Ploidy of Bacillus subtilis, Bacillus megaterium, and three new isolates of Bacillus and Paenibacillus

Benjamin Böttinger, Karolin Zerulla, Jörg Soppa

Bacteria were long assumed to be monoploid, maintaining one copy of a circular chromosome. In recent years it became obvious that the majority of species in several phylogenetic groups of prokaryotes are oligoploid or polyploid, e.g. in halophilic and methanogenic archaea, proteobacteria, and cyanobacteria. The present study aimed at investigating the distribution of ploidy in an additional group of prokaryotes, i.e. in the gram-positive genus Bacillus. First, the numbers of origins and termini of the two laboratory strains Bacillus subtilis and Bacillus megaterium were quantified using an optimized real time PCR approach. B. subtilis was found to be mero-oligoploid in exponential phase with, on average, 5.9 origins and 1.2 termini. In stationary phase the average numbers of origins per cell was considerably smaller. B. megaterium was found to be polyploid in exponential phase with about 12 copies of the origin and terminus. Again, the ploidy level was down-regulated in stationary phase. To verify that oligo-/polyploidy is not confined to strains with a long history of growth in the laboratory, three strains were newly isolated from soil, which were found to belong to the genera of Bacillus and Paenibacillus. All three strains were found to be oligoploid with a growth-phase dependent down-regulation of the ploidy level in stationary phase. Taken together, these results indicate that oligo-/polyploidy might be more widespread in Bacillus and related genera than assumed until now and that monoploidy is not typical.

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Ploidy of *Bacillus subtilis*, *Bacillus megaterium*, and three new isolates of *Bacillus* and *Paenibacillus*

running title: Quantification of ploidy in *Bacillus*

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1 **Introduction:**

2 Polyploidy, the presence of multiple copies of the genome, is common in eukaryotes like
3 ciliates, fish, amphibians and plants. The advantages and disadvantages of polyploidy have
4 been discussed in several reviews (Comai, 2005; Neiman, Kay & Krist, 2013), most often
5 polyploidy is viewed as a positive trait that can provide fitness advantages (te Beest *et al*.,
6 2012).

7 In contrast to eukaryotes, prokaryotes have long been assumed to be monoploid and contain
8 one copy of a circular chromosome. This assumption originated from the best studied gram-
9 negative bacterium, *Escherichia coli,* which contains one copy of the chromosome when its
10 generation time is longer than the time required for chromosomal replication and segregation.
11 However, under optimal laboratory conditions the generation time of *E. coli* is smaller than
12 the replication time, and a new round of replication is initiated before termination of the
13 previous round. The number of replication origins per cell is then larger than the number of
14 termini and the cell becomes mero-oligoploid (Bremer & Dennis, 1996; Pecoraro *et al*.,
15 2011). Therefore, *E. coli* is not a true monoploid species. Several monoploid prokaryotic
16 species exist, which contain one copy of the chromosome irrespective of the growth rate, e.g.
17 *Caulobacter crescentus* or *Wolinella succinogenes* (Pecoraro *et al*., 2011).

18 However, many prokaryotic species have been shown to be oligoploid or polyploid. For
19 example, the extreme radiation-resistant bacterium *Deinococcus radiodurans* contains about 8
20 copies of the chromosome (Hansen, 1978; Bentchikou *et al*., 2010). It is extremely resistant to
21 DNA-shattering treatments such as ionizing radiation or desiccation and can regenerate a
22 functional genome from hundreds of chromosomal fragments. Another bacterium, *Thermus*
23 *thermophiles HB8*, which is closely related to *D. radiodurans*, contains 4-5 copies of the
24 chromosome and the oligoploidy has been discussed to be important for genome maintenance
25 and repair at elevated growth temperatures (Ohtani, Tomita & Itaya, 2010). An extreme
26 example is a symbiont of surgeonfish; *Epulopiscium spp*. The cells can reach lengths in

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excess of 600 µm and they contain thousands of genome copies. The copy number is positively correlated with cell volume and it has been argued that cells of this size could not be monoploid due to diffusion limitations (Mendell *et al*., 2008; Bresler et al., 1998).

In recent years several groups of prokaryotes have been investigated and it was found that the fraction of oligo-/polyploid species is high in halophilic archaea, methanogenic archaea, cyanobacteria, and proteobacteria (Soppa, 2011; Hildenbrand *et al*., 2011; Griese, Lange & Soppa., 2011; Pecoraro *et al*., 2011). In contrast to these groups, information about the ploidy distribution in gram-positive bacteria has been sparse. Genome copy numbers have been experimentally determined for *Lactococcus lactis* and for *Bacillus subtilis*. *L. lactis* was found to have two copies of the chromosome when it is grown very slowly, which were replicated into four chromosomes during the C period of the cell cycle. Therefore, this species is diploid without overlapping chromosomal replication cycles (Michelsen *et al*., 2010). For *B. subtilis* several experimental approaches have shown that the ploidy level depends on the growth rate and that it is monoploid during very slow growth and mero-oligoploid during fast growth, similar to *Escherichia coli*. For example, tagging the replication origin with the green fluorescent protein yielded one fluorescent spot before and two spots after replication during slow growth, but two or four spots during fast growth (Webb *et al*., 1998). Quantifying the number of replication origins per cell by blocking initiation, incubation to allow run-off of replication, and subsequent analysis of the DNA content of individual cells by flow cytometry revealed that the cells had two origins and four origins (Kadoya *et al*., 2002) or four origins and eight origins (Moriya *et al*., 2009). Quantification of the average genome content by fluorescence microscopy and by a chemical method revealed that cells growing with a doubling time of 73 minutes contained about 1.5 genomes, while cells growing with a doubling time of 30 minutes contained 3.2 genomes (Sharpe *et al*., 1998).

These results motivated us to choose *B. subtilis* as a first gram-positive species for the quantification of the copy numbers of origins and termini using an additional method, i.e. a

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real time PCR method that enables to quantify different parts of the chromosome or different replicons simultaneously. The method was used in recent years to analyze the ploidy of various archaea and bacteria (Breuert *et al*., 2006; Hildenbrand *et al*., 2011; Pecoraro *et al*., 2011; Griese, Lange & Soppa, 2011; Zerulla *et al*., 2014). *B. megaterium* was chosen as a second species because it has been reported that spores of *B. megaterium* contain two genomes, in contrast to those of *B. subtilis* (Hauser & Karamata, 1992), and of its large cell size. These two species have been cultivated in the laboratory for decades under optimal conditions, what might have changed the ploidy level. Therefore, three new spore-forming aerobic strains were freshly isolated from soil, and their ploidy levels were also determined.

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Materials and Methods

Bacterial species, media and growth conditions

Bacillus subtilis 168 (DSM strain No. 23778) was obtained from Karl-Dieter Entian (Goethe University, Frankfurt, Germany). It was grown in a medium recommended by the German Culture Collection (DSMZ; www.dsmz.de; medium No. 1), with 0.5% (w/v) peptone, 0.3% (w/v) meat extract and 0.5% (w/v) NaCl. 30 ml cultures were grown in 100 ml Erlenmeyer flasks at 37°C with a rotating frequency of 200 rpm.

Bacillus megaterium DSM32 was obtained from the German Culture Collection (DSMZ; www.dsmz.de; strain No. 32). It was grown in a medium recommended by the DSMZ (www.dsmz.de; medium No. 1) as described above. 30 ml cultures were grown in 100 ml Erlenmeyer flasks at 37°C with a rotating frequency of 200 rpm.

Isolation and characterization of aerobic spore-forming bacteria

For the isolation of new strains a soil sample was taken near the Biocentre of the Goethe-University of Frankfurt. 1 cm^3 of soil was transferred to a 15 ml falcon tube and thoroughly mixed with 10 ml of sterile water. 1 ml of the suspension was transferred to a 1.5 ml Eppendorf cup and was heated for 10 min to 80°C to kill all vegetative cells. Serial dilutions in sterile water were prepared and were plated on LB-Miller agar plates (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, and 1.2% (w/v) agar), and were incubated at 37°C overnight. Several colonies were re-streaked to guarantee that colonies represent pure clones. Individual colonies were used to inoculate liquid LB-Miller medium and were analyzed microscopically. Three clones were chosen arbitrarily that seemed to represent different species based on colony and cell morphology.

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1 For sequencing part of the 16S rRNA gene 1 ml aliquots of the cultures were removed, cells
2 were harvested by centrifugation, and resuspended in 1 ml lysis buffer (10 mM Tris/HCl pH
3 7.2, 1 mM EDTA, 10 mg/ml lysozyme). They were incubated for 30 minutes at 37°C and 1.15
4 g silica beads A3B (Analytik Jena, Germany) were added. Cells were lysed by shaking three
5 times for 40 seconds in a FastPraep (MP Biomedicals, Solon, USA). The beads and cell debris
6 were removed by centrifugation, and aliquots of the supernatants were used as template in
7 PCR reactions to amplify part of the respective 16S rRNA genes using the primers "16S1kin"
8 and "16S2kin" (Table S1). The resulting PCR fragments were sequenced from both ends
9 using the above mentioned primers, and the sequences were combined using Clone Manager
10 (Scientific and Educational, Cary, USA).
11 A multiple sequence alignment of the three new sequences and the sequences of selected
12 species of different genera of gram-positive bacteria was generated using ClustalOmega
13 (compare results) at the website of the European Bioinformatics Institute (Sievers and
14 Higgins, 2014; EBI; www.ebi.ac.uk/Tools/msa/clustalo/). The program "ClustalW2
15 phylogeny" was used to generate a neighbor joining tree using the ClustalOmega output as
16 input (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny/). The tree was visualized
17 using the program "TreeView" (Page, 1996;
18 http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

19

20 **Sequencing part of a single copy gene of the new isolates**

21 The real time PCR method for ploidy determination requires the presence of sequence
22 information. Of course no sequence information apart from the 16S rRNA sequences were
23 available. The 16S sequence could not be used because the copy numbers of the ribosomal
24 RNA operons of the isolates were unknown, and many species contain more than one copy.
25 Therefore, the aim was to generate sequence information of a single copy protein-encoding

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gene. The *sigL* gene encoding the sigma factor 54 was chosen, and *sigL* sequences of ten species of the genus *Bacillus* and two species of the genus *Paenibacillus* were retrieved from the database and a multiple sequence alignment was generated using ClustalW. Two highly conserved regions were chosen and degenerated oligonucleotides were designed (sequences see Table S1). The oligonucleotides were used for amplification and sequencing of a *sigL* fragment of about 1 kbp using standard PCRs with the respective genomic DNAs of the three isolates. Based on the sequences of the three *sigL* genes oligonucleotides were designed for the amplification of standard fragments and analysis fragments for the three new isolates (Table S1). Quantification of the ploidy levels was performed as described below.

Growth curves and quantification of cell densities

For the generation of growth curves cultures were grown in 30 ml medium in 100 ml Klett flasks (37°C, 200 rpm). Growth was recorded using a Klett Colorimeter. In each case, three biological replicates were performed. Average values of the optical densities and their standard deviations were calculated. The doubling time was determined by fitting a straight line to the half-logarithmic representation of the optical densities in exponential phase. All growth curves are shown in the Supplementary Material.

For the quantification of genome copy numbers cell densities were determined microscopically using a Neubauer counting chamber.

Preparation of cell extracts

Aliquots of 1×10^8 to 5×10^8 cells were withdrawn from cultures in exponential or stationary phase, and cells were harvested by centrifugation (5 min 13000 rpm) and resuspended in 190

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µl LPT buffer (1.2% (v/v) Triton-X-100, 20 mg/ml Lysozym, 2 mM EDTA and 20 mM Tris/HCl, pH 8.0). For isolate 2 additionally 50 U (exponentially growing cells) or 100 U (stationary phase cells) Mutanolysin (M9901, Sigma-Aldrich) were added to the LPT buffer. The cells were incubated for 30 min at 37°C and subsequently 10 µl Proteinase K (20 mg/ml; Applichem) and 300 µl Lysisbuffer AL (Qiagen) were added to the suspension followed by a second incubation for 20 min at 65°C and a terminal heat-inactivation at 96°C for 5 min. More than 98% of the cells had been lysed, which was verified by determination of the cell densities with a Neubauer counting chamber. Cell debris was pelleted by centrifugation (10 min 13000 rpm) and the integrity of genomic DNA was verified by analytical agarose gel electrophoresis. Aliquots of the cell extract were dialyzed on membrane tubes (Medicell International Ltd; MWCO 12-14 KDaltons) against distilled water. Serial dilutions were generated and 5 µl aliquots were included as template in real time PCR analyses for quantification of genome copy numbers (see below).

Quantification of ploidy levels using a real time PCR method

To determine genome copy numbers, a real time PCR approach was applied (Breuert *et al.*, 2006). At first, fragments of ~1 kbp were amplified using standard PCRs with genomic DNA of *B. subtilis*, *B. megaterium* and the three isolates I1, I2 and I3 as templates. The sequences of the oligonucleotides are included in Table S1. The amplified genomic regions are summarized in Table S2. The PCR fragments were purified by using preparative agarose gel electrophoresis and an AxyPrepDNA gel extraction kit (Axygen Biosciences). DNA concentrations were determined photometrically using a Nanodrop photometer (ND-1000; Nanodrop Tech., Rockland, USA). The numbers of DNA molecules per volume were calculated using the molecular weights of the PCR fragments computed with “oligo calc” (www.basic.northwestern.edu/biotools) and the Avogadro number.

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For each standard fragment, a dilution series was generated and used for real time PCR analysis in parallel with dilution series of the respective cell extract. The "analysis fragments" were about 200-300 bp and exact sizes and genomic localizations (when possible) are summarized in Table S2. The real time PCR analyses were performed as previously described (Breuert *et al*., 2006). By comparison of the threshold cycle (C_T) differences of the different dilutions it was verified that the PCR was exponential at least up to the threshold DNA concentration used for the analysis (i.e., a 10-fold dilution corresponds to a C_T difference of ~3.32). In addition, a no template control was included to ensure that product formation was based on the added template DNA in standard curve and the dilutions of cytoplasmic extracts. Furthermore, correct product formation and absence of byproducts was monitored by the generation of melting curves and checking the products by analytical gel electrophoresis.

A standard curve was generated and used to calculate the genome copy numbers present in the dilutions of the cell extract. In each case three biological replicates were performed. For every biological replicate four dilutions of the cytoplasmic extracts were analyzed in duplicates, therefore the calculated average ploidy levels rest on 24 technical replicates. In combination with the cell densities of the three biological replicates, the numbers of genome copies per cell were calculated.

Results:

Ploidy of *Bacillus subtilis*

B. subtilis has been isolated more than 100 years ago (Perdrix, 1907) and has been cultivated in the laboratory ever since. The strain *B. subtilis* 168 (DSM strain No. 23778) is a widely used strain. The cells were cultivated in the medium recommended by the German Culture Collection (medium No. 1), resulting in fast-growing cultures with a doubling time of 24 minutes. The respective average growth curve of three independent cultures is shown in Supplementary Material Figure S1. For this as well as for all other species of this study the method of cell lysis was optimized to fulfill the following three criteria: 1) more than 95% of all cells were lysed, 2) the genomic DNA remained mainly intact and no fragments smaller than 20 kbp were visible in analytical agarose gels, and 3) the resulting cell extract did not inhibit exponential amplification during real time PCR, which was verified by a ΔC_t value of about 3.32 of serial tenfold dilutions.

The copy numbers of two genomic regions were quantified, which represent the intracellular concentration of the replication origin and the terminus, respectively. The results of three independent cultures in exponential and stationary phase are summarized in Table 1. As expected, in exponentially growing cells the average number of origins (5.9±0.6) was found to be considerably higher as the number of termini (1.2±0.2). The average value of 5.9 indicates that most cells contain 4 or 8 origins, respectively, in congruence with one earlier report (Moriya *et al.*, 2009). The small average number of termini indicates that *B. subtilis* divides soon after replication is complete.

In stationary phase cells the average number of origins per cell was found to be considerably smaller (2.8±0.7) as in exponentially growing cultures, whereas the number of termini per cell (1.3±0.4) is nearly the same in exponential and stationary phase. However, as a culture in late stationary phase (Figure S1) was analyzed, it had been expected that replication had long

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ceased and the average number of origins per cell would have been closer to one and identical to the number of termini.

Taken together, exponential cells contain on average five times more origins then termini due to the intertwined rounds of replication, while stationary phase cells have only twice as many origins as termini. These results showed that *B. subtilis* is mero-oligoploid during fast growth.

Ploidy of *Bacillus megaterium*

The next species analyzed was *Bacillus megaterium*, another widely used species of the genus *Bacillus* (2140 publications with *Bacillus megaterium* in title or abstract in PubMed), which has been isolated more than 60 years ago (Buchanan, Breed & Johnbrool, 1951). *B. megaterium* can be found in many diverse habitats (Vary *et al*., 2007). It was grown in the medium recommended by the German Culture Collection with a doubling time of 27 minutes (growth curve: Figure S2). The levels of the origin region and the terminus region were analyzed separately, as for *B. subtilis*. The results are summarized in Table 2. *B. megaterium* turned out to be polyploid, with average values of about 13 genome copies per cell in exponential growth phase and 6 genome copies per cell in stationary phase. Thus, *B. megaterium* is polyploid in exponential growth phase and oligoploid in stationary phase. Remarkably, the presumed origin and the presumed terminus region had an identical copy number in fast growing cells, which seems to be highly unlikely for a genome with a single replication origin. Possible explanations include 1) that the real origin and real terminus are not at the presumed localizations, but elsewhere, and 2) that the genome of *B. megaterium* has several replication origins, like several archaeal species (Norais *et al*., 2007; Hawkins *et al*., 2013). In stationary phase cells the number of presumed termini is higher than that of the presumed origin, which is also unexpected. Although the variance in absolute numbers were higher for the three stationary phase *B. megaterium* cultures than for any other species and

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1 condition in this study, this is true for all three cultures, indicating that this effect is real, again
2 indicating that the annotation of replication origin and terminus might not be correct.

3 Taken together, *B. megaterium* was shown to be polyploid in exponential growth phase and
4 oligoploid in stationary phase, and the results include unexpected findings that should induce
5 further investigations. To our knowledge these results represent the first direct quantifications
6 of the presumed origins and terminus regions of the *B. megaterium* chromosome.

7

8 **Isolation and characterization of three new species of aerobic spore-forming bacteria**

9 Both *B. subtilis* and *B. megaterium* have been cultured in the laboratory for decades under
10 optimal conditions, and that might have led to mutations that influence the genome copy
11 number. Therefore, we aimed at quantification of the ploidy levels of several freshly isolated
12 species of *Bacillus* or related genera. The isolation of aerobic spore-forming bacteria from soil
13 is straightforward, a soil sample is suspended in sterile water and heated to 80°C to
14 simultaneously kill all vegetative cells and induce germination of spores. After isolation of
15 pure clones and an initial morphological analysis of colonies and cells, three examples, most
16 probably representing three different species, were chosen arbitrarily and further
17 characterized. Table 3 summarizes some characteristics of the three new isolates. A large part
18 of the 16S rRNA gene of the three isolates was amplified and sequenced. A multiple sequence
19 alignment of the partial sequences of the three isolates and 17 sequences of 16S rRNA genes
20 from species of four genera of Firmicutes was constructed and used to generate a
21 phylogenetic tree (Figure 1). All three isolates could be clearly classified, i.e. isolate I1 was
22 closely related to *B. simplex* and *B. infernus*, isolate I2 was most closely related to *B. cereus*
23 and *B. anthracis*, and isolate I3 was closely related to *P. lautus* and *P. polymyxa*. Thus the
24 new isolates represent two diverse positions within the genus *Bacillus* and the genus

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Paenibacillus and are excellently suited to analyze the ploidy levels of gram-positive spore formers freshly taken from soil.

Ploidy levels of the three new isolates

Obviously the genome sequences of the three new isolates are unknown. However, for the application of the real time PCR method for ploidy quantification sequence information is a prerequisite. Therefore, a large part of the single copy gene *sigL*, which encodes the sigma factor 54, was amplified and sequenced for all three isolates (see Material and Methods). These sequences enabled quantification of the copy numbers of the chromosomes of the three isolates, but of course the localizations of the respective *sigL* genes with respect to the replication origins are unknown.

For each isolate three independent cultures were grown in LB-Miller medium. They had doubling times of 26 minutes (isolate I1, growth curve: Figure S3), 24 minutes (isolate I2, growth curve: Figure S4) and 48 minutes (isolate I3, growth curve: Figure S5). The genome copy numbers were quantified for exponentially growing and stationary phase cultures, and the results are summarized in Table 4. Isolate I1 had average genome copy numbers of 4.7 (±1.1) during exponential phase and 2.3 (±0.4) during stationary phase. Isolate I1 is thus oligoploid during exponential phase and diploid during stationary phase. The genome copy number of isolate I2 was also found to be growth phase-regulated, the average genome copy numbers were 6.4 (±1.4) during exponential phase and 2.4 (±0.3) during stationary phase. Thus the values are very similar although the two isolates are only distantly related within the genus *Bacillus*. The average values of the genome copy numbers of isolate I3 were 3.4 (±0.5) during exponential phase and 2.5 (±0.5) during stationary phase. Taken together, all three new

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1 isolates of the genera *Bacillus* and *Paenibacillus* turned out to be oligoploid during
2 exponential phase and diploid at stationary phase.

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Discussion:

Ploidy in *Bacillus subtilis* and *Bacillus megaterium*

In several studies the DNA content of *Bacillus subtilis* cells was investigated by fluorescence microscopy and flow cytometry (Webb *et al*., 1998; Sharpe *et al*., 1998; Kadoya *et al*., 2002; Moriya *et al*., 2009). It could be shown that the DNA content of the cells as well as replication correlate with the growth-rate. In fast-growing cells (generation time ≤ 60 min), the time required for replication, DNA segregation, and cell division is longer than the generation time, and accordingly the cells are mero-oligoploid and contain more origins than termini. In contrast, when the doubling time is longer than the time required for replication, segregation, and cell division, *B. subtilis* is monoploid (Webb *et al*., 1998; Sharpe *et al*., 1998). All these studies were done by fluorescence microscopy or flow cytometry. Therefore, it should be noted that these previous analyses did not quantify specific sites of the chromosome directly, but the bulk DNA was quantified. If the study included an inhibition of replication initiation, the amount of DNA quantified after run-off of replication was taken as being informative about the number of origins at the start of the experiments. The number of termini remained unknown in these studies. Therefore, to our knowledge our analyses are the first direct quantifications of origin and terminus regions of the *B. subtilis* chromosome and yielded experimental evidence not available before. In accordance with previous studies, the real time PCR analyses revealed that fast growing cells of *B. subtilis* are mero-oligoploid, and, in addition, that the cells become monoploid when they enter the stationary phase.

The ploidy regulation of *B. subtilis* is the same as that of *E. coli*. Also *E. coli* cells are mero-oligoploid during fast growth and monoploid during slow growth, and the numbers or origins and termini during fast growth are very similar (Pecoraro *et al*., 2011).

A previous study showed that *B. subtilis* spores are invariably monogenomic. Interestingly, spores of larger bacilli, e.g. *Bacillus megaterium*, *Bacillus cereus* and *Bacillus thuringiensis,*

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1 typically contain two genomes (Hauser & Karamata, 1992). To unravel whether the higher
2 ploidy level of the spores is reflected in a higher ploidy level of vegetative cells, it was chosen
3 to determine the number of origins and termini in *B. megaterium*. It was indeed found that fast
4 growing cells of *B. megaterium* are polyploid and contain 12 copies of the chromosome.
5 Unexpectedly, the numbers or origins and termini were identical, while a higher number of
6 origins had been expected at a doubling time of 27 minutes. Possible explanations for this
7 observation might be 1) that the origin and terminus regions are not at the presumed
8 localizations (the *dnaA* gene for the origin and the opposite site of the chromosome for the
9 terminus), but elsewhere on the genome, 2) that *B. megaterium* possesses several replication
10 origins, like some archaeal species (Norais *et al*., 2007; Hawkins *et al*., 2013), or 3) that the
11 DNA polymerases are much faster than in other species. Also the unexpected finding that in
12 stationary phase cells the number or origins is smaller than the number of termini indicates
13 that replication of the *B. megaterium* genome is not fully understood and should be further
14 investigated.
15 Polyploidy in bacteria is not a seldom exception, but it is widespread in various phylogenetic
16 groups. Examples include the gram-negative bacterium *Pseudomonas putida*, which contains
17 on average about 20 origins and 14 termini during exponential phase (Pecoraro *et al*., 2011),
18 two halophilic archaea with about 30 copies of the chromosome (Breuert *et al*., 2006), the
19 cyanobacterium *Synechocystis* sp. strain PCC 6803 with more than 40 genome copies (Griese
20 *et al*., 2011), the methanogenic archaeon *Methanococcus maripaludis* with about 55 genome
21 copies during exponential phase (Hildenbrand *et al*., 2011), the symbiont *Buchnera* sp. with
22 120 genome copies (Komaki & Ishikawa, 2000), and the giant bacterium *Epulopiscium* sp.
23 with many thousand genome copies (Mendell *et al*., 2008). Various possible evolutionary
24 advantages of polyploidy for prokaryotes have recently been discussed (Soppa, 2013). They
25 include obvious advantages like a low mutation rate or high resistance against radiation and
26 desiccation, but also the usage of genomic DNA as a phosphate storage polymer, in addition

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1 to its many genetic roles in heredity, DNA repair, DNA exchange etc. (Zerulla *et al*., 2014).
2 All these traits might potentially allow for ecological niche expansion or increased flexibility
3 in the organism´s responsiveness to environmental changes (Madlung, 2013).
4
5 **Ploidy in new isolates of the genera *Bacillus* and *Paenibacillus***
6 *B. subtilis* and *B. megaterium* have both been isolated decades ago and have been cultivated
7 under optimal conditions in the laboratory since then. Therefore, due to the absence of natural
8 selection the possibility exists that mutations had occurred and accumulated, including
9 mutations that might affect the ploidy level. Two species exemplify that such an "evolution in
10 the laboratory" can indeed occur. The genomes of two laboratory strains of the haloarchaeon
11 *Halobacterium salinarum* have been sequenced (Pfeiffer *et al*., 2008a; Pfeiffer *et al*., 2008b).
12 Both strains originate from the same natural isolate, that was isolated from salted fish about
13 90 years ago. The chromosomes were found to be still nearly identical and to contain "only"
14 12 differences, including point mutations, frame-shift mutations, and insertions and deletions.
15 In contrast, the plasmids differed considerably. 350 kbp of DNA were nearly identical in
16 sequence, but distributed on two plasmids in one strain and on four plasmids on the other
17 strain. The plasmids of both strains contained additional 215 kbp of sequences that were not
18 present in the other strain, respectively. The second example are laboratory strains of the
19 cyanobacterium *Synechocystic* sp. strain PCC6803, which all originate from one clone
20 isolated from a freshwater lake more than 40 years ago (Stanier *et al*., 1971). Strain-specific
21 phenotypic differences like absence or presence of motility or glucose resistance are well
22 known, and recently parallel whole-genome resequencing of several strains revealed strain-
23 specific sequence differences (Kanesaki *et al*., 2012).
24 Therefore, we decided to quantify the ploidy levels of three fresh isolates in addition to the
25 two long-studied laboratory species. All three species could be clearly classified and represent
26 two diverse positions within the genus *Bacillus* and one position in the genus *Paenibacillus*.

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Of course, the genome sequences of the new isolates are unknown. Therefore, to gain sequence information required for the real time PCR approach a large part of the *sigL* gene was amplified and sequenced for all three isolates. The gene *sigL* encodes the sigma factor 54, it is highly conserved in *Bacillus* and ubiquitously present as a single copy gene (Schmidt, Scott & Dyer, 2011). The analyses of the ploidy levels of the three new isolates of the genera *Bacillus* und *Paenibacillus* revealed that all of them are oligoploid during exponential phase and diploid at stationary phase. These results show that also species freshly isolated from the environment are not monoploid, and therefore, monoploidy seems to be seldom or absent in the genus *Bacillus*.

Growth phase-dependent copy number regulation

In all five species of *Bacillus* and *Paenibacillus* the genome copy number was considerably lower in stationary phase than in exponential growth phase. This behavior has also been observed for other species, e.g. the haloarchaea *H. salinarum* and *H. volcanii* (Breuert *et al*., 2006), and the methanogenic archaeaon *Methanococcus jannaschii* (Malandrin, Huber & Bernander, 1999). However, it is not universal, e.g. slowly growing *Methanosarcina acetivorans* up-regulate the genome copy number from about two in exponential phase to about five in stationary phase (Hildenbrand *et al*., 2011). Unfortunately, in most species the copy numbers are only known for growing, but not for resting cells, so that the distribution of these two different strategies is unknown.

Overview of ploidy levels in different species of gram-positive bacteria

An overview of gram-positive bacteria with experimentally determined ploidy levels is given in Table 5. Among seven species investigated thus far, only four strains of one species are truly monoploid. In contrast, most species are (mero-)oligoploid, one species is polyploid, and one species is hyperpolyploid. Therefore, it seems that oligo-/polyploidy might be more

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1 widespread in *Bacillus* and related genera and that monoploidy is not typical for *Bacillus* and
2 related genera of gram-positive bacteria. A similar large variance of ploidy levels and a low
3 fraction of monoploid species has also been observed for other phylogenetic groups of
4 bacteria, e.g. the cyanobacteria (Griese *et al*., 2011) and the proteobacteria (Pecoraro *et al*.,
5 2011).

6

7

8 **Acknowledgements:**

9 We thank Prof. Dr. Karl-Dieter Entian for supplying the species *Bacillus subtilis 168.*

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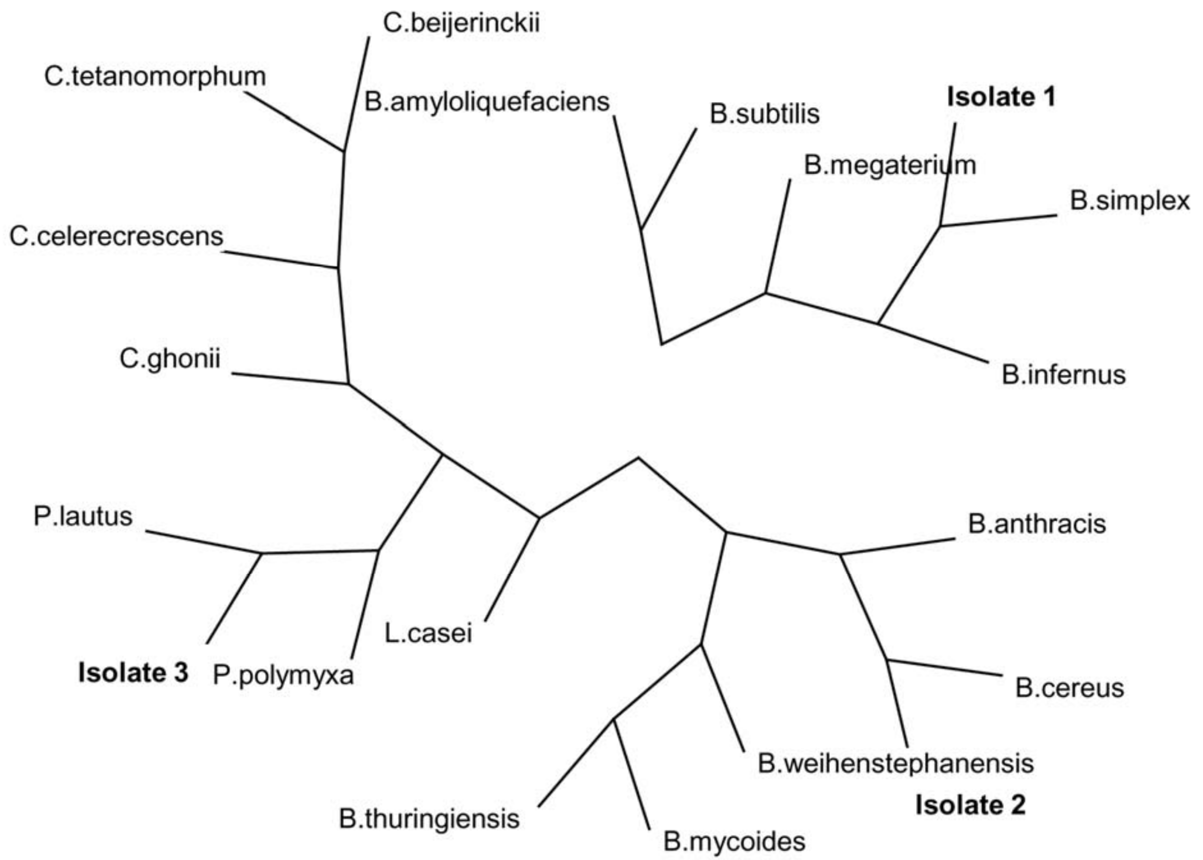
1 **Figure 1:** Phylogenetic tree of the three new isolates and 17 selected species of the genera
2 *Bacillus, Paenibacillus, Lactobacillus*, and *Clostridium*.



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1 **Table 1.** Origin and termini copy numbers in *B. subtilis 168.*

Culture No.	Doubling time [min]	Cell density [cells/ml]	No. origins per cell ±sd	Average value ±sd	No. termini per cell ±sd	Average value ±sd
1	24	$3.4x10^8$	5.3±1.7		1.2±0.1	
2	24	$3.3x10^8$	6.0±1.3	5.9±0.6	1.1±0.2	1.2±0.2
3	24	$1.6x10^8$	6.4±1.5		1.3±0.3	
1	stationary	$1.6x10^9$	2.0±0.2		1.2±0.4	
2	stationary	$1.5x10^9$	3.4±1.0	2.8±0.7	0.9±0.3	1.3±0.4
3	stationary	$1.6x10^9$	3.0±0.9		1.7±0.6	

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1 **Table 2.** Origin and termini copy numbers in *B. megaterium.*

Culture No.	Doubling time [min]	Cell density [cells/ml]	No. origins per cell ±sd	Average value ±sd	No. termini per cell ±sd	Average value ±sd
1	27	$2.5x10^8$	10.7±6.8		15.1±7.2	
2	27	$1.8x10^8$	15.1±3.3	12.4±2.4	12.7±1.6	12.9±2.1
3	27	$1.9x10^8$	11.4±2.2		10.9±5.1	
1	stationary	$2.9x10^9$	3.0±1.1		4.9±2.3	
2	stationary	$2.5x10^9$	3.5±1.0	4.3±1.8	7.0±1.3	7.3±2.6
3	stationary	$2.4x10^9$	6.3±1.3		10.0±1.5	

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1 **Table 3.** Cell characteristics of the three new isolates.

Species	Growth-phase	Cell shape	Length	Filamentous	Motility
Bacillus sp. I1	exponential	rods	2-5 µm	short filaments	yes
Bacillus sp. I1	stationary	rods	2-5 µm	short filaments	yes
Bacillus sp. I2	exponential	rods	5-10 µm	filaments	yes
Bacillus sp. I2	stationary	rods	2.5-5 µm	short filaments	yes
Paenibacillus sp. I3	exponential	rods	5 µm	short filaments	yes
Paenibacillus sp. I3	stationary	rods	2 µm	short filaments	yes

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1 **Table 4.** Ploidy levels of the three new isolates.

Culture No	Doubling time [min]	Cell density [cells/ml]	Genomes per cell ±sd	Average value ±sd	Cell density [cells/ml]	Genomes per cell ±sd	Average value ±sd
I1-1	26	$1.4x10^{8}$	4.0±0.6		$9.5x10^{8}$	2.0±0.4	
I1-2	26	$1.3x10^{8}$	4.1±0.4	4.7±1.1	$1.2x10^{9}$	2.0±0.5	2.3±0.4
I1-3	26	$1.4x10^{8}$	6.0±0.9		$9.6x10^{8}$	2.8±0.6	
I2-1	24	$2.8x10^{8}$	4.8±1.3		$7.0x10^{8}$	2.6±0.5	
I2-2	24	$3.1x10^{8}$	7.3±1.8	6.4±1.4	$7.5x10^{8}$	2.5±0.6	2.4±0.3
I2-3	24	$2.8x10^{8}$	7.0±2.2		$8.7x10^{8}$	2.0±0.3	
I3-1	48	$5.3x10^{8}$	2.8±0.6		$6.9x10^{8}$	2.9±1.0	
I3-2	48	$4.5x10^{8}$	3.9±0.4	3.4±0.5	$7.1x10^{8}$	1.9±0.1	2.5±0.5
I3-3	48	$4.5x10^{8}$	3.7±0.6		$7.3x10^{8}$	2.6±0.7	

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1 **Table 5.** Overview of ploidy levels in different species of gram-positive bacteria.

Species	Number of genomes per cell	Ploidy	References
Lactococcus lactis			
5 strains	2-4	Diploid	Michelsen *et al*., 2010
4 strains	1-2	Monoploid	Michelsen *et al*., 2010
Bacillus subtilis	4-8	Mero-Oligoploid[a]	Webb *et al*., 1998
Bacillus subtilis	4-8	Mero-Oligoploid[a]	Moriya *et al*., 2009
Bacillus subtilis	6/3[b]	Mero-Oligoploid[a]	This study
Bacillus megaterium	12/4[b]	Polyploid	This study
Wild-type isolate I1 *Bacillus sp.*	5/2[c]	Oligoploid	This study
Wild-type isolate I2 *Bacillus sp.*	6/2[c]	Oligoploid	This study
Wild-type isolate I3 *Paenibacillus sp.*	3/3[c]	Oligoploid	This study
Epulopiscium spp.	10,000-100,000	Hyperpolyploid	Mendell *et al*., 2008

2 [a] during fast growth

3 [b] number of origins per cell in exponential and stationary phase

4 [c] number of genomes per cell in exponential and stationary phase

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1 **Supplemental Information:**

2 Supplemental Table S1. Sequences and applications of oligonucleotides used in this work.

Species	Oligonucleotide	5´-3´	Application
B. subtilis	standard ori for	caagatgtattaaaagcagtttcatccagaaccacgattcc	standard fragment ori
	standard ori rev	gcgcctgtaaagcttacgcgtatttctgctcc	
	analysis ori for	gcagattgaagagcatcatgcgattcagatccc	analysis fragment ori
	analysis ori rev	caggctggttgtgtctggataattcccgtcc	
B. subtilis	standard term for	ggaggaaatgctattgacgcagcggttgc	standard fragment term
	standard term rev	gtgggttgtttggccttctactttgtctttcggc	
	analysis term for	gcactctgaaagggctggaagaagccttgg	analysis fragment term
	analysis term rev	ccaaatccttttgaataagggtatctccttctttaagcgg	
B. megaterium	standard ori for	gaaagctgtatcttctagaacaacaattccaattttaacgggg	standard fragment ori
	standard ori rev	cagcattgaatcatcctctaatgttcaaatgacaaatgg	
	analysis ori for	gtttagatgcagaagagtatcctcatttgccacagatcg	analysis fragment ori
	analysis ori rev	cggtggctgtcagtggcaatgcacg	
B. megaterium	standard term for	ccattatcgcagggattatggtttcaatattaggcgg	standard fragment term
	standard term rev	gctaaaagtagacccacgcctacacctgttgttaaatcg	
	analysis term for	ggtcagatcgctaactttttaggactaaaaggaattgcaaagc	analysis fragment term
	analysis term rev	ggaaatagcatcacgatgttatcccatgttaaatctggg	
Isolates I1, I2, I3	16S1kin	gagagtttgatcctggctcag	sequencing of the 16S rRNA gene
	16S2kin	acgagctgacgacagccatg	
Isolates I1, I2, I3	*sigL* for Sq	atgagcaakcgytdgaraaycc	sequencing of the *sigL* gene
	sigL rev Sq	gcmcgrctvayygtngaytcatg	
Isolate I1	standard for	gaatcaccagcagttttccggtgagcagc	standard fragment
	standard rev	gccaagttcgtccgcgatttctttcatcg	
	analysis for	gcatcggagcgaggaatcttcaagagtgcc	analysis fragment
	analysis rev	cctgaaaaaagagtgatgccggacgcgg	
Isolate I2	standard for	caaatacagtattataaaatagaagaagaagag	standard fragment
	standard rev	gagtggcttcaaatacgctggaccttccc	
	analysis for	gccagcaggggtaggagcacgtaatattcagg	analysis fragment
	analysis rev	ggctgcaatgatgtaatacagttcaccgccg	
Isolate I3	standard for	caactgcgtatgctcaccctgccaaggg	standard fragment
	standard rev	catcggtgcaattcccttgattccttcaacc	
	analysis for	ctggcatacgggaagctggagaagatcgc	analysis fragment
	analysis rev	ggagagcttggggagattttccggatggatc	

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1 Supplemental Table S2. Standard and analysis fragments used for copy number
2 quantifications.

Species	Fragment	Size [nt]	Genomic localization
B. subtilis	standard ori	1018	1982-3000
	analysis ori	385	2320-2704
	standard term	1017	2005849-2006865
	analysis term	231	2004333-2004563
B. megaterium	standard ori	1054	1586-2639
	analysis ori	199	1882-2080
	standard term	1045	2563924-2564968
	analysis term	332	2564178-2564509
Bacillus sp. I1	standard	718	*sigL*
	analysis	221	*sigL*
Bacillus sp. I2	standard	696	*sigL*
	analysis	207	*sigL*
Paenibacillus I3	standard	699	*sigL*
	analysis	216	*sigL*

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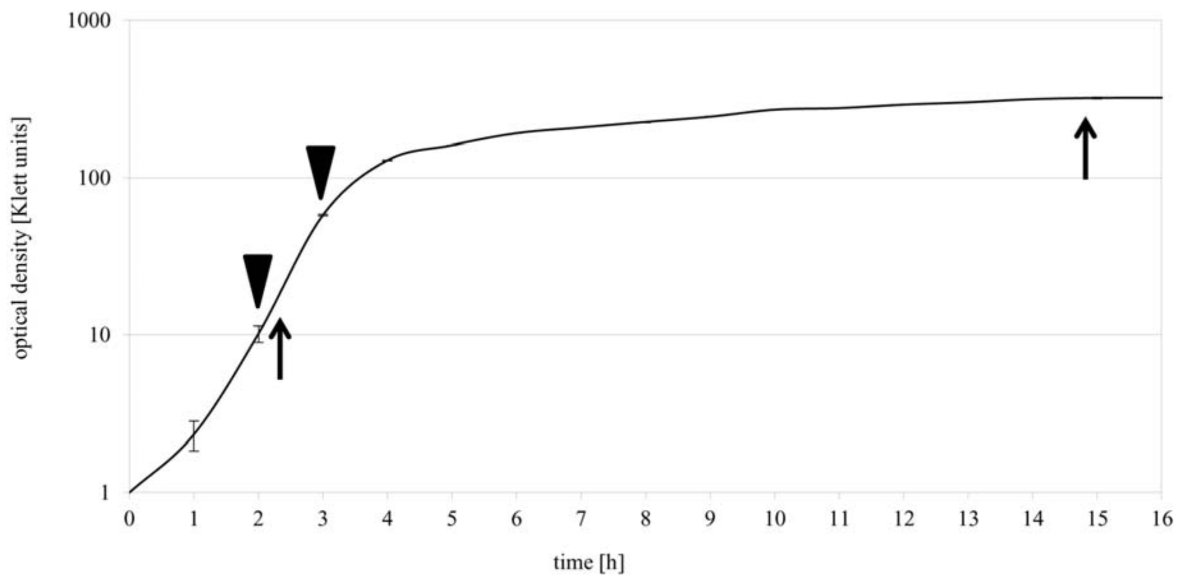
1 Supplemental Figure S1. Growth-curve of *Bacillus subtilis*. Average values of three biological
2 replicates and their standard deviations are shown. The arrows indicate the times of aliquot
3 removal for the analysis of exponentially growing and stationary phase cells, respectively.
4 The triangles indicate the time window used to calculate the doubling time.



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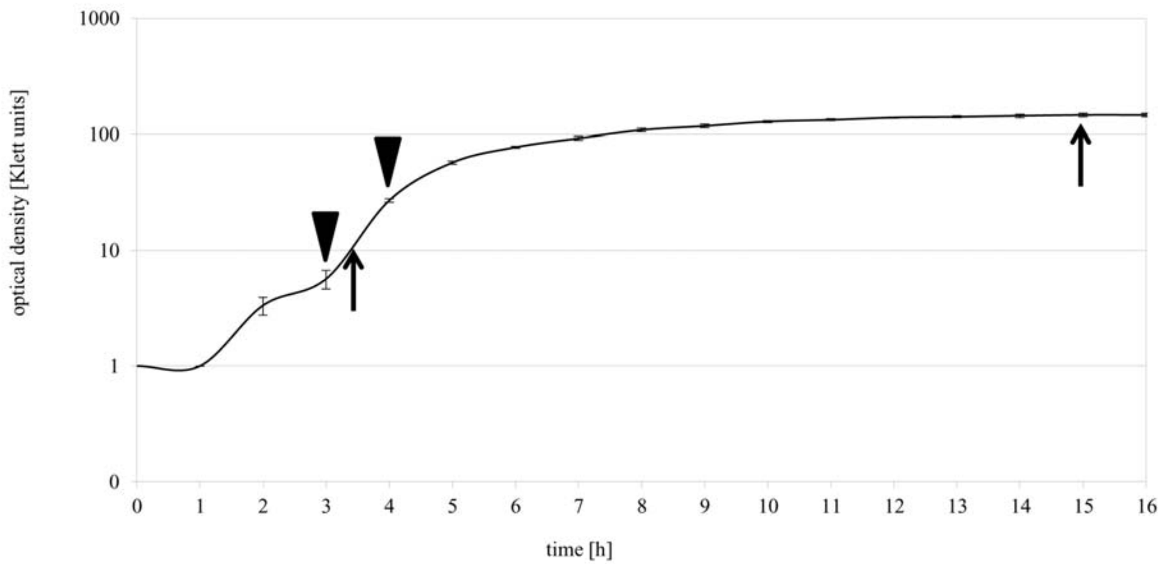
1 Supplemental Figure S2. Growth-curve of *Bacillus megaterium*. Average values of three

2 biological replicates and their standard deviations are shown. The arrows indicate the times of

3 aliquot removal for the analysis of exponentially growing and stationary phase cells,

4 respectively. The triangles indicate the time window used to calculate the doubling time.



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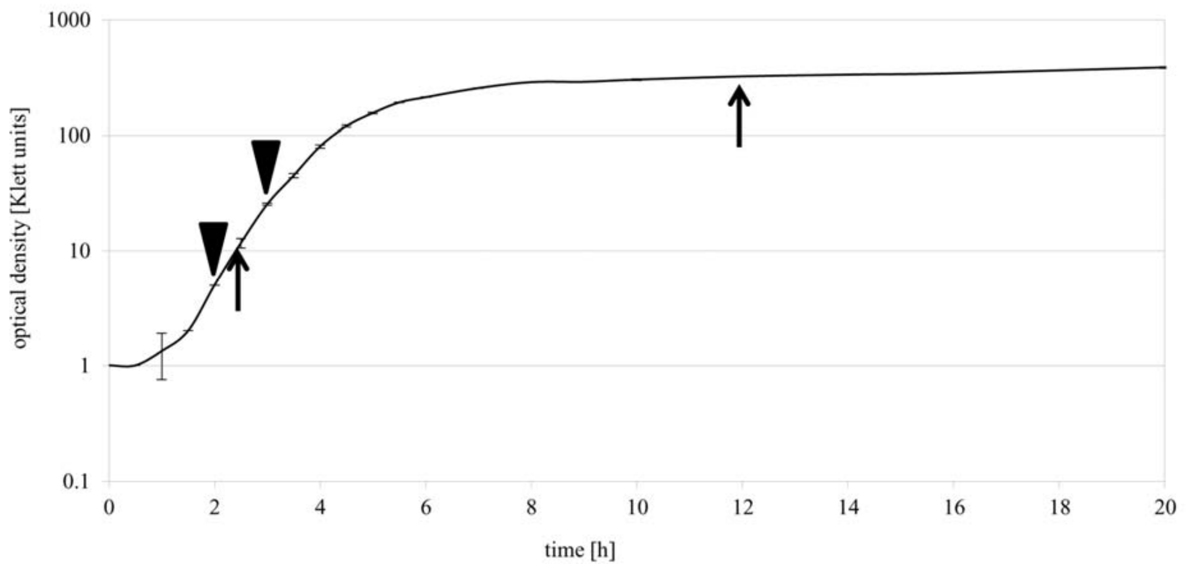
1 Supplemental Figure S3. Growth-curve of *Bacillus sp*. isolate I1. Average values of three

2 biological replicates and their standard deviations are shown. The arrows indicate the times of

3 aliquot removal for the analysis of exponentially growing and stationary phase cells,

4 respectively. The triangles indicate the time window used to calculate the doubling time.



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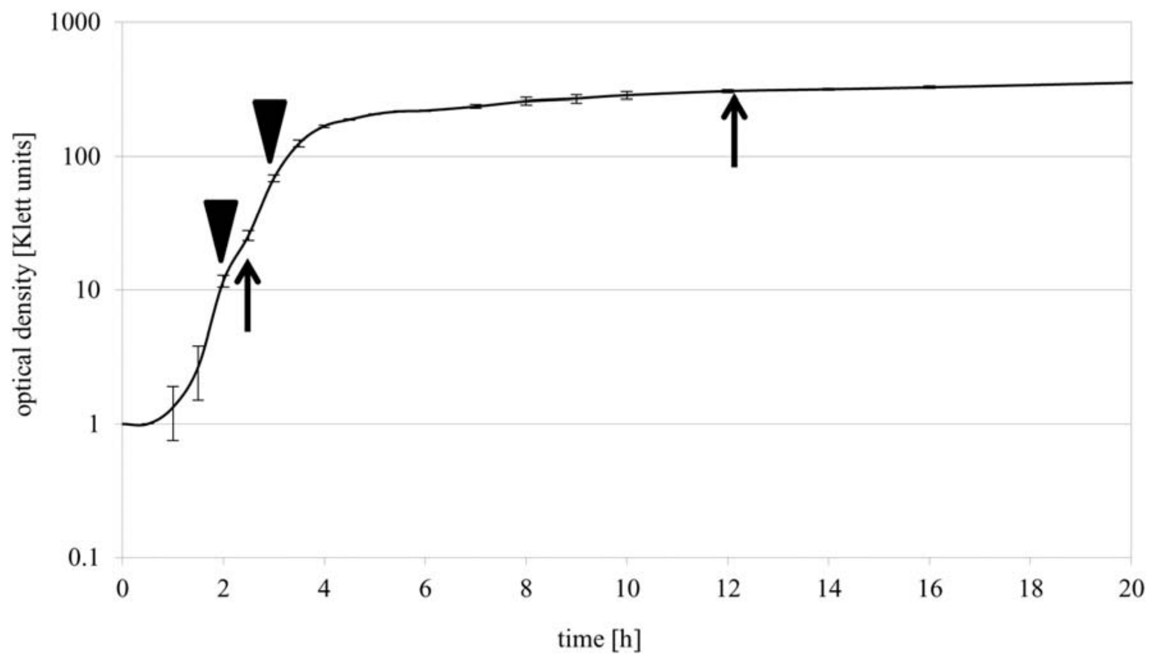
1 Supplemental Figure S4. Growth-curve of *Bacillus sp*. isolate I2. Average values of three
2 biological replicates and their standard deviations are shown. The arrows indicate the times of
3 aliquot removal for the analysis of exponentially growing and stationary phase cells,
4 respectively. The triangles indicate the time window used to calculate the doubling time.



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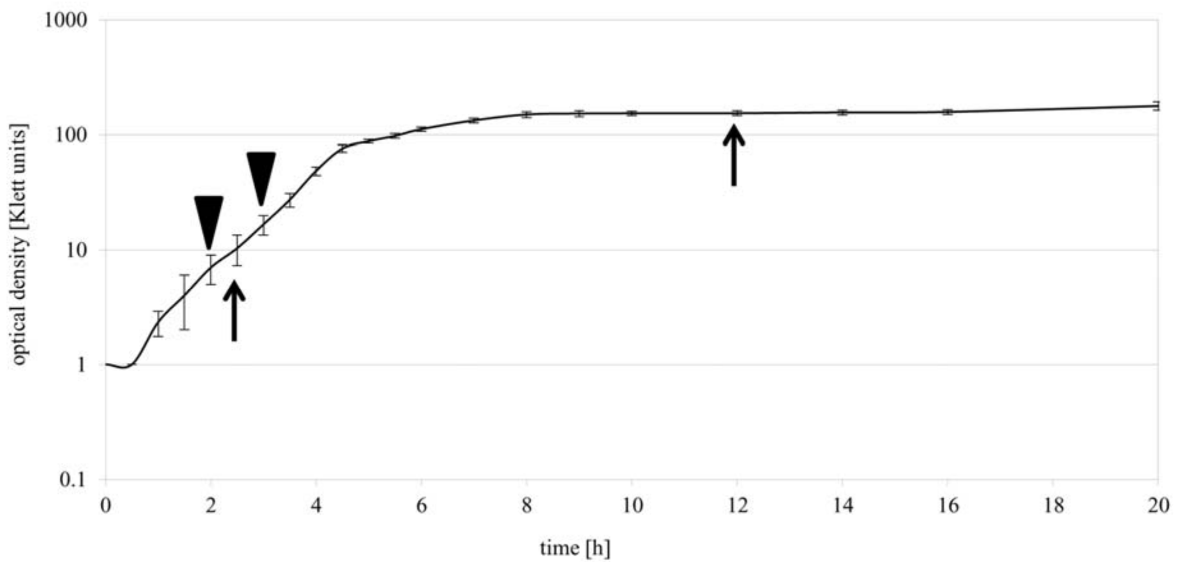
1 Supplemental Figure S5. Growth-curve of *Paenibacillus sp*. isolate I3. Average values of
2 three biological replicates and their standard deviations are shown. The arrows indicate the
3 times of aliquot removal for the analysis of exponentially growing and stationary phase cells,
4 respectively. The triangles indicate the time window used to calculate the doubling time.



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