Physiological and morphological characterization of two Bacillus strains

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Abstract

Organisms of the genus Bacillus have been described more than 100 years ago and cultivated, engineered and used as a model system in microbiology for many decades. Despite their ubiquitous and successful use in wide areas of research and industry little was known about some of the most basic rules concerning their cell development and cell differentiation as well as biofilm and colony formation. This project describes the behaviour of two strains of Bacillus under different growth conditions. The responses of the strains toward certain conditions turned out to be very different, which might be linked to their function or survival strategy within the natural environment. One strain seemed to follow the r- another the K-strategy. Furthermore, it was observed that cell motility within a colony can vary significantly depending on nutrient availability and is likely triggered at certain developmental stages of the colony.

Introduction

Strains of the genus Bacillus have been isolated for over 150 years, with the first scientific description dating back to 1872[1]. They are aerobic endospore forming bacteria belonging to the Firmicutes that live mostly in soil, but also occur in animal guts and other environments[2]. Bacillus species are also known to be human pathogens, which is part of the reason why they are not only well studied and understood, but also used as a widespread model system in microbiology, e.g for cell development and spore formation[3]. However, it was not until recently that cell development and arrangement within Bacillus colonies and biofilms has been elucidated[4]. Over the last ten years many exciting findings concerning cell development[5] and biofilm formation[6] have been discovered, but comparatively little research has been done connecting those cell capabilities to ecosystem function and microbial ecology. This project was aimed at describing cell physiology and colony morphology in relation to different nutrient and agar conditions. Observing responses

of the organisms toward these conditions might reveal some information about their metabolic capabilities or life styles.

Materials and Methods

Sampling and isolation

2 g of soil from Bell Tower Field was suspended in 10 ml ultrapure water (18 MΩ - Milli-Q) by thorough vortexing and incubated at 80 °C for 10 min. 100 µl each of four dilutions (10^{-1} – 10^{-4}) of this suspension were plated on Nutrient agar plates (Difco) and incubated at 30 °C over night (o.n.). 12 colonies were chosen according to their colour and morphology, restreaked for isolation on Nutrient agar plates and incubated at 30 °C o.n. This procedure was repeated to assure isolation of clonal strains.

Cultivation media

Normal LB broth:	2.5% LB powder (Difco) in Milli-Q (0.5% yeast extract, 1% NaCl, 1% peptone from casein)
Low nutrient LB broth:	0.25 % LB powder (Difco) in Milli-Q

Cultivation plates

Nutrient agar plates:	0.5% sodium chloride (NaCl) 0.5% Peptone 0.3% yeast extract 1.5% agar (Difco)
Low agar/ high nutrient (LAHN) plates:	1% agar 2.5% LB powder (Difco)
Low agar/low nutrient (LALN) plates:	1% agar 0.25% LB powder
Low agar/double nutrient (LADN) plates:	1% agar 5% LB powder
High agar/low nutrient (HALN):	2.5% agar 0.25% LB powder
High agar/high nutrient (HAHN)	2.5% agar, 2.5% LB powder

Cell counts and growth rates

Cell numbers in 0.1 µl of liquid cultures were assessed using a Neubauer chamber and then extrapolated to cells/ml. Depending on the cell density the cultures were diluted before counting. Growth rates and doubling times of the liquid cultures were calculated using the absorption of the culture as measured by a photometer at a wavelength of 600 nm.

Microscopy

Colony and cell morphology, as well as size and motility, was observed and measured via binoculars (Zeiss) and microscopy (Zeiss Discovery.V8 SteREO; Zeiss Imager.A2; Zeiss C-LSM 700). The pictures and movies were acquired and processed digitally using the implemented software AxioVision.

Embedding, Cryo-Sectioning and Staining

Bacterial cultures were cut out of agar plates using a sterile scalpel, placed in a silicon Cryo-mold and fixed in a 1× PBS solution containing 4% formaldehyde and 0.5% glutaraldehyde for 1 h at RT. The fixative was removed with a pasteur pipette and the cultures washed for 30 min in 1× PBS. After removing the PBS the cultures were embedded in O.C.T. Tissue Tek (Sakura, CA, USA) and incubated for several hours. Then the mold was shock frozen in liquid nitrogen, transferred to -80°C for several hours and then stored at -20°C for at least another few hours. Embedded colonies were sectioned with a cryo-microtome into 20 µm sections and placed onto polysine-covered glass slides (Thermo Fisher Scientific Inc., Schwerte, Germany). Slides were stored at -20°C until used.

Slides were stained with Alcian Blue solution (2.5% Alcian Blue powder in 3% actic acid). The solution was dropped onto the sections, incubated for 30 min at RT and rinsed carefully twice with 1× PBS and Milli-Q. The slides were air dried, embedded in mouting medium (Citifluor:Vetashield, 4:1), that contained 1µg/ml DAPI and stored at -20°C until used.

Microsensor

Measuring oxygen consumption by the liquid cultures was carried out using a Microsensor system (Unisense) and the software MicOx. Measurements in mV were converted to µmol/l by the software and the data processed with Xcel (Microsoft).

Data Analysis

The pictures taken by the microscope were processed with iPhoto (v6.0.6; Apple Computer, Inc.) and the movies animated with ImageJ (v1.38x; www.rsb.info.nih.gov/ij). Statistical analysis of the data was carried out using R (v2.7.0; The R Foundation for Statistical Computing)

3. Results and Discussion

Isolation and identification of different Bacillus strains

Pasteurization of 2 g soil and subsequent cultivation of the viable spores it contained, yielded 12 different colony morphotypes of aerobic spore forming bacteria. These morphotypes were isolated and their 16S rRNA amplified and identified. All 12 isolates (ASF1-12 (B11, D12, E01-E10); see Suppl. CD) belonged to the genus Bacillus (Figure 1). Two of these organisms were chosen for further characterization based on their identity, cell and colony morphology. The first organism (isolate E05) was closely related to *Bacillus cereus* (ASF6), the second one (isolate E10) was a close relative of *Bacillus pumilus* (ASF12). Cells of ASF6 were around 10 µm long and slightly motile (Figure 2a, Suppl. Movie 6_LAHN). Cells of ASF12 were around 2.5 µm long and highly motile (Figure 2b).

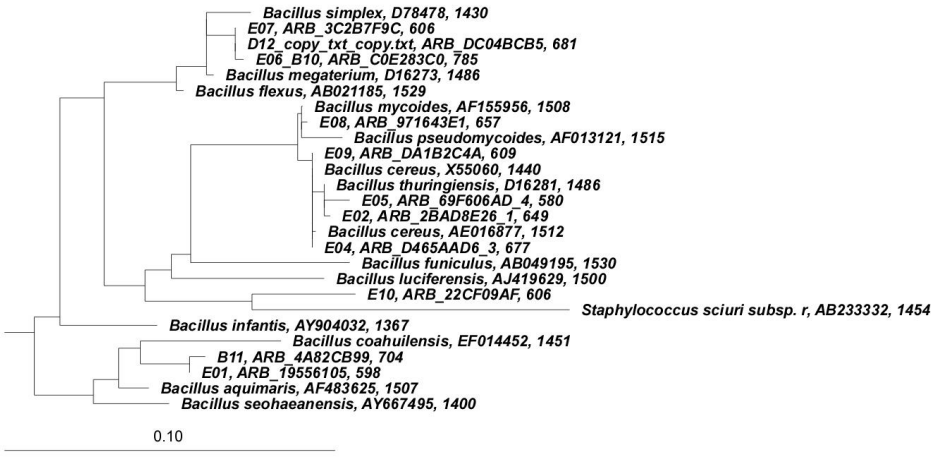

Figure 1:

Phylogenetic tree of the strains isolated from Bell Tower Field soil. The tree was build in ARB using the SILVA database

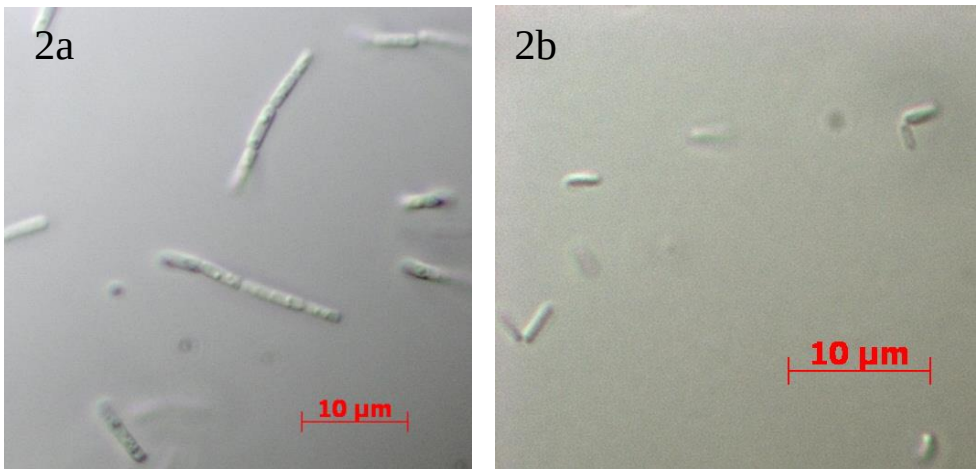

Figure 2:

Cells of the two strains as observed at 400x magnification.

Growth in liquid culture

Cultivation of the two strains in liquid culture revealed significant differences concerning growth rates and metabolic capabilities. Two types of growth media, a normal LB medium and nutrient depleted LB medium were prepared and each inoculated with a silmilar amount of cells (Figure 4) of one of the strains. In all

cultures cell numbers and absorbance seemed to correlate quite well (Figure 3). Within the first 3 hours after inoculation the largest change in absorbance and thus the fastest growth was observed in the high and low nutrient ASF6 culture (Fig 5, Fig 6). With 20 min and 21 min, respectively, the minimal doubling time of the organism was very similar in both conditions, although in the low nutrient condition it was delayed by one hour occuring between 5 and 6 hours after inoculation. In the nutrient depleted medium ASF6 used up most of the nutrient shortly after it entered the exponential phase.

The minimal doubling time of strain ASF12, although it had a longer lag phase, was similar to those of ASF6 (18 min) and surprisingly occurred under the nutrient depleted growth condition. Moreover, strain ASF12 finally grew to a higher cell density under both conditions. This suggests that the two strains have different life styles. ASF6 seems to be metabolizing fast, which might enable it to take over under high nutrient conditions out-growing its competitors. At the same time it does not cope so well with low nutrient conditions be it from the beginning, as in the low nutrient medium or when a formerly rich environment is nutritionally exploited, as in the high nutrient medium after 20 hours. Reasons for that could be found in a poor affinity to the substrate or a less efficient metabolism. This behaviour follows the r strategy, where a population is successful under favorable conditions because it is fast and produces more offsprings that other organisms. ASF12 seems to be rather a K strategist. It might have a slower metabolism and growth rate and hence produces less offsprings in a given time, but eventually outnumbers the competitors by a more efficient use of nutrients. It seems more adapted to quality than quantity.

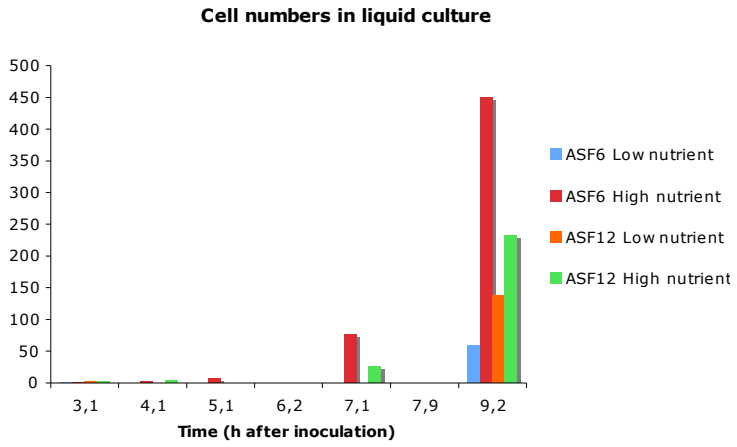

Figure 3:

Cell counts of the liquid cultures at different time points after inoculation as assessed by the Neubauer chamber.

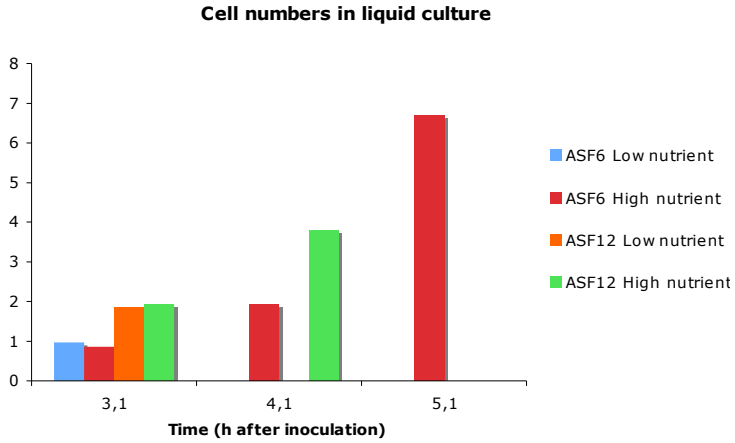

Figure 4:

Cell numbers in the liquid cultures at the first three time points between 3 and 5 hours after inoculation.

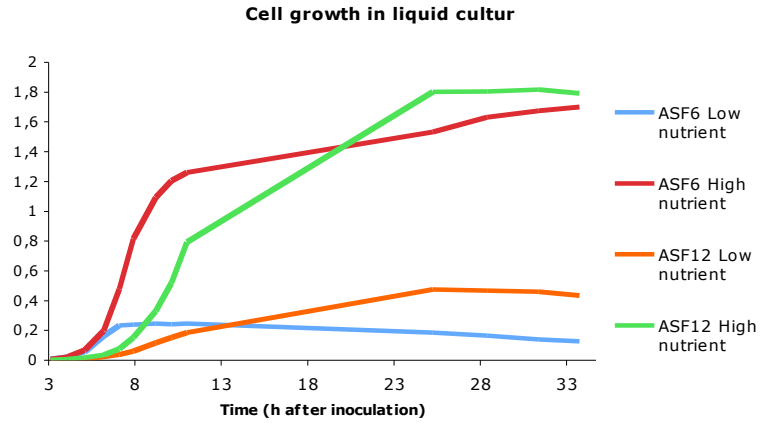

Figure 5:

Absorbance of the liquid cultures over time as measured with a spectrophotometer .

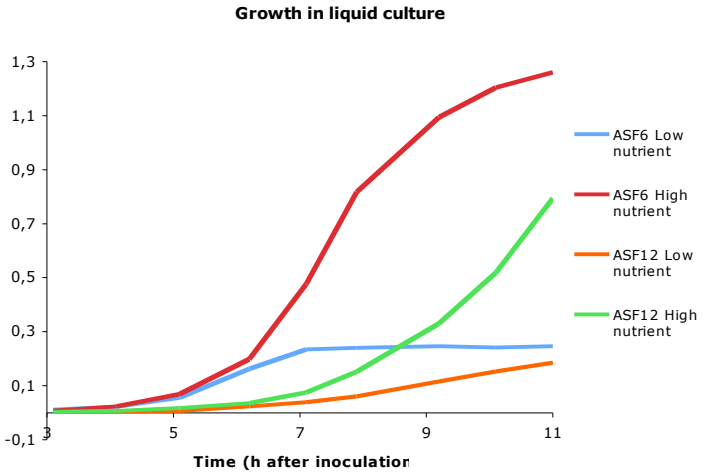

Figure 6:

Detail of figure 5 depicting the first 5 timepoints.

Growth on hard substrate

The growth of the two strains was also observed on different hard substrates. Two agar concentrations (1% and 2,5%) were therefore combined with 3 nutrient conditions (0.25%, 2.5% and 5% LB). The diameter of the colonies was chosen as a proxy for growth and performance. 20 colonies were chosen per strain and condition and their diameter measured 3 to 4 times over the course of 72 hours. The data show marked differences between ASF6 and ASF12 that might be due to different life styles resulting from the occupation of different ecological niches. ASF6 grows significantly faster than ASF12 as shown by the slope of the regression curve that was fitted on the dataset (Figure 7). A more detailed look at the dataset reveals preferences of the strains concerning nutrient and agar concentration and indicates different strategies how the organisms cope with these growth environments (Fig lm).

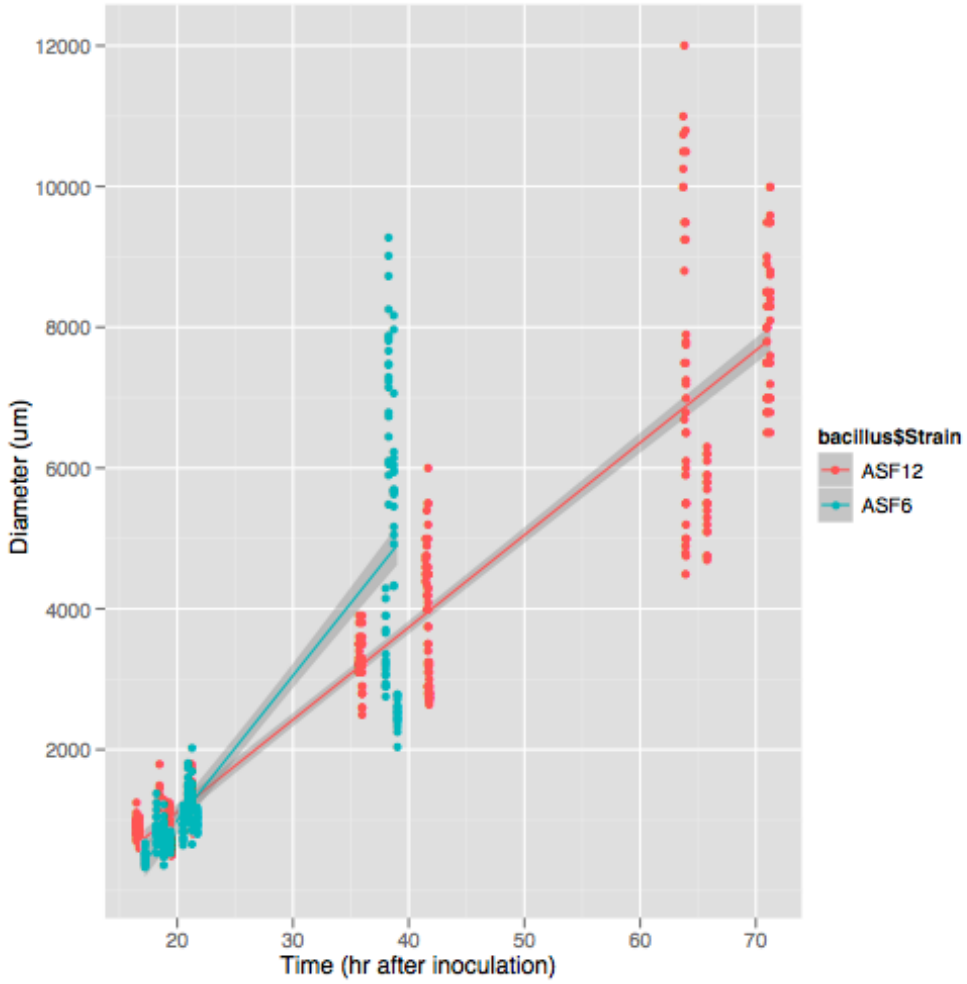

Figure 7:

Visualization of colony diameter over time. A linear regression shows the confidence of the trend including 95% confidence intervals.

To visualize patterns in the dataset, each datapoint, which is the diameter of a single colony at a given time, was tagged with information about the condition it was obtained from. To clarify the trends within the data, the timepoints 1 and 2, 3 and 4 were plotted as 3 single graphs (Figure 8-10). Colony size of the strain ASF6 correlated positively with nutrient availability and negatively with agar concentration. Both correlations were significant as shown by the results of the linear regression (Fig. 11). Growth behaviour of ASF12 differred substantially, since it correlated negatively with agar concentration and it showed no effect towards agar concentration. Unfortunately the double agar condition was only included in the analysis of strain ASF12, which hampers the comparison of both strains. The results of the linear regression supported not only the inevitable correlation of time and growth but also the finding that ASF6 grows faster than ASF12, at least within the observed 72 hour time period.

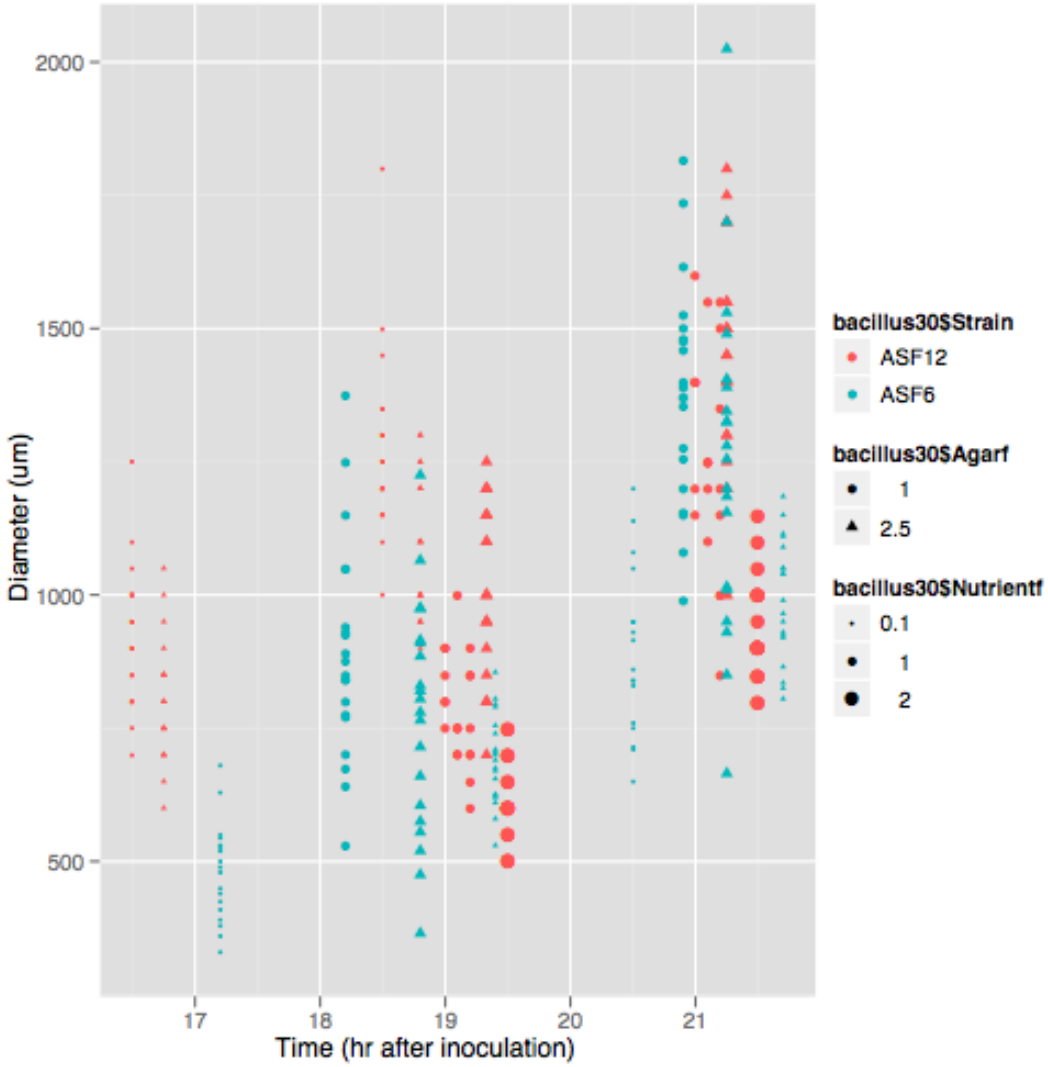

Figure 8:

Zoom-in on the first two datapoints. The points include information about strain, agar concentration and nutrient concentration. Trends of the strains concerning usage of nutrients and agar preferences are easier to detect.

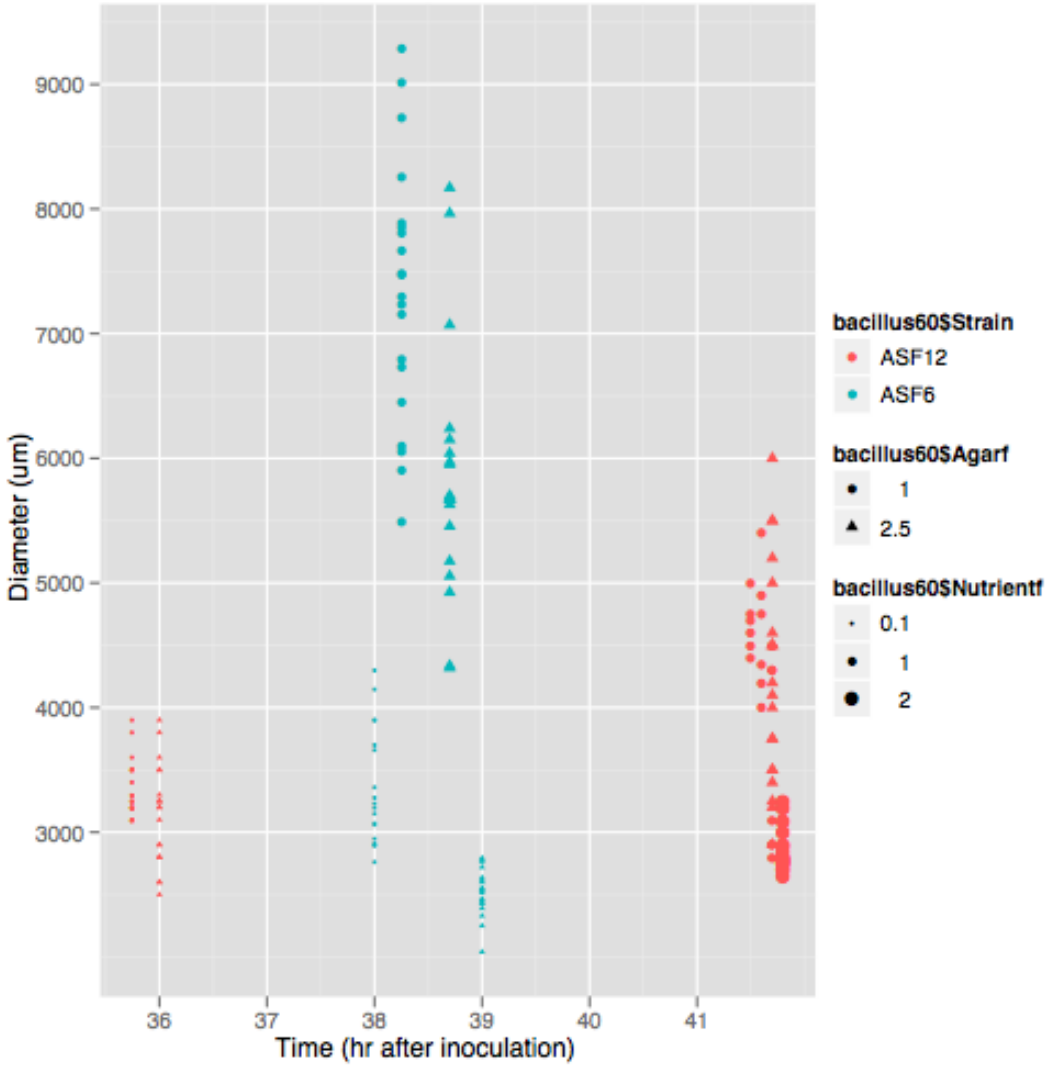

Figure 9:

Figure 9 includes the same information as figure 8, but shows timepoint 3 instead.

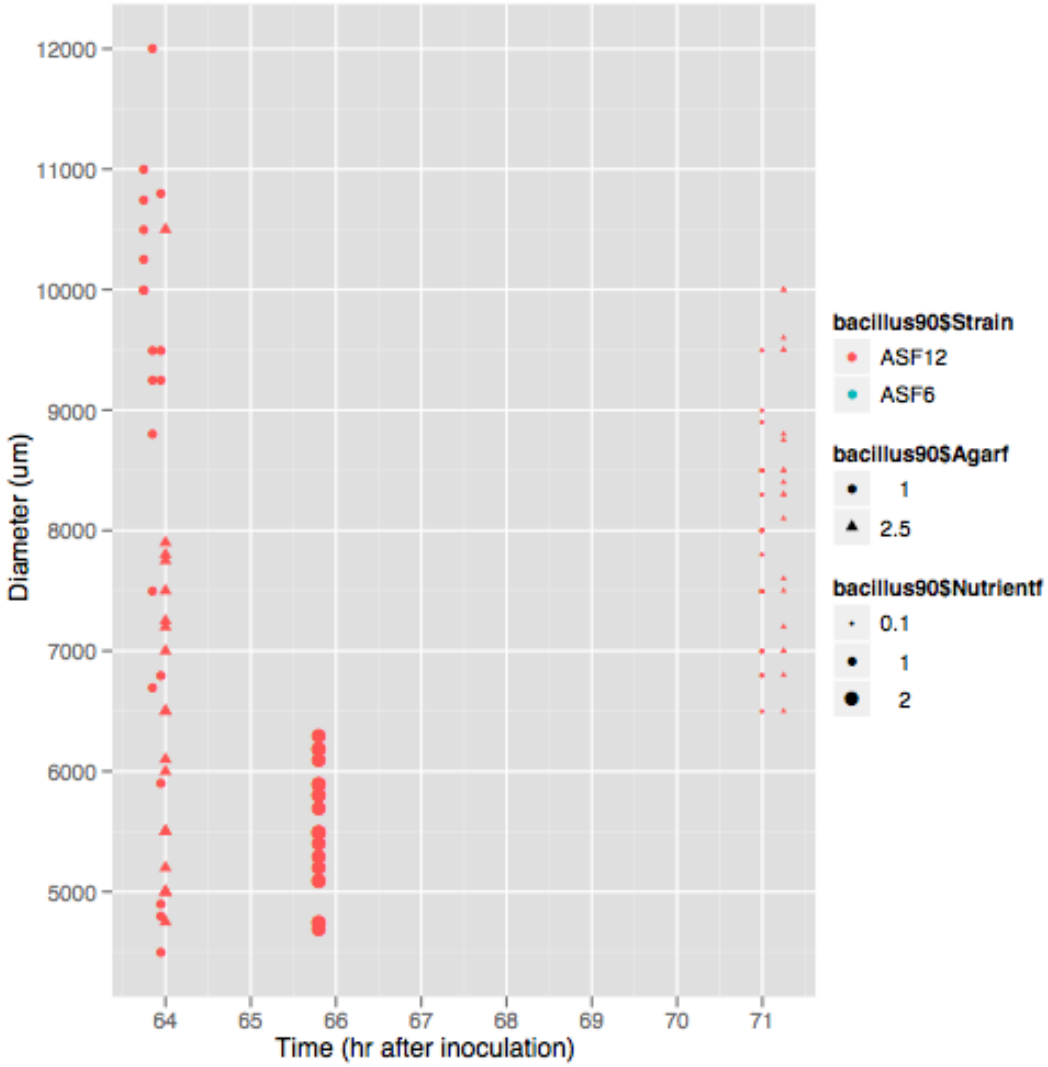

Figure 10:

Depiction of all information about the colonies at time-point 4.

ASF6	Estimate	Std Error	t-value	p value
Time	207.59	7.16	28.992	< 2e-16 ***
Agar	-583.16	127.73	-4.565	8.17e-06 ***
Nutrient	1568.72	141.82	11.061	< 2e-16 ***

ASF12	Estimate	Std Error	t-value	p value
Time	131.699	2.201	59.840	< 2e-16 ***
Agar	-75.850	94.073	-0.806	0.421
Nutrient	-495.055	64.788	-7.641	1.52e-13 ***

Figure 11:

Values of the linear regression as calculated by R.

Respiration measurements

Oxygen consumption was measured at 3 hours after inoculation (timepoint 1) and again at 7 hours after inoculation (timepoint 4) (Figure 12). At timepoint 1 (t1) it took about 35 min until the culture was completely anoxic. This equals an average consumption of 0.11 µmol oxygen per liter liquid culture per second. Considering the number of cells in one liter of liquid culture, which was approximately 10^9, an oxygen consumption of 0.11 fmol oxygen per cell per second can be calculated. Given this number is true, it would mean that a single Bacillus cell is reducing about 10^8 oxygen molecules per second. At timepoint t5 it took the liquid culture roughly 14 minutes to turn anoxic at an average oxygen cosumption rate of 0.28 µmol/l per second. With 6×10^9 cells/l this equals an oxygen cosumption of 0.02 fmol/cell per second. Although being in the same order of magnitude, these values don't match very well. Reasons for that might be found in the inaccuracy of the oxygen measurements or cell counts. To assess valid rates, measurements of more timepoints have to be carried out and more importantly they have to be replicated. However, these results might give a rough estimate as to how much oxygen can be expected in a liquid culture and how many molecules of oxygen can be consumed per cell in the exponential growth phase.

The decrease in oxygen concentration was also measured for a single colony (Figure 13). The ASF6 colony had a diameter of around 3 mm and was grown on a LADN plate. Interestingly, the concentration did not fall below 140 µmol/l in both replicates that were measured. The reasons for this behaviour remain elusive but could be assessed by a larger screening of colonies and more replicates.

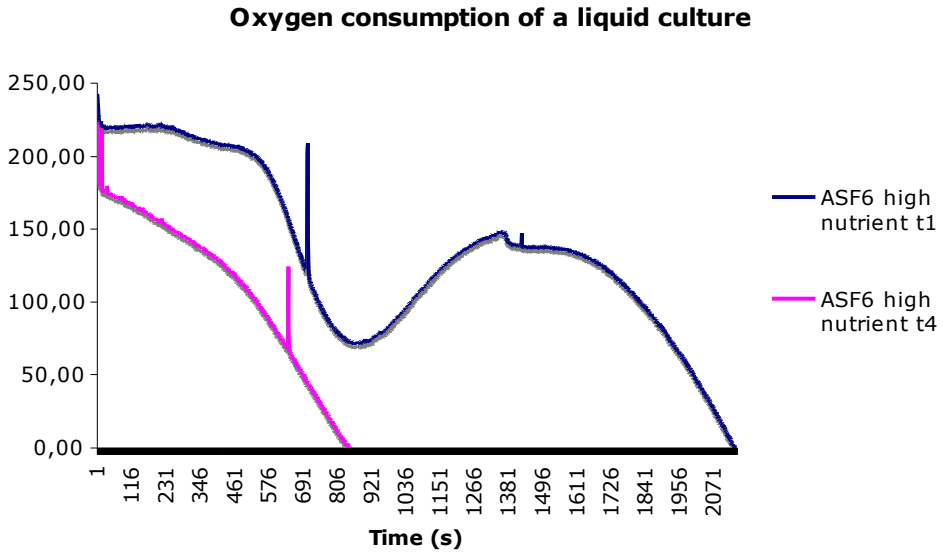

Figure 12:

Figure 12 shows two oxygen profiles as measured with a microsensor probe. The reason fort he dent in the curve of timepoint 1 is unclear, but might be due to a failure of the stirrer bar, which happens occasionally or due to a bubble that could block the sensor tip.

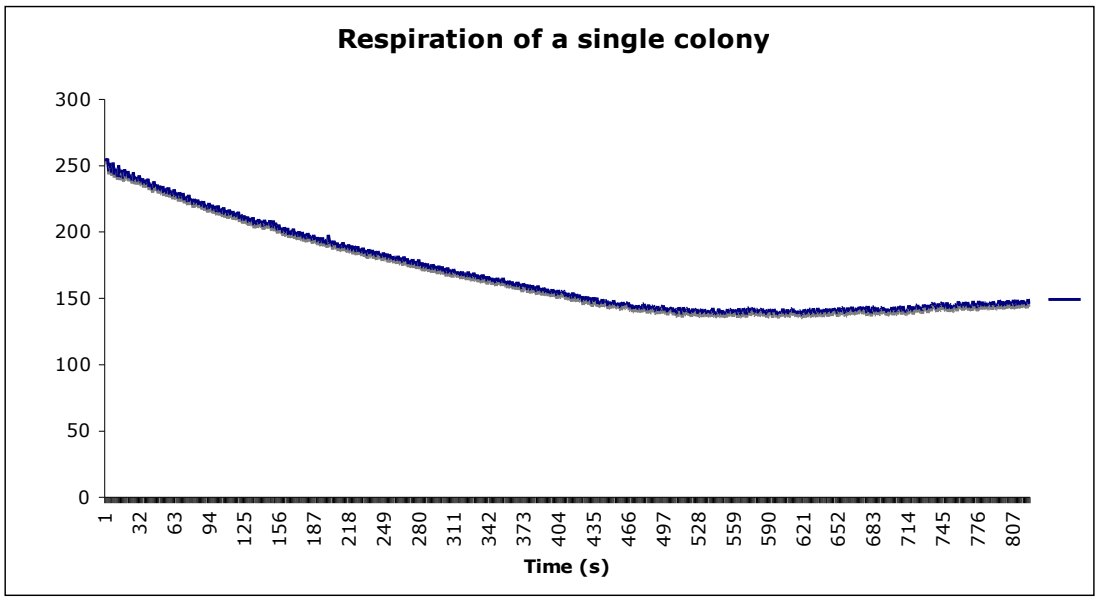

Figure 13:

Oxygen profiles of a single ASF6 colony.

Observation of the structure and growth of Bacillus colonies using microscopy

The quasi crystalline structure of cells within colonies grown at different conditions was observed using cell dyes and confocal laser scanning microscopy. Unfortunately the embedding of colonies, the cryo-sectioning and subsequent staining with Alcian Blue and DAPI did not yield satisfying results. Although, the structure of the colony and the arrangement of cells was visible, good pictures could not be obtained. However, it seemed like there are two major types of structure, a crystalline type, where the rods a lined up more or less straight and an amorphous type, where bundles of rods were arranged in loops, waves and knots.

Colonies of both strains at different growth conditions and timepoints revealed very different morphologies and behaviour when they were observed over longer periods of time. To visualize cell movement at the edges the colonies were illuminated with a bright field and phase contrast, movements within the colonies were observed using a dark field. A picture was taken every 10 s for 20 min and then animated to a movie. Colonies of ASF6 showed movement of cells and growth mainly on the edges when they were grown on normal LB plates (Movie 6_LAHN.avi). On double nutrient plates (5% LB) the movement of cells was restricted to the interior part of the colony, creating a circular flow that resembled convection (Movie 6_LADN_20min_oxygen.avi). This observation indicates that cells have different motilites and orientation within a colony depending on external factors such as nutrient concentration. Hence, this finding supports our recently established understanding of heterogeneity and cell differentiation within clonal cell colonies[5]. To see whether or not oxygen availability could be a reason to create a current within the colony, which would ensure a constant mixing of air, the colonies were incubated in a mixture of N_2/CO_2 (4:1). No response was observed within 30 min (Movie 6_LADN_20min_N2/CO2.avi), which could be either because the organism needs a longer response time or because the current was not established to mixing of oxygen, but rather of nutrients or else.

Other interesting motions and wavelike movements were observed in some colonies at certain growth stages. The four movies (12_LAHN_movie1-4) depicting these motions are from the same spot recorded in a bright and dark field. Notably they are not time-lapsed but in real time, which shows that colonies can be very dynamic systems.

Conclusion and Outlook

This work has tapped into a great diversity of behaviour, carried out by the two strains ASF6 and ASF12 in response to changes in environmental conditions. It revealed the organisms strategies to make a living on the plates, which could be a good approximation of their life styles in the environment. ASF6 seems to follow the r strategy of success through quantity, whereas ASF12 seems to be rather a K strategist. Cells and colonies grow and act differently under different conditions concerning cell size and motility among others. Cell differentiation and heterogeneity, hence, could to be a much more relevant and ubiquitous trait of microorganisms thriving everywhere out there.

To follow up on these experiments and confirm some of the preliminary findings it is necessary to create larger and thus more stable datasets. More replicates of the tested conditions need to be included and more strains tested. The protocols for embedding, staining as well as measuring respiration rates have to be optimized not only for a culture approach but also for assessment and observations of single cells. The dawning era of single cell methods will greatly improve our understanding of individual cells and their interactions with each other and their environment.

Acknowledgements

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