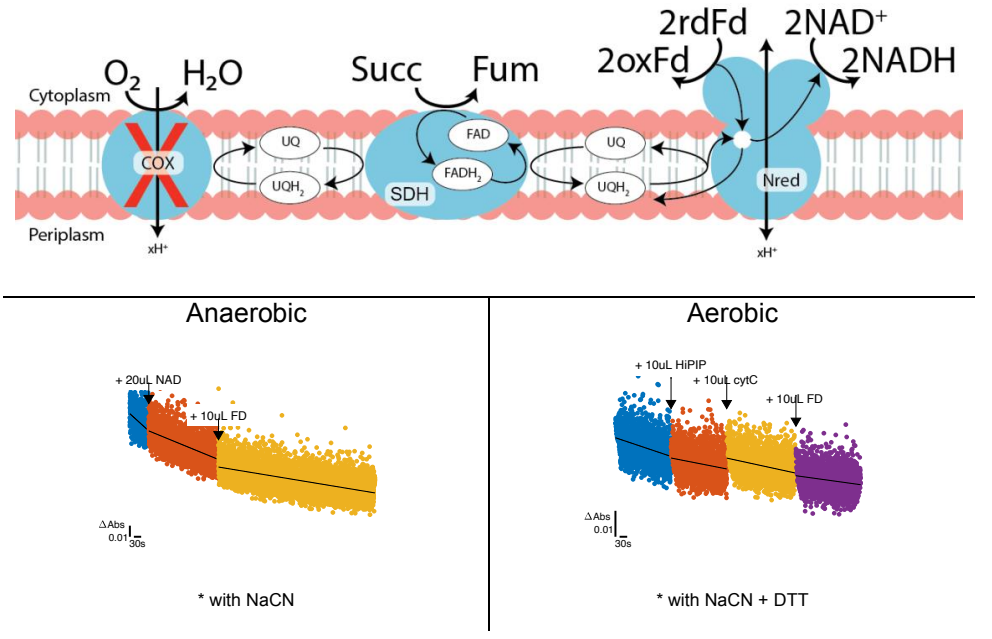


Figure 5.12: Reduction of NAD^+ by succinate and other electron donors (HiPIP, cytC, and Fd) in Accumulibacter membrane vesicles monitored at 340 nm. Cyanide (NaCN) was used to inhibit cytochrome oxidase activity (COX). **Top)** reaction scheme – electrons are transferred from succinate to SDH, then to FAD, then the quinone pool and finally to NAD^+.**Left)** Nred activity of Accumulibacter harvested during the anaerobic phase. Reaction was triggered by the addition of ferredoxin (Fd). **Right)** Nred activity of Accumulibacter harvested during the aerobic phase. Here the reaction was triggered by HiPIP, then cytC, and finally Fd. However, for all cases, the signal-to-noise ratio was too low to allow for accurate slope calculations and hence why activities could not be calculated and compared.

PROTON MOTIVE FORCE-DRIVEN UPHILL/REVERSED ELECTRON TRANSFER

In uphill/reversed electron transfer (RET), electrons are exchanged from a high potential electron donor, to a lower potential electron acceptor and usually these processes are possible at the cost of proton motive force (PMF) or sodium motive force (SMF).

A similar uphill electron transfer has been experimentally demonstrated in *Thiobacillus ferrooxidans* where electrons are transferred from a high potential electron donor Fe^{2+} through a bc_1 complex and NDH to finally reduce NAD^+ (Elbehti, Brasseur, and Lemesle-Meunier, 2000). The authors claim ATP hydrolysis is responsible for generating an electrochemical proton gradient, which supports the PMF cost of RET. However, the ATP needed to maintain PMF in this way is likely prohibitive for cells and it is more likely that membrane potential, $\Delta\Psi$, is what sustains RET. The feasibility of an ATP-driven RET can be assessed by an ATP balance over the cell, *i.e.* ATP-driven RET is only possible when anaerobic phosphate-associated proton efflux and glycogen degradation by Accumulibacter generate more ATP than that required for acetate uptake and activation. Typically, this is not the case, leaving no ATP left to drive RET (Smolders et al., 1994b). Therefore, we hypothesize that, in Accumulibacter, RET is sustained by the generation of membrane potential, $\Delta\Psi$.

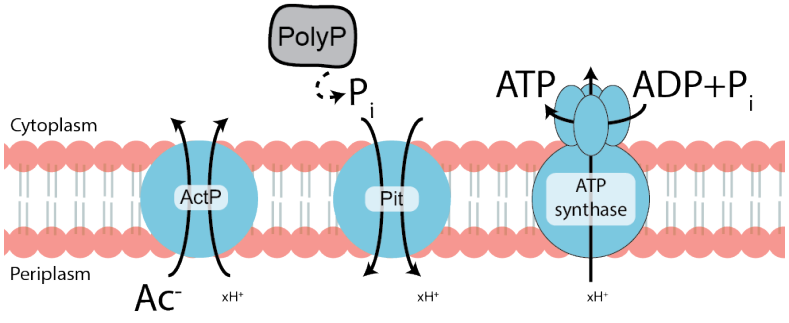


Figure 5.13: Acetate uptake driven by the membrane potential in anaerobic conditions: **1)** ActP-mediated symport of acetate is energized primarily by the electrical membrane potential. **2)** Pit efflux of H^+ in symport with inorganic Pi generates an electrochemical gradient and is used for acetate uptake. **3)** Energy is conserved by the PMF-driven proton influx through a H^+-ATPase. Figure adapted from (Burow et al., 2008).

In a bioenergetics study, Burow and colleagues demonstrated that anaerobic acetate uptake and phosphate release were coupled and depended on PMF, specially on $\Delta\Psi$ (Burow, Mabbett, and Blackall, 2008). Both processes were negatively affected by the uncoupler CCCP (76 and 78% respectively), which affects PMF, and by the inhibitor valinomycin (36 and 32% respectively) that affects $\Delta\Psi$. The inhibition of the sodium motive force (SMF) by monensin was also evaluated but had a minor impact (7 and 1% respectively on the anaerobic acetate uptake and phosphate release).
In agreement with previous findings (Saunders et al., 2007; Van Veen et al., 1994), the authors also hypothesized that membrane potential, $\Delta\Psi$, was actually generated by phosphate efflux *via* a H^+ symporter (Pit) and thus could drive ATP production by the ATP synthase. The inhibition of the ATP synthase using DCCD led to higher substrate-level phosphorylation, *i.e.* higher glycogen degradation, supporting the notion ATP synthase plays a role in ATP generation anaerobically (Figure 5.13).

Thus, in PAOs, $\Delta\Psi$ is generated *via* phosphate-efflux and is distributed between acetate import, ATP generation and, potentially, also in RET (Figure 5.14).

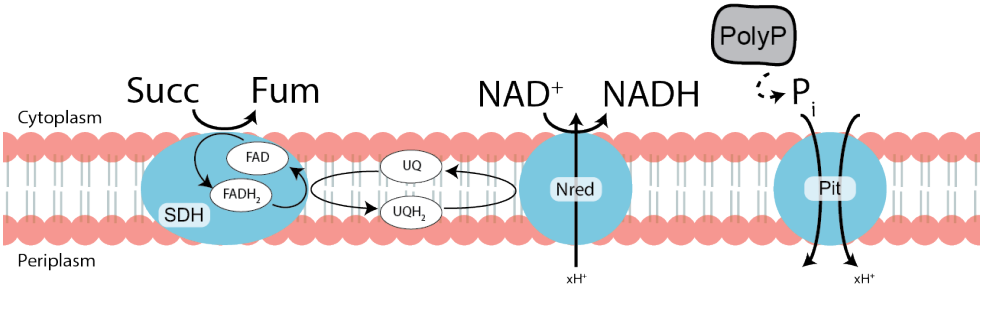


Figure 5.14: Proposed model for reversed electron transfer from succinate to NAD^+ at the cost of membrane potential, $\Delta\Psi$, generated by Pi-efflux.

Given the nature of Accumulibacter as a PAO, we also support the hypothesis that the export of Pi outside the cell is done *via* the inorganic phosphate transport system, Pit, in symport with a proton and thus generates $\Delta\Psi$(Veen, 1997). A direct way to test this hypothesis is to monitor the activity of the ATP synthase while Pi is being transported from the reaction mixture into membrane vesicles. Beforehand, we recommend assaying ATPase to confirm it remains active in these vesicles (McMillan et al., 2013; Tomashek, Glagoleva, and Brusilow, 2004). The observation of membrane potential, $\Delta\Psi$, generated by Pi-efflux alone as done for *Acinetobacter johnsonii* (Van Veen et al., 1994) would also be very interesting to corroborate the observations in (Burow et al., 2008) as it would be obtained by an independent method without the side-effects that inhibitors might have on other cellular activities.

ANOTHER QUESTION REMAINS...

What would be the real advantage of having a full TCA cycle operating without an external electron acceptor?

When oxidative phosphorylation is possible, an organism will benefit from fully oxidizing substrate to CO_2 to harvest the most energy possible for its cellular activities. When this is no longer possible and ATP becomes a scarce resource, a coupling arises between ATP generation from fermentative pathways and ATP demand from anabolic processes. Thanks to the capacity of PAOs to maintain a large polyphosphate storage pool, the metabolism is likely organized in such a way that they depend less on fermentation of glycogen to PHA to survive. When glycogen is used, ATP is produced in glycolysis. *But if glycogen is not available, how can PAOs still harvest some ATP from substrate-level phosphorylation?* In a full TCA cycle, one GTP is produced from succinate-CoA to succinate. This CoA could also be directly transferred to acetate converting it to acetyl-CoA and this would represent one ATP saving. Thus, it only depends on the ATP cost of reverse electron transfer to determine whether this scenario is indeed more attractive than relying only on the glyoxylate shunt as currently assumed in PAOs biochemical model.

FUTURE WORK

With this study we aimed to give the first steps towards demonstrating the existence of a mechanism in an Accumulibacter enrichment that allows for FAD regeneration without an external electron acceptor.
We successfully prepared inverted membrane vesicles from an Accumulibacter enrichment culture and we were able to detect activity of NADH dehydrogenase (NDH) and Succinate dehydrogenase (SDH) using conventional assays for these enzymes. To prevent the use of oxygen as electron acceptor, two cytochrome oxidase (COX) inhibitors were tested, azide and cyanide. Since we observed that SDH is sensitive to azide, we recommend the use of cyanide as inhibitor. As alternative to using cyanide, we propose to run the assays inside an anaerobic chamber. Inhibiting COX is important to simulate the anaerobic condition in which the NAD reductase (Nred) is expected to operate. Unfortunately, with the current designed assay for Nred, the signal-to-noise ratio was too low to draw any conclusion at this point on its existence and mechanism. Since Nred likely requires membrane potential investment in order to operate, we recommend, as a next step, to assess how PMF can be maintained in these vesicles. We propose:

- assaying for ATPase activity,
- test Nred with either ATP- or Pi efflux-driven membrane potential,
- test buffers with different pH and establish a ΔpH across the membrane,
- perform all assays in an anaerobic environment and taking care that any NADH-consuming processes are inhibited (*i.e.* membrane-bound hydrogenase), and
- further explore the possibility of an electron bifurcation mechanism by using different types of ferredoxins (*e.g.* ferredoxin from Accumulibacter).

Accumulibacter dominate EBPR communities thanks to their winning metabolic strategy under fluctuating anaerobic/aerobic conditions. While we are getting closer to understanding satisfactorily their storage polymer cycling competitive strategy, we are still far from understanding many subtleties of their bioenergetics, mainly when it comes to the intricate coordination of many of their membrane processes.

SUPPLEMENTARY INFO

CULTIVATION PARAMETERS, PERFORMANCE AND CHARACTERIZATION OF THE ENRICHMENT OF ACCUMULIBACTER USED IN THIS STUDY

This study was carried out using an Accumulibacter enrichment culture continuously cultivated in a sequencing batch reactor (SBR-4). A summary of the main process parameters and key performance indicators are found below in Table 5.2 and microbial community characterization by 16S rRNA and FISH is presented in Figures 5.15 and 5.16.

Table 5.2: Process parameters and key performance indicators of the Accumulibacter enrichment understudy.

Parameters	**SBR-4**
pH	7.6
Temp	20^{o}C
Carbon source	400 mg_{COD}/L (65:35 $Acetate_{COD}$:$Propionate_{COD}$)
Phosphate load per C-fed	0.1 Pmol/Cmol
Broth volume	1.5 L
Cycle time	Total – 6 h: Settling – 30 min; Anaerobic – 120 min; Aerobic – 180 min
Hydraulic retention time (HRT)	12 h
Solids retention time (SRT)	5 days (Aerobic: 2.5 days)
KPIs	
Anaerobic P-release per C-fed	~0.76 Pmol/Cmol
Anaerobic-feast length	~45 min (total anaerobic period was 120 minutes)
FISH	PAO
16S rRNA gene amplicon sequencing (dominant OTUs)	Dominant: Ca_Accumulibacter Sub-populations (<10% of rRNA count): Pseudoxanthomonas, Chryseobacterium

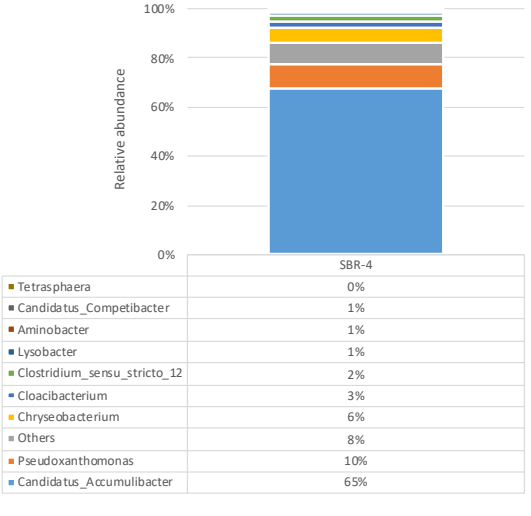


Figure 5.15: Taxonomic distribution of microbial community based on 16S rRNA gene copy numbers. The 16S rRNA gene amplicon libraries have been deposited under project PRJNA490689. SBR-4 was used in this study.

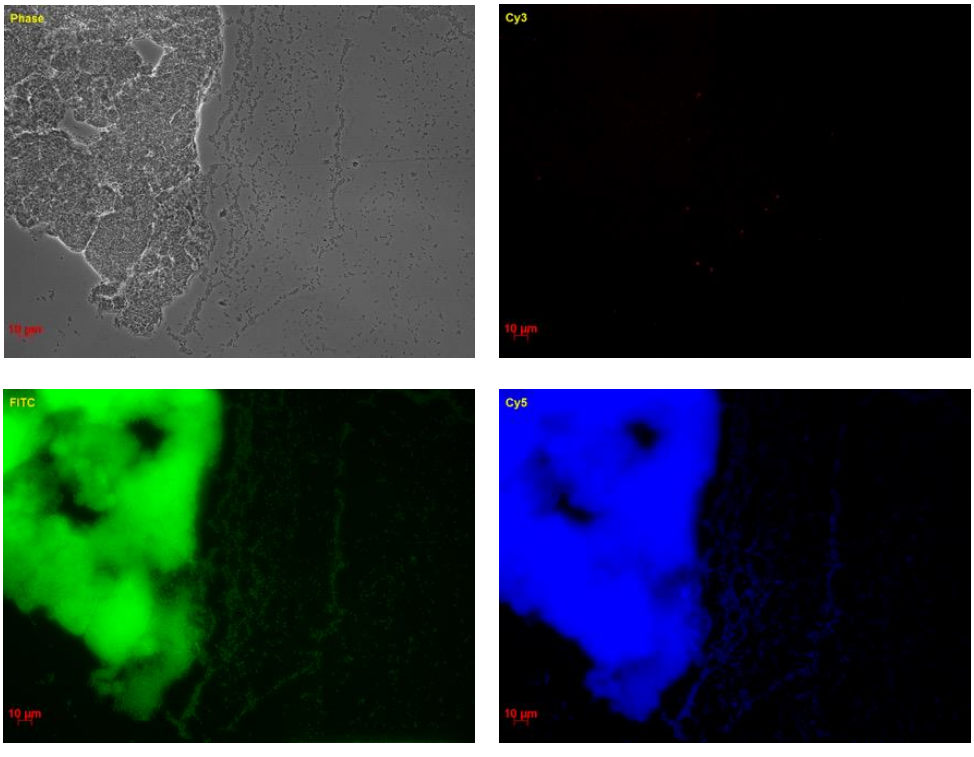


Figure 5.16: Standard fluorescence *in situ* hybridization (FISH) images from the microbial community present in SBR-4: **Top-left** Phase contrast channel; **Bottom-left** Cells hybridized with PAOmix probe (mixture of probes PAO462, PAO651 and PAO846) (Crocetti et al., 2000); **Top-right** Cells hybridized with GAOmix probe (mixture of probes GAOQ431 and GAOQ989) (Crocetti et al., 2002); and **Bottom-right** Cells hybridized with the general bacteria EUB338 probes.

E. coli AS POSITIVE CONTROL FOR NADH DEHYDROGENASE ASSAY

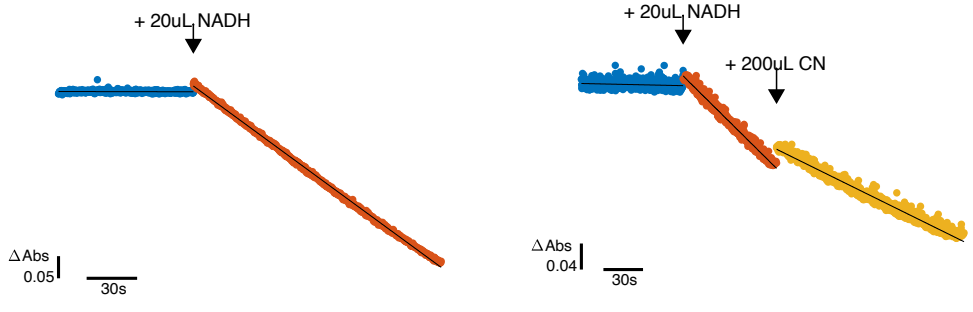


Figure 5.17: Consumption of NADH by *E. coli* MG1655 (WT) membrane vesicles monitored at 340 nm. Both oxygen and menadione (MD) were used as final electron acceptors. Cyanide (CN) was used to inhibit cytochrome oxidase activity (COX).

6 CHAPTER

Concluding remarks & Outlook

In this doctoral dissertation we explored the potential of integrating and cycling through different approaches of systems biology, such as bottom-up biochemistry and molecular biology, top-down -omes and -omics analyses and the integration of data and observations from both approaches into computational models. With each iteration, we were able to advance the *mechanistic* understanding of the functionality and regulation of storage and central carbon metabolism of the PAO *Candidatus* Accumulibacter phosphatis (hereafter referred to as Accumulibacter) under dynamic conditions. While this bacterial community member was the cellular system example studied here, the developed and applied approaches are not specific to this microorganism and they can be applied to other cell systems and communities exposed to changing environments.
The fluctuations of nutrients and oxygen in the environment surrounding the cells lead to intricate metabolic strategies. The overall metabolic traits of Accumulibacter are well described, however the flexible coordination of these traits in slightly different experimental conditions has led to controversial hypotheses on which metabolic pathways are used - *structure*, when are these pathways active - *function*, and what mechanisms control the operation of these pathways - *regulation*.

In **Chapter 2**, we observed experimentally using enzymatic assays that PHA production in Accumulibacter is in fact NADH-preferring and not NADPH as previously assumed in literature. This clarifies previous concerns on how NAD^+ can be regenerated and how NADPH is formed and alleviates the presumed need for H_2 release and energy-dependent transhydrogenase activity. With this new insight on cofactor preferences of oxidoreductases, a (traditional) flux balance analysis revealed how reducing equivalents can be differently balanced anaerobically given the proportion of acetate-to-glycogen consumed. Varying proportions of acetate and glycogen consumed result in different optimal metabolic flux distributions through glycolysis, glyoxylate shunt or oxidative and reductive branches of the TCA cycle. This metabolic flexibility can explain the heterogeneity of experimental observations found in literature regarding the anaerobic metabolism of Accumulibacter. In other words, there is no controversy, only a very flexible metabolism that adapts depending on the environment (acetate uptake) and on intracellular storage compounds availability (polyphosphate/glycogen). On this topic, still some questions remain open:

- There are genetic and metabolic observations that suggest an even more flexible metabolic network since ferredoxin-preferring oxidoreductases, hydrogenases and carbon fixating enzymes are annotated for Accumulibacter. The impact of these sources/sinks/carriers of reducing equivalents on the metabolism of Accumulibacter is still unknown.
  - Hydrogen gas (H_2) production has been observed in Accumulibacter enrichments, however a confirmation of its origin or role is still unknown.
  - *What is the role of carbon fixating enzymes in heterotrophic bacteria like Accumulibacter?*
- The observation of a NADH-driven PHA accumulation leads to re-thinking the ecophysiological role of PHA accumulation. *Could it be regarded as a fermentation product under anaerobic conditions instead of a stress response under unbalanced growth conditions as postulated for aerobic NADPH-driven PHA accumulation?*

- Next to balancing of redox metabolism, future studies should also include the ATP stoichiometry and a description of the associated membrane transport mechanisms and ions transport to further constrain the analysis.

In **Chapter 3**, we applied a recently developed dynamic, conditional flux balance analysis to qualitatively predict which energy allocation strategy is most competitive in an environment where oxygen is periodically unavailable. Storage metabolism revealed itself as a selective advantage in situations where robustness and competitiveness are key, however trading itself off for biomass yield. Another interesting outcome of this analysis is how using a meta-network in this model would still simulate the distinct metabolic strategies of known functional groups of bacteria under different environmental conditions. This can be seen as a fusion of *"Unity in biochemistry"* by A. Kluyver with a derived interpretation of *"Everything is everywhere, but the environment selects"* by L. Baas-Becking in which every metabolic strategy is everywhere but the environmental conditions are selecting. This highlights how microbial ecosystems may be better described by the energy allocation phenotype (what is happening) instead of a genotype-based description of each organism (who is present). The full potential of this modeling approach is yet to be unleashed since many questions and ideas were generated from this first analysis:

- Currently a minimum acetate uptake rate is set as a constraint. Ideally, the model would be able to predict what this rate should be, based solely on environmental constraints. This raises the question: *what limits rates in cellular systems?*
  - If transport processes are limiting, putative rate/yield trade-offs imposed by the membrane could be identified by setting additional constraints, such as:
    - diffusion limitations of substrates defined by the surface/volume ratio, or
    - membrane space limitations, *e.g.* balance between electron transport chain enzymes, phosphate and ion transporters, acetate transporters, among other transporters.
  - If thermodynamics is dictating, then mass-action descriptions could be integrated to further limit fluxes depending on metabolite levels.
  - If kinetics is dictating, more complex kinetic expressions for "bottleneck" enzymes could be used rather than the simpler upper-bound assumption for maximum rate based on enzyme concentration and its roughly estimated specific enzyme activities (k_{cat}).
- The model was still too coarse to describe the competition between PAOs and GAOs. The description of regulatory mechanisms and transport processes including membrane bioenergetics is essential to quantitatively predict competition in a microbial community.
- Even though energy was mostly spent on storage polymer cycling and the model outcomes were not sensitive to the k_{cat} values, these likely play an important role on the simulated proteome allocation.

  - Experiments should be carried out to validate proteome allocation, preferably both cytoplasmatic and membrane proteomes. These experiments should be designed in such way that result in discriminating proteomes under modified conditions such as temperature or other carbon sources.
  - Alternatively, both data-driven and knowledge-driven computational approaches could be developed to predict specific enzyme activities of various organisms, thus allowing for better estimation of the 'cost' of metabolic fluxes.

In **Chapter 4**, a stable isotope labeling experiment was conducted using ^{13}C-acetate to monitor intracellular switches of carbon flows of an enrichment culture of Accumulibacter upon changes in acetate and oxygen availability. Distinct ^{13}C patterns in intermediate metabolites can reveal different metabolic modes of operation of the TCA/glyoxylate reactions. From the insights generated, hypotheses are generated for further investigation of regulatory mechanisms governing the lifestyle of Accumulibacter, which may enable the design of novel control strategies to improve EBPR processes. Stable isotope tracing methods can be used to generate insight on actual intracellular metabolic modes and switches of microbial communities cultured under many various dynamic environmental conditions, *e.g.* other carbon sources, pH, T, phosphate/glycogen availability, aeration intensity. The use of other ^{13}C or ^{15}N labelled metabolites to investigate specific questions would also be interesting, such as the role of carbon fixation in anaerobic redox balancing or to investigate which and when are EPS precursors, proteins or lipids synthesized during the EBPR cycle using untargeted metabolite analyses. While there is plenty to explore using this method, there is also potential for improvement:

- Quantitative flux analysis: In this study, ^{13}C enrichment patterns were measured and analyzed qualitatively. Absolute, intracellular concentration of metabolites are still not available due to different experimental challenges. Once metabolite concentrations can be measured, then computational approaches for ^{13}C-fluxomics can also be applied to estimate intracellular fluxes.
  - The feasibility of quantitative dynamic ^{13}C analysis to study metabolic flux switches in PHA accumulating organisms was shown using another model organism (Verhagen, 2017).
  - Accumulibacter granules pose a challenge on quantitative metabolomics as broth heterogeneity affect sampling reproducibility (Berg, 2017). To overcome this effect, larger samples could be processed, however proper quenching of a large sample needs to be validated. Consequentially, either larger reactor volumes or lower sampling time-resolution would be needed. Alternatively, we started exploring ways of eliminating selective pressures for granulation such as removing or replacing the settling phase by a membrane process for biomass/liquid separation or changing the medium composition by removing granule-forming promoters, *e.g.* calcium ions (Janssens, 2018). However, there seems to exist a very strong motive for these cells to aggregate together.

- Beyond contributing to broth heterogeneity, these granules also contribute to cellular heterogeneity. They harbor multiple types of bacteria with distinct functional roles. And even when granules are mainly formed by Accumulibacter, not all cells are exposed to the environment in the same way. It is important to realize that this experiment is capturing the average metabolome of the broth and it is very difficult, if not impossible, to deconvolute it. *If cells contain different amounts of storage polymers or are exposed to different amounts of acetate or phosphate, do they still have all the same metabolic strategy?* A kinetic metabolic model including the different metabolic strategies discussed in **Chapter 2** and the regulatory mechanisms proposed in **Chapter 4** could be used to explore the effect of storage variability in metabolic strategy switching. Then, a two-compartment model could be used to simulate the effect of micro-environments and resulting average metabolism.
- The effect of the heterogeneity in polymer ^{13}C-labelling (PHA and glycogen) should be assessed as these cannot be considered perfectly mixed pools as assumed in current ^{13}C models. Similar, metabolite channeling of some intermediates can lead to biases in the flux estimation (Winden, Verheijen, and Heijnen, 2001).

Membrane bioenergetics is an essential piece of the metabolism of Accumulibacter. These bacteria import and export a great deal of molecules and ions in short periods of time, making them an interesting candidate as general model organism to study *in vivo* membrane processes, certainly in the anaerobic conditions. In **Chapter 5**, we gave the first exploratory steps towards studying membrane bioenergetics processes using membrane vesicles of Accumulibacter enrichments. As first question, we explored the problem of FAD regeneration under anaerobic conditions, however this approach can also be used to investigate ATP generation, denitrification, H_2 production/utilization, among other processes. The following improvements are suggested:

- Improve experimental conditions: The experimental conditions used during the assay may not have been optimal for Accumulibacter. Optimization of the protocol is needed, *i.e.* buffers used, but also fully anaerobic conditions must be guaranteed, *i.e.* by carrying the experiment in an anaerobic chamber to avoid the following doubts:
  - *Are there metabolic processes in Accumulibacter that are sensitive to O_2 alike in strict anaerobes?*
  - *Do Accumulibacter have a particularly high affinity for O_2 to be competitive under micro-aerobic conditions?*
- Membrane proteomics: To know what membrane proteins are present in the membrane vesicles (cell free extracts), membrane proteomics should be carried out in parallel. Enzymatic activities together with abundance of these membrane proteins could then be integrated in cFBA modeling approaches alike in **Chapter 3**, in particular to analyze any constraints imposed by membrane space allocation.

Beyond the challenge of studying metabolism under dynamic conditions, also the fact that no isolates of Accumulibacter are available added an extra layer of complexity to these studies. Efforts to isolate or the study of an already isolated PAO are recommended to eliminate uncertainties arising from microbial heterogeneity, at least while developing

methodology. In particular when using an isolate, one could then extend the experimental space by designing different cycles or even use steady conditions, explore limits of polyphosphate, PHA and glycogen accumulation and evaluate what are the rate limiting processes in each phase of the cycle.

Finally, among many mysteries related with Accumulibacter lifestyle that keep intriguing the author of this doctoral dissertation, one was selected to end this outlook with:

Why do Accumulibacter invest a considerable amount of their resources into producing biofilms in order to aggregate into granules?

Cellular activity in biofilms is necessarily different from a suspended planktonic state. Cells are more protected from environmental aggressions such as inhibitory concentrations of nutrients, harmful chemical agents, predators. On the other hand, diffusion of substrates or undesirable products is limited. Furthermore, production and excretion of extracellular polymeric substances (EPS) is relatively costly for cells. To justify such resource allocation, it is logical that cells must have a strong advantage when forming these granules. Despite our efforts to cultivate Accumulibacter in a planktonic state, or of other researchers to isolate these bacteria (personal communication, unfortunately not reported), one must wonder why these bacteria are so strongly attached together in granules. In **Chapter 2**, we analyzed and discussed that metabolic flexibility can explain the heterogeneity of experimental observations found in literature regarding the anaerobic metabolism of Accumulibacter. These are bulk observations and using these experimental methods we will inevitably only measure the average metabolic response: *What if what we have been observing all along is the combination of two or even multiple metabolic modes, each running in a different cell?* Examples of this are known and have been nicely modelled in (Picioreanu, Xavier, and Loosdrecht, 2004). In aerobic granular sludge, oxygen does not diffuse all the way to the core, creating an anaerobic microenvironment where strict-anaerobes can proliferate but also other spatially located microenvironments where PAOs and GAOs thrive (Figure 6.1). On another example, in anaerobic granules, acetogens and hydrogenotrophs benefit from a synergistic association due to the proximity conferred by living together in a biofilm. It was this last example together with the observation of H_2 production by Accumulibacter enrichments under anaerobic conditions (Oyserman et al., 2016b) and in our own lab (Berg, 2017) that motivated the following hypothesis linking H_2 syntrophism, the flexible anaerobic metabolism of PAO and their superior capacity to aggregate in biofilms.

In this hypothesis, H_2 could serve as an electron sink to regenerate NAD^+ under anaerobic conditions when the cell is too reduced such as in acetogens or in a glycogen accumulating metabolism (GAM, high glycogen-to-acetate consumed). This would give purpose to the ferredoxin-preferring oxidoreductases and the hydrogenases annotated in Accumulibacter's genome and transcriptome (García Martín et al., 2006; Oyserman et al., 2016b). To prevent thermodynamic inhibition from H_2, either hydrogen is released to and continuously removed from the bulk (possible in continuously sparged bioreactors) or, to make advantage of the proximity created by the biofilm, H_2 is consumed by another cell with

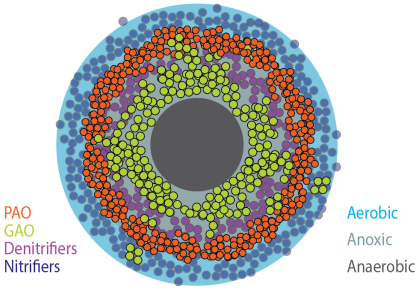


Figure 6.1: Stratified metabolic functionalities across a granule from aerobic granular sludge systems. Here PAO and GAO were meant as different bacteria, but this could also be regarded as Accumulibacter performing polyphosphate accumulating metabolism (PAM) in an outer layer and Accumulibacter performing glycogen accumulating metabolism (GAM) in an inner layer. Adapted from (Winkler et al., 2013) and based on the agent-based modelling study by (Xavier et al., 2007).

the need for NADH. This would be the case of a hydrogenotroph or a more polyphosphate accumulating metabolism (PAM, low glycogen-to-acetate consumed) while converting acetate into PHB. In fact, in our reactors we only observed a small amount of H_2 once acetate was depleted, potentially pointing to hydrogen consumption associated to acetate consumption. In theory, this could happen in between two Accumulibacter cells with similar enzymatic machinery but in slightly different micro-environments and/or with different internal reducing states (Figure 6.2).

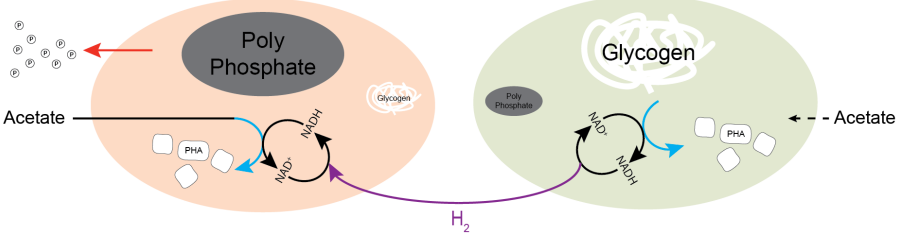


Figure 6.2: Illustration of how Accumulibacter cells with a different metabolism could profit from syntrophy. **Left:** cells with a dominant polyphosphate accumulating metabolism (PAM) consume acetate and require NADH for PHA accumulation. **Right:** cells with a glycogen accumulating metabolism (GAM) convert glycogen (and likely also a small amount of acetate) to PHA but need to release H_2 to balance the internal redox state.

In a recent review by Takhaveev and colleagues, metabolic heterogeneity among clonal microbial cells is discussed and examples are provided where populations tend to show phenotypic variability that occur on the level of metabolism (Takhaveev and Heinemann, 2018). Experimentally, this may be challenging to capture as it would require single-cell technologies and a way of tracking H_2 exchange. Computationally, this hypothesis can be tested by using mathematical models describing cell population dynamics (Charlebois and Balázsi, 2019). In a planktonic state, this syntrophic relationship would likely not be possible, thus offering an explanation why Accumulibacter benefit from forming granules and why Accumulibacter have not yet been isolated to date. All methods applied in this dissertation with a focus on the role of H_2 would help getting a better understanding of the potential link between H_2 metabolism and granulation in Accumulibacter cultures.

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CURRICULUM VITAE

"...Empanel:Empaled.STOP.LGGdS.thesis.ready.for.review.STOP.Nallary..."
This coded telegraphic message surprised the narrator of this section in the early hours of a cloudy and rainy dutch-like summer day. Leonor, which is a Cascalense, from the Portuguese Riviera, where the sun, beach and water sports last the whole year round was sending me this text meaning her thesis was done. With my eyes filled with tears of joy, I went to my mailbox and saw her invite to write this, short but yet rich, bio...

Leonor is a Biological-to-Digital translator who believes that cells - these fascinating, versatile systems created by Nature - have an incredible potential to provide for sustainable solutions to improve our lives. She is currently assuming, at the DSM Biotech Center in Delft, the challenge of working within multi-disciplinary teams to develop and apply computational technologies, including mathematical models, advanced data analytics, and data visualisation to support business driven R&D projects.

She thrives in dynamic and collaborative environments, where people are committed, inspired and supported to perform at their best. This was precisely her motivation during the four years where she worked as a PhD researcher, at Delft University of Technology, in a project that combined systems biology approaches to study environmentally-relevant microorganisms under the supervision of Prof. dr. ir. Mark van Loosdrecht and Dr. Aljoscha Wahl. During this immersive and rich period she acted as representative of PhD candidates at the department and faculty levels, she collaborated as teaching assistant on the academic units of "Analysis of Metabolic Networks" and "Introduction to Algorithmic & Programming Skills" and she also chaired several group meetings and symposia. Still, when you ask her what she cherishes most about that time, she will tell you about her seven MSc. students, four Erasmus students and one MBO intern that she supervised/mentored. Collaborating with them gave her immense positive energy, and she loved being able to share her experience with them as the natural mentor that she is.

As a Biological Engineer (BSc and MSc) from Técnico Lisboa, Lisbon University, specialized in fermentation and modelling, Leonor is driven by tackling challenging problems at the interface between biology and engineering where rational, computational-based approaches and/or the integration of knowledge from several disciplines are required. That was the rational that motivated her to embark in February 2014 in an overseas adventure at Johns Hopkins University where she took the challenge of seeking for a phenotypic signature of prostate cancer cells response to drug therapy under the supervision of Prof. Denis Wirtz and Dr. Jude Phillip. During this intensive, but exciting adventure she mentored and coordinated four undergraduate summer interns who contributed to her Master thesis by generating and processing data of hundreds of single cells' movements in time! The same motivation for research at the interface led her to skip 2013 summer vacation to pursue an internship at INESC-MN where she researched on magnetoresistive chips for biological applications under the supervision of Dr. Filipe Cardoso and Dr. Tomás Dias. Her pioneering mindset led her in 2012 to do an Erasmus exchange at TU Delft where she took courses but also researched "On downstream processing for advanced biofuel production" under the supervision of Prof. Luuk van der Wielen, Dr. Maria Cuellar and Dr. Arjan Heeres.

Leonor loves to communicate. Besides the multiple tech-languages, she is able to confidently interact in three different spoken-languages: while hanging out with friends in her favourite *"Bij Best"* brunch place in Delft the conversation flows in English, but it quickly changes to Dutch when she is ready to order her food, only to be delighted by the nicest waitress who always greets her in Portuguese, *"Olá, bom dia!"*. This communication appetite has driven her to exhaustively prepare numerous oral presentations and posters, always being careful with her audience background and always irradiating enthusiasm in every story she tells.

She also engaged in several non-profit social organisations, where she often finds the purpose and space to initiate, push forward ideas and activate movements for noble causes. In the last few years, she has contributed to strengthen the global network of Portuguese biological engineering *alumni* and students, with the aim of narrowing the gap between academia and professionals. This same spirit of community development and exchange comes from her times at NAPE (Técnico's Student Support Unit) where she assumed several roles, ranging from freshmen and international students mentorship, coordination of mentors, organisation of networking activities, and guiding prospective students through the different university degree options.

She also loves to compete in team sports, namely volleyball, which she plays since her high school times at the Escola Secundária S. João do Estoril, while studying Science and Technology. The drive for high performance is evident in her academic track where individually and collectively she won, in the Science and Innovation area, six honors and awards ranging from Academic Merit, Poster Prizes, Best Pitch and first place in TU Delft LST MSc. Design Competition.

- A proud parent, 2020

MASTER THESES SUPERVISED

Conditional Flux Balance Analysis in *Candidatus* Accumulibacter phosphatis: Search for an optimal resource allocation in anaerobic/aerobic and feast/famine cycles.

Sergio Tomás Martínez (May 2018)

The pitfalls of being a Polyphosphate Accumulating Organism

Marissa Janssens (April 2018)

Anaerobic ATP generation in *Candidatus* Accumulibacter phosphatis

Lenno van den Berg (September 2017)

Grow or store? Exploring metabolic decision making under feast/famine conditions using ^{13}C tracer

Koen Verhagen (June 2017)

Development of a metagenome-based central carbon metabolic network model for *Candidatus* Accumulibacter phosphatis

Hein van der Wall (June 2016)

Quantitative Targeted Metabolomics in Storage Accumulating Prokaryotes Case Study: Plasticicumulans acidivorans TUD YJ-37

Andy Wiranata Wijaya (May 2016)

Analysis of redox metabolism in PAO I: Reassessing the current metabolic models of Phosphate Accumulating Organisms

Kasper M. Akkermans (November 2015)

Reports available upon request.

PRESENTATIONS & POSTERS

Life in changing environments: The intriguing cycles of Polyphosphate Accumulating Organisms (presentation)

Guedes da Silva L, Wahl SA

BioSB research school - Integrated Modeling and Optimization 2020, NL (online)

The environment selects: Modeling intracellular energy allocation in microbial communities under dynamic environments (poster)

Guedes da Silva L, Tomas Martinez S*, van Loosdrecht MCM, Wahl SA*

Metabolic Pathways Analysis 2019, Riga, LV

Understanding PAOs metabolic decisions using Conditional FBA (poster)

Guedes da Silva L, Tomas Martinez S*, van Loosdrecht MCM, Wahl SA*

MB5.0, Delft, NL, 2018

Academic *versus* Industrial PhD (presentation)

Guedes da Silva L, Fernandes D

LinkTo, Apr 2018, Técnico Lisboa, PT

Grow or store? Exploring metabolic decision making under feast/famine conditions using ^{13}C tracer (presentation & poster)

Guedes da Silva L, Verhagen K, Wijaya AW, Kleerebezem R, Loosdrecht MCM, Wahl SA

NBC-18: Biotechnology in Harmony, Ede, NL, 2018
Microbiology Centennial Symposium, Wageningen, NL, 2017 (only poster)
Metabolic Pathway Analysis, Bozeman, Montana, USA, 2017 (only presentation)
Microbiological Methods for Waste & Water Resource Recovery, Delft, NL, 2017

Store or Perish: Storage polymers as sources of energy and redox power in anaerobic conversions (poster)

Guedes da Silva L, Gomes JC, van de Wijgaart R, van den Berg L, Akkermans KM, Verhagen K, Gamez KO, Welles L, van Loosdrecht MCM, Wahl SA

Microbiology Centennial Symposium, Wageningen, NL, 2017
Microbiological Methods for Waste & Water Resource Recovery, Delft, NL, 2017
Dutch Bioinformatics & Systems Biology conference, Lunteren, NL, 2017
TUDelft BioDay, Delft, NL, 2017 **(Best poster award)**

Analysis of metabolic functionality and regulation of Polymer Accumulating Microorganisms under dynamic environments (presentation)

Guedes da Silva L, Kleerebezem R, van Loosdrecht MCM, Wahl SA

Soehngen Institute of Anaerobic Microbiology (SIAM) symposium, Utrecht, NL, 2016
TUDelft Biotechnology department symposium, Delft, NL, 2016

Metabolic analysis of Enhanced Biological Phosphorus Removal (EBPR) phosphate and glycogen accumulating organisms (poster)

Guedes da Silva L, Akkermans KM, van Loosdrecht MCM, Wahl SA

Metabolic Pathways Analysis 2015, Braga, PT **(Best poster award)**

The Portuguese Bio-Discoveries: Experiences ERASMUS & Summer internship (PEN) & American master thesis (presentation)

Guedes da Silva L

Biological Engineering symposium (jornadas) 2015, Técnico Lisboa, PT

ARTICLES

An NADH preferring acetoacetyl-CoA reductase is engaged in poly-3-hydroxybutyrate accumulation in *Escherichia coli*

Olavarria K, Carnet A, van Renselaar J, Quakkelaar C, Cabrera R, Guedes da Silva L, Smids AL, Villalobos PA, van Loosdrecht MCM, Wahl SA (2021)

J Biotechnol 325:207–216. https://doi.org/10.1016/j.jbiotec.2020.10.022

Revealing metabolic flexibility of *Candidatus* Accumulibacter phosphatis through redox cofactor analysis and metabolic network modeling

Guedes da Silva L, Gamez KO, Gomes JC, Akkermans KM, Welles L, Abbas B, van Loosdrecht MCM, Wahl SA (2020)

Appl Environ Microbiol 86. https://doi.org/10.1128/AEM.00808-20

Quantitative profiling of microbial communities by de novo metaproteomics

C Kleikamp HB, Pronk M, Tugui C, Guedes da Silva L, Abbas B, Mei Lin Y, van Loosdrecht MCM, Pabst M (2020)

bioRxiv 2020:2020.08.16.252924. https://doi.org/10.1101/2020.08.16.252924

The environment selects: Modeling energy allocation in microbial communities under dynamic environments.

Guedes da Silva L, Tomás-Martínez S, Loosdrecht MCM van, Wahl SA (2019)

bioRxiv 689174. https://doi.org/10.1101/689174

AWARDS

Best pitch *in SIAM Entrepenurial day 2017, Delft, NL*

Best poster *in BioDay 2017, Delft, NL*

Best poster *in Metabolic Pathways Analysis 2015, Braga, PT*

1st place *in Bioprocess Design Competition TU Delft LS&T MSc. 2013, Delft, NL*

Academic Merit Recognition *by Técnico Lisboa in 2011/2012 and 2012/2013, Lisboa, PT*

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that supported me in this journey and who
gave me a hand, a word, a smile or a hug
whenever my path needed to be (en)lightened.
- Leonor, 2020

A FIRST BIG THANKS GOES TO... THE HAMSTEREEEN TEAM!

This dissertation, this journey, this would have not been possible without you, dear team.

Mark, thank you for giving me the opportunity to let my curiosity drive this project and for providing such a wonderful environment to do it. Thanks to you, our team could wonder, explore, experiment, and simply diverge to the infinite! It is a privilege to be given so much freedom in research, but you really make sure we have the space to try, fail, learn, grow, and succeed! Then, when the time came to wrap-up and converge into this dissertation, your support was invaluable to me. With so many mysteries still to solve, this task was at times very difficult. Thanks for your patience, kindness and understanding during this period. And I will never forget the day we opened that bottle of wine to celebrate that this dissertation was finally *DONE*!

Aljoscha, it all started with back in my Erasmus master student years, during the course you were lecturing back then - Analysis of Metabolic Networks. Your enthusiasm for the art of mathematically describing cellular physiology was (and is) inspiring. Despite challenging, your proposals for group assignments based on real biological investigations were always very intriguing puzzles. Later, I was looking forward to solving more of these

puzzles, so I reached out to you looking for PhD opportunities. I could feel butterflies in my stomach while you were telling me about a possible project on the metabolic analysis of polyphosphate accumulating organisms under dynamic conditions. *"That's the one."* Years have passed, and despite all the experimental hurdles presented by these intringuing bugs, I'm proud of how far we reached on *"the one"* and I'm grateful for this opportunity you gave me. Thank you.

Robert, thank you for always bringing me back to reality, not always an easy task, but an essential one. **Dr. PAO** (aka Laurens) thanks for helping me getting started in the fantastic world of PAO. It is an honor to finally join you as Miss Dr. PAO! **Karel**, my dear friend, what a fantastic collaboration we had, with loads of dancing, music and love for physiology. Thank you for introducing and guiding me through the world of enzymes! **David** and **Thomas**, there are no enzymes without DNA, and your help was essential to acknowledge and understand better that side of Life! **Robin**, my favourite blond office mate, my brother in science, thank you for all the support, discussions and guidance. I'm happy you are back in the same time zone! **Gerben**, it was a privilege to run my fermentations in your super-duper systems. Thank you for all the support and discussions, I'm super looking forward to reading your thesis and to witness your future endeavours. **Duncan**, latest acquisition to this team, but great contribution and support on the quest to find that special enzyme that will make everything work! It is remarkable how you make membrane bioenergetics sound easy, I hope one day it will also make that much sense to me as well. Until then, I will just keep asking you, so thanks (in advance) for your patience!

My dear students, I cannot imagine this journey without you. You made it special, warm, adventurous, exciting! **Kasper**, **Joana** (*minha afilhada*), **Andy**, **Hein**, **Alex**, **Leonor** (junior), **Jo** (my Joy), **Koen**, **Roel**, **Lenno**, **Marissa**, and **Sergio**, THANK YOU all for your commitment and dedication in unraveling the secrets of these intriguing hamstereen-bacteria!

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The fantastic design of this dissertation cover, defence invitation, and chapter separators is thanks to the talented and very patient **Inês**. Thanks for making my book beautiful!

A GREAT ENVIRONMENT IS ESSENTIAL FOR THRIVING...

Dear **CSE rockstars**, many thanks for the *"gezelligheid"*, we had so many nice moments together, Christmas markets in Germany, great retreats, breakfasts, muffins! A special word goes to my officemates, **Eleni**, vizinho **Hugo**, **Jinrui**, **Mariana**, **Koen**, thanks for being there through all the ups and downs, always nice to have your warm support. Also, to our neighbouring ladies, **Flo**, **Francisca** and **Yaya**, thanks for your kindness and sweet gifts. There are of course many more people in the group to thank, because we may have not

shared offices, but we for sure shared unforgettable moments in and outside the lab! The most marked memories I will never forget are all of our ^{13}C sampling experiments: that was ultimate coordination and collaboration culminating in a true winning team-spirit! Thanks to you all for your great sample passes and receptions! As there would not be sampling without fermentation, big kudos go to the **fermentation team**, who always made sure I had the right tool to fix my reactor, thanks! Also there would be no fermentation without media, so a big thanks to the **media kitchen team** for guaranteeing food for all the greedy bacteria! Sampling without analysis would not make any sense, so a big thanks to the **analytical team** for processing the gazillion samples we produce - respect! Last, but not least, all this would not work smoothly without the great support of our dear **Jenifer**, thank you!

If one group is fun, two groups is a party - dear **EBTers**, lab-life with you never gets boring! After careful analysis, I came to the conclusion the EBT law is *"thou shall compensate all the time spent in the lab with fun social activities"*: ping-pong in the corridor, coffee-breaks with celebration cakes every week, lunches at the sports center, drinks at t' Keldertje and later botanical garden, dinners, Tango-tuesdays, most creative outfits for department events, sports&culture on the weekends, great lab retreats, ... It will be hard to top this, THANK YOU ALL!
Danny, my other blond brother, so many great moments we shared, both in the lab with our PAO granules, but also kareokeing, sporting, eatin' and chillin'! Thank you for your friendship and support. **Simon**, my dear friend, thanks for being always so kind and supportive, specially with your positive-energy hugs which always made my days brighter! My SIAM buddies, **Julius**, **Laura**, and **Marissa** thank you for our shared PhD moments filled with your kindness, support, and friendship. **Jelmer**, I will also keep nice memories of both our *Plasticicumulans* and surfing moments! Thank you for all the guidance and support. Also at EBT, lab support is great: **Udo**, thanks for teaching me how to use my common sense in the lab, and thanks to you, I will never forget how to FISH and take good care of a microscope. **Ben**, *muito obrigada por todas as análises às comunidades microbianas*, it was always nice to receive your Portuguese messages! **Dimitry**, thank you for all the patience and support with cultivating *Plasticicumulans* (unfortunately, it did not make it to this dissertation), and also for all the critical questions you asked during my presentations. Last, but not least, a big thanks to **Miranda** who makes sure everything runs smoothly, thank you!

However, I will never forget my first group ever at BT, even before my PhD: **BPErs**, thank you for welcoming me to the research life. Despite your attempts to warn me to stay away from the dark-side, I always felt it had the best muffins! In your honor, I shall bring this tradition anywhere I work in Delft. **Susana** thank you for your hospitality in my first homeless weeks in Delft. **Arjan**, thanks for setting a great example as student supervisor, and giving me a solid kick-start on research. **Maria**, my guiding star, my mentor, thank you for all the support and guidance. Wherever you go, there is a high chance I'll follow, so I'm curious what your next endeavour will be! And finally, **Kawieta**, you're awesome!

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... BUT CHANGING ENVIRONMENT IS BETTER FOR GROWTH.

Even though I enjoyed my days at the university, I always wondered how was life in the other side of the city center of Delft. I am grateful for the opportunity I am given at DSM, not only because of allowing me to witness first-hand the industrial perspective, but also for allowing me to grow and experience the exciting digital transformation journey! To my **dear colleagues**, thank you all for your contributions, be it in actual scientific support, or just a kind word from time to time. These past (almost) 2 years were intense, but you greatly helped me and energized me to make this dissertation possible! I would also like to thank to my **mentor** for being my sparring partner and helping me shaping *"what I would like to do when I grow up"*.

AND WHAT'S LIFE WITHOUT COMMUNITY PURPOSE?

While I was growing up, I watched my parents and their friends coming together, after work, to collaborate in creating nice experiences for others. They lead by example, and I follow. For me, it all started at the the student support unit (NAPE) at Técnico. There I met a great group of inspiring, big-hearted people eager to help and create great experiences for new and prospective students. Thanks to our team, I could witness the power of multi-perspectives in identifying and solving problems as we were all studying different engineering degrees. I learned a lot from you, ***NAPEanos***, thank you! And so it was here that my interest in mixing up things to better tackle challenges began. At the beginning of my PhD, I had the honor to co-host the Metabolic Engineering Colloquia alongside my dear friend **Xavier**. Our motivation was to bridge the gap and bring closer the research done in both Cells System Engineering and Industrial Microbiology groups and it seems we were just anticipating what is now an actual merger! Xavier, thanks for all the nice discussions, collaborations in assignments and, specially, for all your support throughout the years (including also hosting me in my first homeless weeks in Delft). Next to these monthly *colloquia*, **Hugo** and **Francisca** nicely passed me their baton as CSE representative at the BT PhD committee. As a member, I co-organized three yearly department *symposia* with my dear committee members with whom I learned a lot! Thanks for sharing your experiences and for all the critical discussions about the life quality of PhD candidates. These motivated me, in my last year, to join the recently founded Applied Sciences (TNW) faculty PhD council together with **Arthur** and **Vic**. It was a pleasure collaborating with you two, always advocating for better. Together, we brought our concerns about PhD duration and opened up the conversation, hopefully for the benefit of future generations. With **Flo**, **Kevin**, and others, we also had nice experiences with creating an active feedback loop to the graduate school, proposing several improvements and initiatives. Many thanks to **Ans** and **Laurens** for hearing us patiently. Finally, another big achievement of the council was the first TNW PhD Alumni event, which I am eager to be part of in the next editions to come! And why? Because I am inspired by the co-founders of Técnico's Biological Engineering Alumni group, **Cátia** and **Daniel**, who organized the first Alumni networking event in 2015. Thanks to them, today I am grateful for all the opportunities to meet, collaborate and learn from professionals of bioengineering working on the most diverse topics around the globe. This is how I met **Bárbara** who advised and encouraged me to

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TEAMIES AND FRIENDS ARE IMPORTANT TO KEEP SANE...

Volleyball (indoor and beach) was a major part of my life in Delft. It gave me so much energy! I am grateful for the opportunity to play at PUNCH and VVV Delta for 5 years, with great **teams**, ***dames*** and **coaches**. Thank you all for the great support, team spirit and coaching. Looking forward to playing again soon! I would also like to thank my current coach, **Jeannot**, for helping me stay fit and get stronger, but also for reminding me the importance of recovering, relaxing and resting.
And to all my friends that go a looong way back, thank you all for your support and for always being there: my bioengineers bonitos **Ana**, **André**, **Fi**, **Gonçalo**, **Meggy**, **MJ**, my Hopkins lab- and housemates **Angela**, **Lena**, **Tânia**, my 't Keldertje-mates, los Mexicanos and my Buddies of Design, my first (and forever) mentor **António**, my BFFs from high-school **Bez**, **Mendes**, **Morgi**, **Ritinha**, **Zé** - I miss you all!

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