

Minireview

Martin Lempp, Paul Lubrano, Gert Bange and Hannes Link*

Metabolism of non-growing bacteria

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Abstract: A main function of bacterial metabolism is to supply biomass building blocks and energy for growth. This seems to imply that metabolism is idle in non-growing bacteria. But how relevant is metabolism for the physiology of non-growing bacteria and how active is their metabolism? Here, we reviewed literature describing metabolism of non-growing bacteria in their natural environment, as well as in biotechnological and medical applications. We found that metabolism does play an important role during dormancy and that especially the demand for ATP determines metabolic activity of non-growing bacteria.

Keywords: antibiotics; industrial biotechnology; metabolism; non-growing bacteria; starvation.

Introduction

Bacterial metabolism and growth are mutually connected: metabolic activity is high in growing cells, while non-growing cells are metabolically less active. To which extent metabolic activity decreases in non-growing cells is currently not clear, because it is experimentally difficult to measure metabolic flux in non-growing cells that hardly exchange nutrients with the environment. Yet, the majority of bacteria exist in a non- or slow-growing state, either because nutrients are lacking or other conditions are unfavorable (Bergkessel et al. 2016; Gray et al. 2019). However, even in the

absence of biomass formation, non-growing bacteria synthesize macromolecules and they have a basal metabolism (Anderson and Domsch 2010; Yin et al. 2019). For example, bacteria that account for the absolute minority of species in the biosphere have continued metabolic activity at zero growth (Hausmann et al. 2019). In particular one member of this rare biosphere, a *Desulfosporosinus* species, showed a considerable expression of genes and proteins, implying that these cells make nucleotides and amino acids.

Compared to our knowledge about metabolism of (fast-) growing bacteria, metabolism at zero growth is less understood. A problem is that metabolic models can simulate the complete metabolism of a bacterium like *Escherichia coli* (Monk et al. 2017) and various other bacteria (Kavvas et al. 2018; Nogales et al. 2020), but they can only predict metabolic fluxes during exponential growth. The reason is that during exponential growth, metabolism is geared towards optimal supply of biomass building blocks and energy, which constrains the overall distribution of metabolic fluxes and makes them predictable with models (Orth et al. 2010). In contrast, metabolism of non-growing cells is less predictable with metabolic models, because the metabolic objectives of these cells are not well defined. As a consequence, current approaches to model metabolism of non-growing *E. coli* are based on small-scale phenomenological models, which capture for instance *E. coli* metabolism during stationary phase (Schink et al. 2019). Schink and coworkers combined a small-scale model of *E. coli* with experimental data to quantitatively describe how non-growing *E. coli* cells recycle metabolites from dead cells and how this maintains viability of the overall culture. Finally, it remains to be shown to which extent metabolism of a given bacterium in stationary phase, which follows the fast-growing logarithmic one, equals that of cells that were non-growing for a longer period of time.

In this review we ask if non-growing cells are metabolically active and which objectives define their metabolism. Therefore, we collected recent literature describing metabolism of non-growing bacteria in nature, and non-growing bacteria that are relevant for industrial biotechnology and infectious diseases. Because a wide range of

*Corresponding author: Hannes Link, Max Planck Institute for Terrestrial Microbiology, D-35043 Marburg, Germany, and SYNMIKRO Research Center, D-35043 Marburg, Germany, e-mail: hannes.link@synmikro.mpi-marburg.mpg.de. <https://orcid.org/0000-0002-6677-555X>

Martin Lempp and Paul Lubrano, Max Planck Institute for Terrestrial Microbiology, D-35043 Marburg, Germany; SYNMIKRO Research Center, D-35043 Marburg, Germany

Gert Bange, SYNMIKRO Research Center, D-35043 Marburg, Germany; Department of Chemistry, Philipps University Marburg, Hans-Meerwein-Strasse 6, C07, D-35032 Marburg, Germany

Table 1: Overview of metabolic activity in non-growing bacteria.

Growth arrest	Reference	Organism	Characteristics of metabolism
Carbon limitation	Lempp et al. (2019)	<i>Escherichia coli</i>	Continued metabolic activity and precisely tuned degradation of macromolecules to keep energy levels high.
	Sekar et al. (2020)	<i>Escherichia coli</i>	Significant metabolic activity by fast degradation of glycogen as short-term storage compound.
Nitrogen limitation	Chubukov et al. (2017)	<i>Escherichia coli</i>	Glucose uptake rate only 6% of exponential growing cells. Energy charge slowly decreasing and high alpha-ketoglutarate levels.
	Masuda et al. (2017)	<i>Escherichia coli</i>	In a mevalonate producer, glucose uptake rate reaches 27% of exponential growing cells. Continued flux through TCA cycle.
Phosphate limitation	Chubukov and Sauer (2014)	<i>Escherichia coli</i>	Glucose uptake rate only 10% of exponential growing cells. Energy charge strongly decreased.
Oxygen limitation	Rittershaus et al. (2018)	<i>Mycobacterium tuberculosis</i>	High flux through the reductive TCA cycle reactions and lipid anabolism to maintain redox state.
Temperature	Fleury et al. (2009)	<i>Staphylococcus aureus</i>	Switch from nucleotide <i>de novo</i> synthesis to nucleotide salvaging. Increased expression of biosynthesis of lysine, glutamate, histidine and branched chain amino acids.
	Dong et al. (2018)	<i>Bacillus licheniformis</i>	Increased levels of proline, glutamate and lysine.
pH	Sun et al. (2012)	<i>Escherichia coli</i>	ATP synthase activity ensures survival and pH maintenance.
	Du et al. (2020)	<i>Escherichia coli</i>	Upregulation of genes in TCA cycle, respiration chain, ATP synthase and glutamate biosynthesis
	Wilks et al. (2009)	<i>Bacillus subtilis</i>	Increased expression of ATP synthases upon pH upshift with accompanied growth defect. Arginine catabolism could be a strategy to counteract base stress.
Reactive oxygen species	Christodoulou et al. (2018)	<i>Escherichia coli</i>	Increase of flux to the PPP for NADP ⁺ reduction and ROS scavenging. Decrease of flux to lower glycolysis.
Inhibition of translation	Lobritz et al. (2015)	<i>Staphylococcus aureus</i>	Decreased oxygen consumption rate, TCA metabolites, amino acids, nucleotides, lipids and respective precursors accumulation
Inhibition of translation	Diaz-Pascual et al. (2019)	<i>Vibrio cholerae</i>	Unchanged glucose uptake and fluxes through glycolysis. Increase in cell volume. No change in energy charge, contrasted metabolite accumulation/depletion.

conditions result in zero growth, we structured the review according to the reason for the growth arrest Table 1.

Growth arrest due to starvation

Starvation is one of the main reasons for a growth arrest in natural environments. Most bacteria in the biosphere do not grow because they lack essential nutrients like carbon, nitrogen, phosphate, oxygen or an important trace element (Chubukov and Sauer 2014). Bacteria constantly transition between conditions where nutrients are abundant and conditions where nutrients are scarce. Thus, they should generally be able to switch between dormancy and growth within a few minutes. For example, shifting *E. coli* cells from a medium with high glucose levels to a medium without glucose caused profound changes of the metabolome and transcriptome within less than 5 min (Lempp et al. 2019). However, despite the lack of carbon *E. coli* retained a surprisingly high CO₂ production rate and a high energy level during

the first hour into starvation. Metabolome and transcriptome data revealed that during this phase *E. coli* feeds on internal resources like glycogen, proteins and RNA. This means that non-growing bacteria can retain a high metabolic activity even if no external carbon sources are available. Especially, the short-term storage compound glycogen seems to be important to maintain metabolic activity directly after removal of a carbon source. This result was supported by a study that used metabonomics to compare metabolism of wild-type *E. coli* and a glycogen deficient mutant (Sekar et al. 2020). The study showed that glycogen utilization plays an important role in providing energy during the very early phase of carbon starvation and that this enabled non-growing *E. coli* to quickly resume growth when glucose became available. Readiness for growth resumption was also associated with the accumulation of the glycolysis metabolite phosphoenolpyruvate (PEP) in glucose starving *E. coli* (Xu et al. 2012). In *E. coli*, PEP is required for glucose uptake by the phosphotransferase system (PTS), and therefore cells accumulate PEP within

seconds after removing glucose from the medium (Lempp et al. 2019; Xu et al. 2012). The reason for PEP accumulation is that PEP carboxylase (a major sink of PEP) is allosterically inactivated when flux through glycolysis is low. *E. coli* mutants with allosterically dysregulated PEP carboxylase do not accumulate PEP and show slower growth resumption than the wild-type (Xu et al. 2012). Thus, regulatory mechanisms enforce metabolite changes in starved cells, such that they have a competitive advantage during growth resumption. These studies support the hypothesis that metabolism during starvation affects metabolism during growth resumption. But vice versa, metabolism during growth affects metabolism during starvation. A recent study in *E. coli* showed that the pre-starvation growth rate determines the death rate during carbon starvation (Biselli et al. 2020). The authors explore how the resulting trade-off between fast growth and longer survival impacts feast-famine cycles, and their results highlight the importance to investigate metabolism under dynamic conditions.

Nitrogen is another important nutrient, which makes up approx. 12% of the biomass in bacteria and external supply of nitrogen is essential for growth (Bauer and Ziv 1976; Chubukov et al. 2017). Because nitrogen limitation is often used to control growth of bacteria in industrial bio-processes, there are many studies that characterized metabolism during nitrogen starvation. Although *E. coli* cells stop growing without nitrogen, they continue to consume glucose with an approximately 20-fold lower rate than during exponential growth (Chubukov et al. 2017; Masuda et al. 2017). A long standing question was, which mechanism enabled the 20-fold reduction of glucose uptake and recent metabolome data showed that nitrogen limited *E. coli* accumulate the TCA metabolite alpha-ketoglutarate, which feedback inhibits glucose uptake by the PTS in *E. coli* (Doucette et al. 2011). However, over-expressing the PTS increased glucose uptake in nitrogen starving *E. coli* by more than 4-fold, suggesting that accumulation of alpha-ketoglutarate cannot completely inhibit the PTS (Chubukov et al. 2017). Apart from lower overall fluxes in central carbon metabolism, flux distributions in the tricarboxylic acid (TCA) cycle during nitrogen starvation are comparable to non-starved conditions (Masuda et al. 2017). This suggests that even during no growth cells have substantial energy demand, which is fulfilled by the TCA cycle.

Fewer studies investigated metabolism during phosphate starvation. One study measured metabolite concentrations of phosphate starving *E. coli*, and found that the largest concentration changes occur for phosphorylated metabolites like sugar-phosphates and ATP (Chubukov

and Sauer 2014). Glucose uptake during phosphate starvation was only 10-fold lower than in growing cells, and therefore higher than in nitrogen starved cells. A possible explanation for the higher glucose uptake is that it compensates the loss of energy, which is caused by the low ATP levels in phosphate starving *E. coli*.

Oxygen is rarely essential, because many bacteria switch to fermentative metabolism in the absence of oxygen. However, obligate aerobe bacteria stop growing without oxygen. *Mycobacterium tuberculosis* is a well-characterized example of an obligate aerobe bacterium and it enters a non-growing state when no oxygen is available (Rittershaus et al. 2018). *M. tuberculosis* encounters hypoxic conditions upon infection of its host, since hypoxia is often a consequence of an immune response. To identify the genes that affect fitness during hypoxia-induced growth arrest in *M. tuberculosis*, Rittershaus and co-workers used a transposon library and cultivated the mutants for up to 6 weeks under hypoxic conditions. They sequenced the library to identify mutants with a fitness defect. The identified genes were then characterized further by metabolomics and flux measurements. They showed that during a hypoxic growth arrest, *M. tuberculosis* maintains its NAD+/NADH ratio by inhibiting fatty acid catabolism and shifting carbon flux towards the reductive TCA cycle. This matched previous metabolomics studies in non-growing mycobacteria, which showed that the reductive TCA cycle contributes to membrane polarization via succinate secretion in hypoxic conditions (Zimmermann et al. 2015).

In summary, starvation results in different metabolic phenotypes and they depend on the nutrient which is missing. Starving bacteria remain metabolically active, but their metabolic activity is markedly reduced compared to growing cells. One can expect that the ability to decrease metabolic activity under starvation impacts bacterial fitness, e.g. commensal bacteria must halt metabolism via the stringent response to survive carbon starvation and persist in the gut (Schofield et al. 2018).

Growth arrest due to stresses

The pH of the environment can also arrest growth, primarily because it affects the proton gradient across the cell membrane and thereby the proton motive force. Although a wide-range of metabolic responses are capable to maintain growth at critical pH values, their common function is to increase the proton motive force. For example, upregulation (Wilks et al. 2009) or mutation of ATPases (Sun et al. 2012) can compensate for a less efficient energy generation at low

pH values. Also adaptive laboratory evolution discovered that mutations of genes in the TCA cycle, the respiratory chain and ATP synthase restored growth of *E. coli* at pH 5.5 (Du et al. 2020).

High salt concentrations in the environment and hyperosmotic stress arrest growth, because this conditions result in a release of intracellular water into the extracellular medium. A study measured the proteome and transcriptome of stationary *Bacillus subtilis* in the presence of 6% NaCl (Hahne et al. 2010). Many proteins and transcripts that were upregulated belonged to the SigmaB regulon, which is responsible for the general stress response in *B. subtilis*. Moreover, the study reports that a salt-specific stress response enables first import of K⁺ ions, which is then followed by synthesis of the osmo-protectant proline. Proline synthesis normally starts from glutamate and requires the enzymes ProB and ProI. In case of stress the isoenzymes ProJ and ProH are upregulated and used instead, providing the cell with a so-called “osmo-adaptive route”.

Finally, reactive oxygen species (ROS) can also induce growth arrest. The problem with ROS is that they can immediately damage cellular components, and therefore bacteria need fast defense mechanism against ROS. *E. coli*, for instance, responds within less than 5 s to addition of H₂O₂ by increasing the flux through the pentose phosphate pathway more than 2-fold (Christodoulou et al. 2018). This overcapacity of the pentose phosphate pathway produces the extra amount of NADPH that is needed to regenerate the antioxidant defense system in *E. coli*. This shows that metabolism affects detoxification of ROS, but in turn, ROS also affects metabolism. Especially, enzymes in the TCA cycle are sensitive against ROS, as recently shown by proteomics and metabonomics measurements in *Salmonella enterica* serovar Typhimurium (Noster et al. 2019).

Non-growing bacteria and treatment of infections

Metabolism of non-growing bacteria is also relevant to treat infections. Antibiotics for example enforce a growth arrest, which leads to cell death (bactericidal drugs) or retains cells in a non-growing state (bacteriostatic drugs). *Vice versa*, many antibiotics require an active metabolism including e.g. cell wall and protein biosynthesis in order to execute their function (Wilson 2014). Several studies showed that metabolism impacts efficacy of antibiotics, and that antibiotics impact metabolism (Lopatkin et al. 2019; Stokes et al. 2019). The impact of antibiotics on

metabolism was recently studied with metabonomics methods, which showed that metabolite concentration changes are remarkably specific and localized (Campos and Zampieri 2019). The metabolome data was so specific that it identified drug targets and revealed the most direct drug effects on metabolism.

Although antibiotics perturb the metabolome and stop growth, it seems that cells remain a high metabolic activity. In one study, *E. coli* and *Staphylococcus aureus* treated with the translational inhibitors Chloramphenicol and Linezolid continued to consume oxygen with half the rate that they had during growth (Lobritz et al. 2015). Additional metabolome data showed an accumulation of TCA cycle intermediates, amino acids and nucleotides, indicating severe imbalances between supply and demand of these metabolites. Similarly, non-growing *Vibrio cholerae* cells that were treated with tetracycline had almost the same glycolysis flux as untreated and growing cells (Díaz-Pascual et al. 2019). The continued metabolic activity of *Vibrio cholera* in the presence of tetracycline was then linked to a 2-fold increase in cell size. Metabonomics data showed again accumulation of certain metabolites, like amino acids and nucleotides. Thus, it seems that bacteria cannot coordinate protein synthesis with metabolism if the ribosome is blocked by a drug. Future studies should clarify if the excess of metabolic activity has negative effects and contributes to cell death.

The presence of antibiotics often selects for a small subpopulation of cells that were already stochastically non-growing before addition of the drug, and therefore these cells survive the treatment. These so-called “persister cells” resume growth upon removal of an antibiotic, causing failure of treatments and chronic infections. To identify and isolate persisters, it is possible to label cells with fluorescent proteins that report either single cell growth rates (Peyrusson et al. 2020) or the activity of stationary phase promoters (Conlon et al. 2016). With the latter approach it was possible to measure intracellular ATP levels of *S. aureus* persisters and show that low ATP levels lead to persisters formation (Conlon et al. 2016). This means that persisters have a naturally low metabolic activity and the accompanying loss of energy enforces a non-growing state. The observation that *S. aureus* persisters have a low metabolic activity is supported by a study that used a single cell growth rate reporter. The reporter enabled isolating a small fraction of *S. aureus* cells that survived a treatment with ofloxacin in the vacuole of bacteria-killing phagocytes (Peyrusson et al. 2020). The transcriptome of these cells showed downregulation of almost all metabolic processes, thus indicating that overall activity of metabolism decreases in persisters.

In summary, antibiotics enforce a physiological state in which bacteria are non-growing but metabolically active. This leads to metabolic perturbations because there is an excess of biomass precursors and energy in the absence of growth. Persisters, in contrast, are dormant and metabolically inactive, which renders them robust against treatment with antibiotics.

Non-growing bacteria in industrial biotechnology

Non-growing bacteria that are metabolically active play also an important role for industrial biotechnology, because non-growing cells convert the feedstock into the desired product and not into biomass. Therefore, there is currently a general interest in so-called two-stage bioprocess that separate growth and production phases (Burg et al. 2016). The major challenge of two-stage processes is to switch a production strain between a growing state and a state with high product formation. To this end, it is possible to use bacteria that naturally switch between the two states, such as the polyketide producer *Streptomyces coelicolor* (Wang et al. 2020). *S. coelicolor* switches from growth to production of actinorhodin (a polyketide antibiotic) when nutrients deplete in the environment. ¹³C tracer experiments demonstrated that during this switch actinorhodin was produced from internally stored tri-acyl-glycerides. Additionally, tri-acyl-glycerides became a main source of energy and reducing power, which resulted in an increase of the intracellular concentration of ATP and NADH. The consequence of high ATP and NADH levels was that they feedback inhibited enzymes in the TCA cycle, and this redirected Acetyl-CoA away from the TCA cycle and into production of actinorhodin (Wang et al. 2020). The conclusion from this study was that previously synthesized reserves can sustain constant actinorhodin production and a high overall metabolic activity in non-growing *S. coelicolor*.

Apart from such natural growth switches, it is possible to engineer synthetic growth switches, for example by modifying genes in the quorum sensing system (Gupta et al. 2017) or by temperature-sensitive transcription factors (Harder et al. 2018). With a temperature-sensitive transcription factor, Harder and coworkers controlled expression of isocitrate dehydrogenase in *E. coli* to channel TCA metabolites into overproduction of itaconic acid. Another study used temperature to control enzyme activity directly with a thermosensitive variant of an essential enzyme in arginine biosynthesis (Schramm et al. 2020). The

thermosensitive enzyme (ArgG) rendered *E. coli* auxotroph at 39 °C and prototroph at 30 °C, which enabled a growth switch by changing between the two temperatures. During the 39 °C growth arrest phase glucose uptake remained at 45% of that in the growth phase, and the cells overproduced citrulline (the substrate of thermosensitive ArgG).

Knockdowns with CRISPR interference were also used for synthetic growth switches. A CRISPRi library targeting more than 12 000 loci in the *E. coli* genome, identified targets that could efficiently decouple growth from production (Li et al. 2020). Therefore, the authors measured which CRISPRi targets reduce growth and which targets increase production of GFP, and the overlap between these targets were promising targets for an effective growth switch. CRISPRi was also used to modulate the abundance of citrate synthase in cyanobacteria and switch CO₂ fixation from growth into butanol and ethanol production (Shabestary et al. 2018). CO₂ uptake rates were high for the first hours of the growth arrest and gradually decrease thereafter. Production rates however remained stable over time. The authors proposed that biomass formation acted as a sink for reduction equivalents, so the artificially arrested cells had altered NAD(P)/NAD(P)+H ratios that might prevent prolonged metabolic activity.

In conclusion, non-growing bacteria are relevant for industrial processes, especially if they maintain a high metabolic activity. However, metabolic activity usually decreases when bacteria remain in a non-growing state. Strategies to avoid decreases of metabolic activity are to artificially maintain a high demand for energy, e.g. by expressing ATP-hydrolyzing enzymes (Boecker et al. 2019). This so-called ATP wasting enforced the highest glucose uptake rates that are currently reporter for non-growing *E. coli*: 6.5 mmol/gDW/h.

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