Bacillus subtilis at near-zero specific growth rates
Overkamp, Wout

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2015

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Physiological and cell morphology adaptation of *Bacillus subtilis* at near-zero specific growth rates

Part of this chapter was published in:

Abstract

Nutrient scarcity is a common condition in nature and the resulting extremely low growth rates (below 0.025 h\(^{-1}\)) are an unexplored area in *B. subtilis*. To understand microbial life in natural environments, studying the adaptation of *B. subtilis* to near-zero growth conditions is relevant. To this end, a chemostat modified for culturing an asporogenous *B. subtilis* sigF mutant strain at extremely low growth rates (also named a retentostat) was set up and biomass accumulation, culture viability, metabolite production and morphology were analysed. During retentostat culturing the specific growth rate decreased to a minimum of 0.00006 h\(^{-1}\), corresponding to a doubling time of 470 days. The energy distribution between growth- and maintenance-related processes showed that a state of near-zero growth was reached. Remarkably, an aberrant filamentous morphology emerged, suggesting that cell separation is impaired under near-zero growth conditions.

Introduction

Microbial growth is determined by available nutrient concentrations and in nature, nutrient availability varies over time and per environment (Koch, 1971; Demoling et al., 2007). Regardless of whether the environment facilitates a feast and famine lifestyle (Koch, 1971) or an oligotrophic existence (Egli, 2010), the concentrations of assimilable substrates are frequently low but not absolutely zero (Ferenci, 2001). Consequently, the specific growth rate of most microorganisms is far below their maximum during the vast majority of time. High microbial growth rates as achieved in laboratory batch cultures are probably rare in nature (Brock, 1971; Koch, 1997; Ferenci, 2001). Therefore, to better understand microbial life in natural environments, knowledge about physiology at near-zero growth rates is very relevant.

Inside the cell of a microorganism numerous biological processes take place that require energy. These cellular processes can be categorized into growth- and non-growth related processes. The former involve reactions contributing to production of new cell constituents as well as synthesis of the machineries required for cell division. The latter are also referred to as maintenance processes, and involve reactions
associated with functions other than biomass formation (e.g. maintenance of chemiosmotic potential and turnover of macromolecular compounds; van Bodegom, 2007). When nutrients are present in excess and environmental conditions are favourable, microorganisms invest the majority of the available metabolic energy in growth. In nutrient-limiting conditions however, a relatively large fraction of the energy sources has to be used for maintenance-related processes (Pirt, 1965; Nyström, 2004a; van Bodegom, 2007). When the metabolic energy distribution between growth and maintenance has come to a point where available energy is spent exclusively on non-growth associated processes, zero-growth is the result.

Zero-growth is a metabolically active, non-growing state of a microorganism and is fundamentally different from starvation encountered during stationary phase, which involves deterioration of physiological processes. The energetic investment for an organism to remain in a viable and active state without growth is quantified by the so-called non-growth associated maintenance energy coefficient (Pirt, 1965). Resting states as typified by spores, which have little or no metabolic activity, are not considered to be in zero-growth state.

Even in well-studied organisms such as *Bacillus subtilis*, extremely slow growth has not been investigated extensively, mostly due to a lack of experimental accessibility to achieve near-zero growth rates (Pirt, 1987). For example, in batch cultivations, where cells proliferate at their maximum growth rates until nutrients are depleted, the transition from exponential to stationary phase is rapid and involves continuously changing environmental conditions.

Chemostat cultivation allows growth under controlled environmental conditions and at submaximal specific growth rates. The growth rate can be manipulated by varying the dilution rate, making the chemostat a proven setup for studying the effect of growth rate on physiology (Novick and Szilard, 1950; Herbert et al., 1956; Sauer et al., 1996; Dauner et al., 2001). However, due to homogeneity problems at low dilution rates caused by the drop wise feeding of medium, specific growth rates lower than 0.025 h⁻¹ cannot be set-up reliably in chemostats (Herbert et al., 1956; Daran-Lapujade et al., 2009).

To be able to study microbial physiology at extremely low specific growth rates, retentostat cultivation has been developed (Herbert, 1961; van Verseveld et al., 1986). A retentostat, or recycling fermentor, is a chemostat in which all the biomass is
Bacillus subtilis physiology at near-zero growth rates

retained in the fermenter vessel by a filter in the effluent line. Growing a culture at a fixed dilution rate on an energy-limited medium leads to accumulation of biomass and a progressive decrease of energy substrate availability per cell. Consequently, energy available for growth becomes more and more limiting, ultimately leading to zero-growth rates when the substrate consumption rate equals the substrate requirements for maintenance processes. Meanwhile, starvation is prevented in this setup because the substrate supply continues (Herbert, 1961; Chesbro et al., 1979; van Verseveld et al., 1986; Tappe et al., 1996; Boender et al., 2009; Goffin et al., 2010; Ercan et al., 2013).

From previously conducted long-term retentostat cultivation studies (22 - 45 days) it was concluded that Saccharomyces cerevisiae (Boender et al., 2009), Lactobacillus plantarum (Goffin et al., 2010) and Lactococcus lactis (Ercan et al., 2013) exhibit energy distribution- and maintenance characteristics that can be predicted from higher growth rate chemostats. This is in contrast with studies on Escherichia coli (Chesbro et al., 1979), Bacillus polymyxa (Arbige and Chesbro, 1982), Paracoccus denitrificans and Bacillus licheniformis (van Verseveld et al., 1986), which applied short-term setups (3 days) not truly allowing zero-growth conditions to establish.

The physiology of B. subtilis at various specific growth rates has been the subject of numerous studies (Sauer et al., 1996; Dauner et al., 2001; Tännler et al., 2008; Blom et al., 2011). However, retentostat studies with specific growth rates that approximate zero are an unexplored area in the B. subtilis field. B. subtilis is found in a large variety of niches, like soil and water, in which zero-growth is probably a common state. The differentiation strategies that B. subtilis has evolved, such as formation of highly resistant spores, development of natural competence and motility, secretion of exoproteases and biofilm formation (Dubnau, 1991; Msadek, 1999; Branda et al., 2001; Errington, 2003; Kearns and Losick, 2005; Veening et al., 2008) are probably a reflection of its habitat flexibility. They facilitate survival under a large variety of (dynamic) environmental conditions, making it an intriguing subject of study.

Here, we describe the cultivation of an asporogenous B. subtilis sigF mutant strain in an aerobic, glucose-limited retentostat with the aim to investigate the physiological responses to near-zero specific growth rates. The sigF mutant strain is used to prevent undesirable sporulation/germination processes, which would interfere with reaching
near-zero growth rates. During these long-term retentostat cultivations, extremely low growth rates were reached and remarkable cell morphology changes emerged.

## Results

### Implementation of aerobic retentostat cultivation for *B. subtilis*

Defining the experimental set-up for retentostat cultivation of *B. subtilis* required a number of modifications relative to other zero-growth studies (Boender et al., 2009; Ercan et al., 2013). Achieving a zero growth state in a retentostat with a sporulating strain would be impossible. The endospores are dormant and thus for every spore formed, new vegetative cells could grow. Therefore, growth rates will never approach zero in this situation. Additionally, accumulation of spores will continue which makes estimation of culture parameters like maintenance coefficient very difficult as is described in a retentostat study of *A. niger* (Jørgensen et al., 2010). Because sporulation will interfere with reaching a zero-growth state and therefore is undesired, a sporulation-deficient *sigF* mutant was used in this study. This strain can initiate sporulation, but is unable to express the genes necessary for continuation of the process at stage II in the sporulation cascade (Piggot and Coote, 1976; Setlow et al., 1991; Dworkin and Losick, 2005). The use of a knockout strain may impose a limitation in terms of being able to interpret the natural response of the organism. However, this particular mutant is still able to express genes for sporulation initiation (Fawcett et al., 2000; Steil et al., 2005; Wang et al., 2006) and thereby still allows us to see if sporulation is one of the responses *B. subtilis* applies. For retentostat cultivation, bioreactors were equipped with a cross-flow filter (Fig. 1). These filters were chosen for their particular large filtration surface and tangential flow, minimizing the risk of clogging during cultivation (Koros et al., 1996). Complete biomass retention was confirmed by regular plating of effluent. Continuous filtration for up to 42 days was possible without noticeable clogging or observation of growth in the effluent.

Culture purity can be an issue with such long-term cultivations since the risk of contamination by other bacteria is high. To easily identify contamination by microscopic examination, a fusion of green fluorescent protein (GFP) with the promoter of the constitutively expressed ribosomal ribonucleic acid (RNA) operon
**Bacillus subtilis** physiology at near-zero growth rates

*rrnB* (Krásný and Gourse, 2004) was introduced in the strain cultivated. Consequently, cells not expressing GFP could be identified as contamination. The retentostat cultures described here did not experience any contamination.

**Figure 1. Schematic illustration of a retentostat.** A constant flow of air and fresh medium is provided to the culture. Anti-foam is added with timed intervals to prevent foaming. Biomass is retained in the reactor by a cross-flow filter on the effluent line. By means of a pump the culture is circulated over the filter loop to prevent clogging and de-oxygenation in the filter. Not displayed in the figure are the DO-, pH-, temperature- and level sensors used for monitoring of the culture. The pH sensor is coupled to the base feed to maintain a constant pH. Upon contact of the culture with the level sensor, cell-free effluent is pumped out of the reactor to keep a constant culture volume. Samples are withdrawn directly from the culture using an aseptic sampler system. (Figure by Sietse Koenders).
Growth of \textit{B. subtilis} in medium-supply tubing was found to be a potential problem during prolonged (> 7 days) cultivation. The slow feed rate of 35 ml h\(^{-1}\) allowed the bacteria to grow against the current when aerosols or droplets from the culture came in contact with the medium inlet on the fermentor. To prevent growth in the tubing, a dropper was installed between the medium inlet and the medium-supply tubing, preventing direct contact. A sterile airflow in the dropper pushed the droplets to the reactor and prevented cells from entering the dropper.

Vigorous mixing and sparging with air to keep the culture oxygenated in a bioreactor will lead to formation of foam, which can cause problems. Formation of foam needs to be prevented and during trial experiments it became clear that not every anti-foaming strategy was adequate. When anti-foam was premixed with the supplied medium it precipitated in the silicone tubing, partly blocking the inflow of the medium. Not all anti-foam reached the reactor and as a consequence foaming was not prevented properly. Foaming was prevented most effectively by supplying anti-foam on regular intervals. In our setup this was achieved by automatic addition of 5 ml of a 5\% (wt.wt\(^{-1}\)) antifoam solution every 13 minutes via a computer-controlled pump.

**Growth and viability in retentostat cultures**

\textit{B. subtilis} 168 trpC2 sigF::spec amyE::PrrnB-GFP was grown under aerobic retentostat conditions in chemically defined M9 medium with glucose as the growth limiting substrate. Two independent retentostat cultivations were successfully performed for 42 and 40 days (retentostat 1 and 2, respectively) to study the adaptation of \textit{B. subtilis} to near-zero specific growth rates.

Biomass (measured in grams dry weight (g\(_{\text{dw}}\))) accumulated in the bioreactor and asymptotically levelled off to a near zero growth state during a total of approximately 20 days (Fig. 2A). During this period, the calculated specific growth rate (\(\mu\)) decreased from 0.025 h\(^{-1}\) to 0.0004 h\(^{-1}\) (Fig. 2A). At the end of the cultivation, after approximately 40 days, a minimum was reached of 0.00006 h\(^{-1}\). The specific growth rate reached at the end of the cultivation corresponded to a doubling time of approximately 470 days.
The biomass accumulation in the retentostat cultivations can be modelled with the van Verseveld equation (see materials & methods, equation 5) if the maintenance substrate requirement ($m_s$) and the maximum yield of biomass on the substrate ($Y_{sx}^{\text{max}}$) is independent of the specific growth rate (van Verseveld et al., 1986). However, as $Y_{sx}^{\text{max}}$ seems to be varying with the growth rate (see below), modelling with the van Verseveld equation will yield values different from measured values, which is indeed the case in the initial phase of retentostat culturing (Fig. 2A).

**Figure 2. Growth and viability of *B. subtilis* in retentostat cultures.** Steady-state aerobic chemostat cultures ($D = 0.025 \, \text{h}^{-1}$) were switched to retentostat mode at time-point zero. Displayed are data from retentostat cultivation 1 (■) and 2 (□). (A) Measured biomass concentration (g$_{\text{aw}}$ l$^{-1}$). Data points represent mean ± standard deviation of duplicate samples. Additionally, the biomass calculated with the fitted van Verseveld equation for retentostat 1 (---) and 2 (...) is shown, as well as the corresponding calculated specific growth rates (●) and (○), respectively. (B) Cultivability estimated by colony forming units (CFU ml$^{-1}$) on solid medium. Data points represent mean ± standard deviation of triplicate samples.
Next to the assumption of a constant $m_\text{s}$ and $Y_\text{sx}^{\text{max}}$, two requirements are to be met for the van Verseveld equation: all biomass remains inside the bioreactor, and all biomass is viable and metabolically active (van Verseveld et al., 1986). Both requirements are experimentally found to be met: Regular plating of effluent showed that no biomass escaped from the bioreactor and viability of the cultures was shown to be approximately 99% as assessed with fluorescence microscopy.

Colony-forming units (CFU), determined by plating of the culture, followed a trend during the first 20 days in correspondence with the biomass accumulation (Fig. 2B). Surprisingly, after approximately 20 days (between time points 2 and 3) the colony-forming unit count decreased, whilst both the culture dry weight (Fig. 2A) and proportion of live cells (as detected by fluorescence analysis) remained stable. This was a decrease of approximately 30% for retentostat 1 and 70% for retentostat 2.

**Cell morphology**

Cell morphology was monitored using phase contrast microscopy (Fig. 3A). In general, the cell morphology appeared to be heterogeneous. During the initial days of retentostat cultivation, cells were shaped like the typical *B. subtilis* rod with a few cells being shorter than normal. After roughly 20 days, when biomass accumulation reached a plateau and specific growth rates decreased to 0.0004 h$^{-1}$, a different cell morphology emerged. Cells that were longer than the typical rod started to appear, some reaching lengths of more than 10 times that of the usual vegetative single cell at the start of retentostat culturing. Additionally, most of these cells were bent or curved. The length of the cells and frequency of this aberrant morphology seemed to be proportional to the duration of retentostat culturing. In both retentostats these morphologies appeared, but to a greater extent in retentostat 2. The appearance of the elongated cells coincided with the observed decrease in CFU, with retentostat 2 having the most pronounced decrease in CFU and largest number of long cells. When a sample from the retentostat culture was grown overnight as batch in LB medium, microscopic examination showed that the elongated cells were no longer present and that solely the typical *B. subtilis* rod-shape could be observed.
Bacillus subtilis physiology at near-zero growth rates

Figure 3A. Morphological changes of *B. subtilis* during retentostat cultivation. During a period of 42 and 40 days an elongated morphology emerged in retentostat 1 and 2, respectively. First appearance of this morphology was after 18 and 20 days, respectively, coinciding with the biomass accumulation reaching a plateau. Scale bar indicates 5 μm.

Figure 3B. Membrane visualization in aberrant *B. subtilis* cells developed during retentostat cultivation. Staining of membranes with FM5-95 dye reveals that long cells are composed of multiple compartments. Shown are phase contrast- and fluorescent microscopy images (left and right, respectively). Scale bar indicates 5 μm.
In order to determine whether the elongated cells were single cells or composed of multiple undissociated cells, the cell membrane was visualized by incubating with the FM5-95 membrane dye (Invitrogen, UK). From microscopy images (Fig. 3B) it is clear that the filamentous cells are actually septated. Vigorous mixing did not lead to separation of the multiple compartments, confirming that the cells remained attached to each other. Additionally, the individual cells in such a chain appear to be elongated. When estimations of average cell number per chain in retentostat 2 were used to calculate the amount of CFU in a single cell- and chained cell scenario (results not shown), a decrease of 67% with chained cells was the outcome when compared to single cells. This calculated decrease is similar to the observed decrease in retentostat 2.

**Maintenance coefficient and maximum growth yield**

In a retentostat culture, progressive biomass accumulation leads to decreasing substrate availability per amount of biomass. When the substrate consumption rate ($q_s$) is plotted against time (Fig. 4A), it is visible that $q_s$ approaches an asymptote: the value known as the maintenance substrate requirement ($m_s$; Pirt, 1965). Ultimately, when the point is reached where the $q_s$ equals $m_s$, growth ceases. As is described in material and methods, $q_s$ can be calculated from culture parameters.

If the maintenance substrate requirement and the yield of biomass on the substrate ($Y_{sx}^{\text{max}}$) are independent of the specific growth rate, they can be determined from the linear dependence of the glucose consumption rate over the whole range of specific growth rates achieved in the retentostat (Pirt, 1982; Boender et al., 2009) (Fig. 4B). A linear trendline with an $R^2$ of 0.9605 and 0.9661 could be drawn through the data points of both retentostats. At a $\mu$ of zero, $q_s$ equals $m_s$, which is the value at the Y-axis intercept. In the present study, $m_s$ was found to be 0.24 mmol glucose g$_{dw}^{-1}$ h$^{-1}$. The $Y_{sx}^{\text{max}}$, represented by 1/slope of the plot, turned out to be variable among different ranges of specific growth rates. The $q_s$ from our steady-state chemostats at 0.025 h$^{-1}$ was in agreement with data from higher growth rate chemostats (Dauner et al., 2001), yielding a $Y_{sx}^{\text{max}}$ of 64.56 mg$_{dw}$ mmol glucose$^{-1}$. The average $Y_{sx}^{\text{max}}$ over the range of growth rates in the current study (0.025 h$^{-1}$ to 0.00006 h$^{-1}$), was determined to be 47.57 mg$_{dw}$ mmol glucose$^{-1}$. At the lower growth rates, between 0.00275 h$^{-1}$ and 0.00006 h$^{-1}$ a $Y_{sx}^{\text{max}}$ of 16.74 mg$_{dw}$ mmol glucose$^{-1}$ was found.
Although there was a linear dependence of $q_s$ over this lower range of growth rates, the variation found for the different ranges of growth rates indicate a variable $Y_{\text{mx}}$.

**Figure 4.** Substrate consumption kinetics in retentostat cultures of *B. subtilis*. Steady-state aerobic chemostat cultures ($D = 0.025 \, \text{h}^{-1}$) were switched to retentostat mode at time-point zero. Displayed are data from retentostat cultivation 1 (■) and 2 (□). (A) Specific substrate consumption rate ($q_s$) asymptotically approaches the maintenance substrate requirement ($m_s$) during cultivation. (B) Relationship between specific substrate consumption rate ($q_s$) and specific growth rate ($\mu$). Y-axis intersect of the plotted data determines the maintenance substrate requirement ($m_s$).
To be able to calculate the energy produced from glucose during retentostat culturing, residual glucose concentrations and catabolic end products were determined by HPLC analysis. The glucose concentration in the spent medium was below the detection limit. During retentostat cultivation no metabolites were detected in the culture supernatant, indicating complete respiratory dissimilation of glucose. The amount of adenosine triphosphate (ATP) formed with dissimilation of glucose depends on the coupling efficiency of the electron transport chain to ATP synthesis. *B. subtilis* possesses a three-branched electron transfer chain, of which the cytochrome c branch is inactive in glucose grown cells (Winstedt and von Wachenfeldt, 2000) and the *bd* oxidase branch is usually active under low oxygen conditions (Winstedt et al., 1998). Most relevant for our cultures is the *aa3* oxidase branch, which is predominant in *B. subtilis* under oxygen-rich conditions (Winstedt and von Wachenfeldt, 2000). Transfer via this chain is expected to result in formation of 15 ATP per glucose molecule (Dauner et al., 2001). Assuming that complete oxidation of a glucose molecule to carbon dioxide yields 15 ATP, the maintenance requirement for ATP (m<sub>atp</sub>) is equivalent to 3.6 mmol ATP g<sub>dw</sub>⁻¹ h⁻¹.

The values for the maintenance energy coefficient m<sub>s</sub> calculated above showed that during roughly 40 days of retentostat cultivation, the amount of substrate used for maintenance increased from approximately 30% at a growth rate of 0.025 h⁻¹ to approximately 100% of the total substrate consumed at the end of the retentostat cultivation (See Fig. 5). The two independent retentostat cultures displayed highly similar growth kinetics and the distribution of energy between maintenance- and growth-related processes above shows that a near-zero growth state is reached during cultivation.

**Discussion**

A setup for culturing *B. subtilis* at extremely low growth rates was implemented and the adaptation of *B. subtilis* to near-zero growth conditions was studied by analysis of biomass accumulation, culture viability, metabolite production and morphology.

The extremely low specific growth rate (0.0006 h⁻¹) reached at the end of the retentostat cultivation shows that cell retention is an effective way of reaching near-zero growth rates, while maintaining a steady supply of nutrients. At this stage, all
substrate consumed by the cells is used for maintenance purposes, which is estimated to be 0.24 mmol glucose g\textsubscript{dw}^{-1} h\textsuperscript{-1}. The m\textsubscript{s} was comparable to that found in studies on \textit{B. subtilis} at higher growth rates (Sauer et al., 1996; Dauner and Sauer, 2001; Tännler et al., 2008). In these chemostat studies the m\textsubscript{s} was found to be ranging from 0.21 to 0.49 mmol glucose g\textsubscript{dw}^{-1} h\textsuperscript{-1}.

![Graph showing percentage of energy directed towards maintenance versus growth](image)

**Figure 4.** Percentage of energy directed towards maintenance versus growth. Black bars represent energy directed towards maintenance. White bars represent energy directed towards growth. (A) Retentostat 1. (B) Retentostat 2.
Both $Y_{sx}^{\text{max}}$ values found for steady-state chemostat (47.57 mg$_{dw}$ mmol glucose$^{-1}$) and zero-growth retentostat cultures (16.74 mg$_{dw}$ mmol glucose$^{-1}$) were lower than values determined in other chemostat studies that employed higher growth rates (between 67 and 82 ± 2 mg$_{dw}$ mmol glucose$^{-1}$) (Sauer et al., 1996; Dauner et al., 2001; Tännler et al., 2008). The observed variability of the $Y_{sx}^{\text{max}}$ value implies that the conversion of substrate to net biomass does not have a constant efficiency in $B.\ subtilis$. Various explanations may underlie these observations, including partial lysis of cells, or any other factor that results in higher biomass formation than is measured, or specific growth-related processes that require elevated substrate consumption to generate the same biomass amount at low growth rates.

$B.\ subtilis$ is a typical soil inhabitant and while many soils provide limited amounts of carbon sources (Aldén et al., 2001; Ekblad and Nordgren, 2002; Ilstedt and Singh, 2005; Demoling et al., 2007), the maintenance metabolism does not seem to be specifically evolved for near-zero growth conditions under carbon limitation, because the maintenance coefficient calculated from retentostat cultivation and from extrapolated chemostat cultures at higher growth rates are virtually identical. Instead, $B.\ subtilis$ employs other strategies for survival under challenging conditions, such as the formation of endospores. The asporogenous sigF mutant used in this study is only able to express genes for sporulation initiation and thereby still allows us to see if sporulation is one of the responses $B.\ subtilis$ applies. Whether these genes are differentially expressed under retentostat conditions needs to be elaborated by transcriptome analysis.

The occurrence of the cell chains and elongation of cells (most prominent in retentostat 2) is likely correlated with the coinciding decline of CFU enumerations. Since the viability remained high and the culture dry weight did not decrease, it is unlikely that the decrease in colony-forming units is caused either by cell death or lysis. An alternative explanation for the observed decrease of the CFU count would be a change in the morphology of the cells, resulting in an increased weight per cell or chains of cells that form a single colony when plated, while actually representing multiple cells. Taking into account the average number of cells per chain enabled the accurate explanation of the reduced CFU numbers, indicating that chain formation is the predominant explanation for the observed CFU decline. The retentostat-adapted cell chains rapidly reverted to the typical $B.\ subtilis$ rod shape when
inoculated in normal batch culture conditions, illustrating that this condition-dependent morphology is a response to retentostat cultivation. Chained cell morphology has been observed previously for *B. subtilis* (Cozy and Kearns, 2010; Kawai et al., 2003; Ohnishi et al., 1999; Salzberg and Helmann, 2008) and causes were reported to be related with cell division, cell separation and membrane composition. Kawai et al. (2003) showed that expression of the YneA protein resulted in cell elongation and reduced localization of the cell division protein FtsZ to the cell division site. YneA was found to be responsible for cell division suppression during the SOS response in *B. subtilis*. Cozy and Kearns (2010) showed that a mutant of *LytABCDF* leads to a phenotype where conjoined daughter cells are not separated after division by binary fission. The *LytABCDF* operon codes for cell wall-remodelling enzymes called autolysins, which facilitate cell separation (Blackman et al., 1998; Chen et al., 2009; Margot et al., 1999; Vollmer et al., 2008). RNA polymerase with the alternative sigma factor $\sigma^D$ directs expression of the autolysin enzymes (Kearns and Losick, 2005). Similar findings are reported by Ohnishi et al. (1999) in a study where *lytF* and *sigD* mutants form filamentous cells. In a study by Salzberg and Helmann (2008) it was found that mutants with altered membrane composition formed aberrant cell morphologies. Cells with inactivation of three genes *mprF*, *pssA* and *ywnE* involved in biosynthesis of complex lipids, were shown to be filamentous. In this case the SigD regulon possibly plays a role too, since its expression was negatively affected by these mutations. The studies mentioned above indicate that a wide variety of causes for the reported aberrant cell morphology exist. Transcriptome analysis of the *B. subtilis* retentostat culture will provide more insight.

As a consequence of cells experiencing caloric restriction in the retentostat cultivation, the specific growth rate decreased over time and the substrate consumption rate for maintenance-associated processes relatively increased. A state of near-zero growth was reached, yet enough energy was available to keep the cells alive and metabolically active. This work shows that retentostat culturing is (apart from some pitfalls mentioned above) a controlled and stable cultivation condition, suitable for the study of extremely slow growing cultures of *B. subtilis*, which allocate the vast majority of substrate derived energy to maintenance. Future studies will now focus on dissecting the adaptation of *B. subtilis* to near-zero growth rates in retentostat cultures by means of transcriptome analysis.
Material and methods

Strain, growth conditions and media

*B. subtilis* 168 trpC2 sigF::spec amyE::P
rrnB-gfp+ was used for the retentostat experiments in this study. This strain carries a GFP fusion to the promoter of the constitutively expressed ribosomal RNA operon *rrnB* (Krásný and Gourse, 2004; Veening et al., 2009), and is defective in sporulation, caused by a disruption in the *sigF* gene. Precultures for chemostat and retentostat cultivations were prepared by inoculating a single colony from an lysogeny broth (LB) agar plate into 10 ml LB medium (Sambrook et al., 1989). This culture was grown at 37°C until an optical density at 600 nm (OD600) of 0.3 was reached. Subsequently 1000x dilutions were made in 60 ml M9 medium (Miller, 1972) supplemented with 27.75mM glucose and 0.1mM Tryptophan. The M9 minimal medium contained, per liter of deionized water, 8.5 g of Na2HPO4 · 2H2O, 3.0 g of KH2PO4, 1 g of NH4Cl, and 0.5 g of NaCl. The following components were sterilized separately and added per liter: 1 ml of 0.1 M CaCl2, 1 ml of 1 M MgSO4, 1 ml of 50 mM FeCl3, and 10 ml of M9 trace salts solution. The M9 trace salts solution contained (per liter) 0.1 g MnCl2 · 4H2O, 0.17 g of ZnCl2, 0.043 CuCl2 · 2H2O, 0.06 CoCl2 · 6H2O, 0.06 Na2MoO4 · 2H2O. The cultures were grown overnight and used for inoculation of the bioreactors. Chemostat- and retentostat media were acidified to pH 5 by addition of H2SO4 (95 to 97%) to avoid precipitation of medium components. During the cultivation in the bioreactors the pH was maintained at 7.0 by automatic addition of NaOH 5M.

Chemostat cultivation

Duplicate chemostat cultures were performed at a dilution rate, D (defined as the ratio of the medium feed rate (L h⁻¹) and culture volume (L)) of 0.025 h⁻¹. 2.0 L bioreactors (Infors Benelux BV, the Netherlands) with 1.4 L working volume were inoculated with an exponentially growing preculture to start the chemostats. The bioreactors were operated at 37°C under aerobic conditions. An airflow of 0.1 l.min⁻¹ and a stirring speed of 800 r.p.m. was set to keep oxygen levels above 50% of air-saturation.

The working volume was kept constant by means of a conductivity sensor placed at the surface of the culture, activating a peristaltic pump that removed effluent. To
Bacillus subtilis physiology at near-zero growth rates

prevent foam formation, 5 ml of a 5% (wt.wt⁻¹) solution of the antifoaming agent Struktol J673 (Schill and Seilacher AG, Hamburg, Germany) was added per 24 hours, automatically spread over intervals of 13 minutes. Steady state was defined as the condition in which culture parameters were constant for at least 5 volume changes and when optical density at 600 nm (OD₆₀₀) and cell dry weight (CDW) had remained constant (<5% and <10% variation, respectively) for at least two volume changes. Culture purity was routinely checked by phase-contrast- and fluorescence microscopy. The PrmB-GFP fusion allowed for identification of fluorescent cells as being the inoculated B. subtilis. Additionally, cells were plated on LB agar plates to check for possible contaminations.

Retentostat cultivation

A 2.0 L bioreactor (Infors Benelux BV, the Netherlands) was equipped with an autoclavable polyethersulfone cross-flow filter with a pore size of 0.22 μm (Spectrum Laboratories, CA, USA) to retain biomass in the reactor. The filter was connected to the bioreactor via an external loop, through which culture was circulated.

Two individual retentostat experiments were initiated from chemostat cultures at dilution rates of 0.025 h⁻¹. After reaching steady state in the chemostat, the bioreactors were switched to retentstat mode by withdrawing the effluent through the filter instead of through the standard effluent tube. The retentostat cultivations were operated under the same conditions (temperature, pH, medium flow rate, oxygenation, stirring rate, anti-foam addition) as the chemostats. Since withdrawal of biomass from the culture influences the kinetics of biomass accumulation, sampling volumes and -frequency were kept to a minimum. The super safe sampler ports (Infors Benelux BV, the Netherlands) that were used for fast and aseptic sample withdrawal, allowed for accurate control of the sample volume.

Determination of biomass, substrate and metabolites

During chemostat- and retentostat cultivation, samples were withdrawn from the bioreactor to determine biomass-, glucose- and organic acid concentrations. Cell dry weight was determined in duplicate by cooled centrifugation of 5 mL of culture in pre-weighted tubes, washing with 0.9% NaCl and drying at 105°C for 24 h to constant weight. Additionally, the optical density of the culture was determined by measuring the optical density at 600 nm. Glucose and organic acid concentrations in
culture supernatants were determined by high-performance liquid chromatography (Shimadzu Scientific Instruments, MD, USA) using LC Solutions SP1 software from Shimadzu (Kyoto, Japan). Culture supernatants were obtained by centrifugation (10,000g for 10 min at 4°C), filter sterilized and stored at -20°C until HPLC analysis. Samples were separated using an Aminex HPX-87H anion-exchange column (Bio-rad Laboratories Inc., Richmond, CA) with sulphuric acid (5 mM; 0.6 ml · min⁻¹) as mobile phase at 55°C. Detection was performed with a refractive index detector and UV wavelength absorbance detector (Shimadzu Scientific Instruments, MD, USA).

**Assessment of cell viability**

To estimate the fraction of dead cells in culture samples, the red fluorescent compound propidium iodide (PI) was used. PI binds deoxyribonucleic acid (DNA) and can only do so in cells with permeabilized membranes. A 10 µL culture sample was washed and resuspended in 990 µL 0.85% NaCl and 1 µL of PI (20 mM dissolved in dimethyl sulfoxide (DMSO)) was added and incubated for 10 min. The PrmB-GFP fusion this strain carries causes metabolically active cells to produce GFP and these could thus be detected as alive. Enumeration of live and dead cell fractions was performed using these PI and GFP markers, in a Deltavision (Applied Precision) IX7 1DV Microscope (Olympus) which is described in more detail below. Additionally, colony forming unit (CFU) quantification was performed by plating tenfold dilution series of the cultures (5 to 7 dilutions in LB broth) in triplicate on LB agar (1.5% wt/vol) plates. After 24 hours of incubation at 37°C, colonies were counted.

**Microscopy and analysis of cell morphology**

Cell morphology was analysed by phase-contrast- and fluorescence microscopy. In order to visualize the cell membrane, cells were incubated for 1 minute with an ice-cold 5 µg/mL FM5-95 membrane dye solution (Invitrogen, UK) prior to microscopy analysis. Images were taken with Deltavision (Applied Precision) IX71Microscope (Olympus) using a CoolSNAP HQ2 camera (Princeton Instruments) with a 100× phase-contrast objective. Fluorescence filter sets used to visualize GFP (excitation at 450/90 nm; emission at 500/50 nm) and red dyes (excitation at 572/35 nm, emission 632/60 nm) were from Chroma Technology Corporation (Bellows Falls, USA). Exposure time was between 0.2 and 1 s with 32% transmission xenon light (300 W).
Exposure time for phase-contrast images was 0.05 s. Softworx 3.6.0 (Applied Precision) software was used for image capturing. Time-lapse microscopy was performed as described before (de Jong et al., 2011).

**Calculation of retentostat growth kinetics**

The following mass balance equations are used to calculate growth kinetics in retentostat cultivations. Equation 1 is for biomass, assuming complete cell retention and constant volume. Equation 2 is for substrate. Here, $C_x$ is the biomass concentration (g · liter$^{-1}$), $\mu$ is the specific growth rate (h$^{-1}$), $C_s$ is the residual glucose concentration (g · liter$^{-1}$), $D$ is the dilution rate (h$^{-1}$), $C_{s,in}$ is the glucose concentration in the feed (g · liter$^{-1}$), and $q_s$ is the specific glucose consumption rate (g · g$^{-1}$ · h$^{-1}$).

\[
\frac{dC_x}{dt} = \mu \cdot C_x \tag{1}
\]

\[
\frac{dC_s}{dt} = D \left( C_{s,in} - C_s \right) - q_s \cdot C_x \tag{2}
\]

With the assumptions that variation of the glucose concentration in the bioreactor is negligible in comparison to the amount of glucose supplied with fresh medium ($dC_s/dt << D \cdot C_{s,in}$) and that the residual glucose concentration in the broth (consistently below the detection limit) is much smaller than the glucose concentration in the medium, $q_s$ can be calculated with equation 3.

\[
q_s = \frac{D \cdot C_{s,in}}{C_x} \tag{3}
\]

The Herbert-Pirt (Pirt, 1982) equation (equation 4) describes the relation between specific substrate consumption rate ($q_s$), specific growth rate ($\mu$), maximum biomass yield on substrate ($Y_{sx}^{max}$) and maintenance energy coefficient ($m_s$).

\[
q_s = \frac{\mu}{Y_{sx}^{max}} + m_s \tag{4}
\]
Equation 4 was used to determine $m_s$ and $Y_{sx}^{\text{max}}$. This was done by plotting the values of $q_s$ against $\mu$. The intercept of a linear regression line with the y-axis determines $m_s$. $Y_{sx}^{\text{max}}$ was calculated by taking the slope$^{-1}$ of the regression line.

The biomass accumulation during retentostat cultivation can be described by the van Verseveld equation (van Verseveld et al., 1986) (equation 5).

\[
C_x(t) = \left(C_{x,0} - \frac{D(C_{s,\text{in}}-C_s)}{m_s}\right) \cdot e^{-m_s \cdot Y_{sx}^{\text{max}} \cdot t} + \frac{D(C_{s,\text{in}}-C_s)}{m_s} \tag{5}
\]

This equation assumes an ideal situation with no loss of viability and growth rate independent maintenance-energy requirements.

The specific growth rate of the retentostat cultivations is calculated with equation 6:

\[
\mu = \frac{dC_{x,\text{total}}/dt}{C_{x,\text{viable}}} \tag{6}
\]

In order to determine the derivative of the biomass accumulation data ($dC_{x,\text{total}}/dt$), the measured total biomass concentrations (viable and non-viable cells) were fitted with the equation $C_x = A \cdot e^{B \cdot t} + C$, which is of the same shape as equation 5. This was done using GraphPad Prism 6 (GraphPad Software Inc., USA), minimizing the sum of squares of errors by varying $A$, $B$ and $C$. With $A$, $B$ and $C$ known, the derivative ($dC_{x,\text{total}}/dt$) could be determined. Because only viable biomass can replicate, this is incorporated in the equation. The doubling time of the culture is calculated by dividing the natural logarithm of 2 by the specific growth rate.

**Acknowledgements**

We thank Bert van der Bunt, Marjo Starrenburg and Erik de Hulster for valuable help with the bioreactors; Mark Bisschops for valuable help with calculations; Agata Pudlik and Tim Vos for valuable help with HPLC analysis; members of the joint zero-growth project group (Kluyver Centre, the Netherlands) for support and valuable discussions; and Sietse Koenders for the illustration in Figure 1.
This work was carried out within the research programme of the Kluyver Centre for Genomics of Industrial Fermentation which is part of the Netherlands Genomics Initiative / Netherlands Organization for Scientific Research.

References


