Pole age affects cell size and the timing of cell division in *Methylobacterium extorquens* AM1

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Abstract

A number of recent experiments at the single-cell level have shown that genetically identical bacteria that live in homogeneous environments often show a substantial degree of phenotypic variation between cells. Often, this variation is attributed to stochastic aspects of biology – the fact that many biological processes involve small numbers of molecules, and are thus inherently variable. However, not all variation between cells needs to be stochastic in nature; one deterministic process that could be important for cell variability in some bacterial species is the age of the cell poles. Working with the alpha-proteobacterium *M. extorquens*, we followed individuals in clonally growing populations over several divisions, and determined the pole age, cell size, and interdivision intervals of individual cells. We observed the high levels of variation in cell size and the timing of cell division that have been reported before. A substantial fraction of this variation could be explained by each
cell’s pole age, and the pole age of its mother: cell size increased with increasing pole age, and the interval between cell divisions decreases. A theoretical model predicted that populations governed by such processes will quickly reach a stable distribution of different age- and size-classes. These results show that the pole age distribution in bacterial populations can contribute substantially to cellular individuality. In addition, they raise questions about functional differences between cells of different age, and on the coupling of cell division to cell size.

**Introduction**

In several species of alpha-proteobacteria, the two cells emerging from division have been shown to be systematically different: First, experiments with *Caulobacter crescentus* and *Sinorhizobium meliloti* have shown differences in protein localization between cells with new and old poles (13, 29). Second, experiments with several species of alpha-proteobacteria have shown that cells with ‘new’ poles are smaller than cells with ‘old’ poles (13),(12). Third, in *C. crescentus*, cells with increasingly old poles show signs of aging, while new pole cells are rejuvenated (2, 3). Here, we used the alpha-proteobacterium *M. extorquens* to investigate whether the age of a cell’s poles has a systematic effect on its size, and on the timing of cell division.
M. extorquens is a facultative methylotroph that is ubiquitous in aquatic and terrestrial environments (15), and is often associated with plants (8). M. extorquens strain AM1 is a genetic model system (31) that shows substantial variation in cell size and interdivision intervals (26, 27) between genetically identical cells. Motivated by these reports on phenotypic variation, we were asking three questions about cell size asymmetry and the timing of cell division: First, does cell size increase with pole age? Previous experiments with alpha-proteobacteria have only distinguished between ‘old’ and ‘new’ poles (13), (12). If we analyze ‘old’ poles of different ages – do we see a systematic effect of pole age on cell size? Second, how do cells of different sizes ensure proper timing of cell divisions? Do cells adjust their growth period to attain the goal cell size? Or do they adjust the speed at which they grow? Third, if the size of a cell depends on its pole age, how does the distribution of cell size in a population change over time? We are addressing these questions using a combination of long-term time-lapse microscopy, single cell image analysis, statistical analysis, and individual-based theoretical modeling.

Our main finding is that, in M. extorquens AM1, the pole age of each cell, and therefore its cell division history, influences cell size and the interdivision interval. For reference, we also analyzed cell size and interdivision intervals in the gamma-proteobacterium Escherichia coli, and found no substantial effect of pole age on these traits in this bacterium.
Materials and Methods

Strains. Experiments were performed with *Methylobacterium extorquens* AM1 (19) and with *Escherichia coli* MG1655 (6).

Growth conditions. Strains were grown in minimal medium (11) with 120 mM methanol at 30°C (*M. extorquens*) or in LB at 37°C (*E. coli*) on a shaker.

Microscopy. We used an automated Olympus BX81 microscope with a UPLFLN100xO2PH/1.3 phase contrast oil lens for time-lapse experiments; sample and microscope were kept at 30°C (*M. extorquens*) and 37°C (*E. coli*) with a Cube and Box incubation system (Life Imaging Services, Reinach, Switzerland). To prepare agar pads for time lapse analysis, we melted about 20 ml of solidified medium (minimal medium (11) with 1.5% agar for *M. extorquens* and LB with 1.5% agar for *E. coli*) in a microwave oven. For experiments with *M. extorquens*, we added 120 mM methanol after melting the medium. Then, we filled the cavity of a sterile microscope cavity slide (cavity diameter 20 mm, depth 0.5 mm; Karl Hecht GmbH, Sondheim, Germany) with 200 µl of melted medium, and covered it with a sterile cover slip to attain a flat surface. The cavity slide was transferred to a fridge for five minutes to allow the agar to solidify. We removed the cover slip, and used a scalpel to cut and remove all agar except a small pad in the middle of the cavity with a quadratic cross-section (ca. 4*4 mm) and a height of 0.5 mm. Then, we inoculated the pad with bacteria from liquid cultures as described in the next paragraph.
We used strains in exponential growth for time-lapse microscopy: a single colony of *M. extorquens* AM1 was used to inoculate 2ml of minimal medium and grown for 20 hours. *E. coli* cultures were diluted 1:100 from a stationary overnight culture into fresh LB medium and grown for approximately 150 minutes. Then, the cultures from the two strains were diluted 1:500, and 1 µl of the diluted culture was transferred to the surface of previously prepared agar pads. The agar pad was closed with a cover slip and sealed with vacuum grease (Glissel; Borer Chemie AG, Switzerland). Under these conditions, cells can grow exponentially as a monolayer (25). We took images every 20 minutes (*M. extorquens*) or 4 minutes (*E. coli*).

**Image analysis.** Microscopy pictures were analyzed with the Matlab based program ‘Schnitzcell’ (23) (kindly provided by Michael Elowitz). Schnitzcell was used to segment the images, identify cells, and track cells over consecutive images. To extract the pole age, we extended Schnitzcell with Matlab scripts.

**Statistical analysis.** Statistical analysis was performed with SPSS Statistics 19.0.0 (SPSS Inc.). We used SPSS procedure GLM to analyze effects of pole age on cell size and the timing of cell division.

**Individual-based modeling.** We wrote an individual-based model in the programming language PERL. In the model, each individual was characterized by the age of its cell pole, and by the cell pole age of its mother at the time the individual was produced. The interval between divisions depended on the state of these two variables; the interval for each combination of cell pole age
and cell pole age of the mother was based on estimates retrieved from the data presented in Fig. 2 and 3. Individuals divided exactly after the time interval corresponding to their states of the two variables, without variation around the time interval. The program kept track of the time since the last cell division for each individual. The population was updated in time steps of five minutes. At each time step, the time since the last division increased by 5 minutes for each individual, and the individuals whose time since the last division corresponded to their interdivision interval divided to produce two cells, one with a pole age of one, the other with a pole age that exceeded the pole age of the individual that initiated division by one. The population was initialized with one individual, and grew exponentially. Once it reached a size of 10,000 individuals, population regulation was imposed at each time step by removing individuals at random (i.e., irrespective of their state or time since last cell division) from the population. The population reached stable age distribution after about 50 hours. The frequency of the different age-classes was extracted by averaging the frequencies over the time steps from hour 80 to hour 160. To estimate the distribution of cell size in a population at stable age distribution, we used the data on the relationship between pole age and cell size presented in Fig. 2 and 3.

**Results and Discussion**

We used time-lapse microscopy to follow cells growing, dividing and forming clonal microcolonies. Image analysis software allowed the tracking of
individual cells over successive divisions, so that we could extract the lineage
tree of clonal families that were initiated by one single cell (Fig. 1).

Our goal here was to analyze how cell size and the timing of cell division
depends on the age of an individual’s cell pole, or, more precisely, as the age
of its older cell pole. In rod-shaped bacteria like *M. extorquens* and *E. coli*,
every cell has two poles. One pole was formed at the last division; since each
cell has such a newly formed pole, this pole does not contribute to differences
between cells. The other pole is older; we use this other pole to identify
‘individuals’, and to define the age of these individuals.

Of the two cells emerging from a division, one cell has a pole of age one. We
refer to this cell as a new ‘individual’ (Fig. 1). It then grows, and when it
divides, its age-defining pole segregates to one cell (and this pole has now age
two); the other cell emerging from division has a cell pole of age one. The cell
with the age-defining pole has the same cell pole as the cell that underwent
division; we thus refer to these cells as the same ‘individual’ (Fig. 1B). This
individual can be referred to as the ‘mother’ of the other cell that emerged
from division. This other cell has pole age one, and we refer to it as the
‘daughter’ of the first individual. It is important to note that our results and
conclusions do not depend on this terminology; one can formulate all
conclusions simply in terms of the pole age of cells.
Visual inspection of the lineage trees gives a first impression into how the timing of cell division depends on pole age: in *M. extorquens*, cells with new poles (branching off to the left) have longer time intervals to the next division than cells with older poles (Fig. 1A). In *E. coli*, no such effect is discernible (Fig. 1D). To analyze this more systematically, we combined the data from different lineage trees, separated the cells according to pole age, and plotted each pole age group separately (Fig. 2). In *M. extorquens* (Fig. 2A), but not in *E. coli* (Fig. 2D), the age of the cell pole influences cell size (after as well as before division), and it affects the timing of cell division. Cells with new poles, of age one, are born small, just above 2 µm (Fig. 2A). They take longer until they divide for the first time, but this extra time is not enough to compensate for their small size; they enter division at a smaller size than cells with older poles. Cells with increasingly older poles are increasingly larger when they are formed, and they reach the next division increasingly faster. The experiments with *E. coli* did not provide any evidence for such effects of pole age on cell size and interdivision intervals (the effects reported in earlier studies (25) (32) on rates of growth and survival would only manifest for cells with older poles); this is consistent with the notion that the effects we observed in *M. extorquens* are specific, rather than being a consequence of the experimental set-up.

While cells of different pole ages differ in the timing of cell division, they do not differ in the growth dynamics. Analyzing changes in cell length over time,
at the single-cell level, we found that the rate of length increase is
indistinguishable between cells of different pole ages (Duncan post-hoc test;
p= 0.309), and there is no evidence that new-pole cells would initially not
grow for a period of time (Fig. S1); in other words, in contrast to *C.
crescentus*, *M. extorquens* did not show evidence for a ‘juvenile phase’
characterized by a reduced cellular growth rate.

These results are based on experiments that do not maintain constant
conditions – the microenvironment of the cells might potentially change due
to an increase in the number of cells. It is thus important to ask whether the
results still hold when correcting for possible changes in the course of the
experiment. We corrected the size and the interdivision interval of each cell
by normalizing to all other cells that were observed at the same time in the
growing microcolony. These corrected measurements again show that cell size
increases with increasing pole age, and that the interdivision interval
decreases, and that these differences are statistically significant (Fig 3A-C).

This analysis with normalized values produced another interesting and
important insight: the size of a cell not only depends on the age of its own
pole, it also depends on the pole age of its mother at the time the cell was
produced. Cells that were produced by mothers with young poles are smaller,
and they have longer interdivision intervals, than cells produced by mothers
with older poles (Fig. 3D-F). This indicates that pole age of a cell not only affects its own properties, but that these effects extend beyond generations.

Given our observation that the size and the timing of cell division depend on the pole age of each cell, and on the pole age of its mother – can *M. extorquens* maintain a stable average cell size over time? Do such dependencies of cell size on pole age lead to size distribution in the population that is constant over many generations, or rather to a situation where the cell size distribution continues to change over successive generations? The latter case would suggest that the effects we observed can only be transient – that these effects of pole age on cell size might have been induced by our experimental conditions, and that populations governed by such effect would not be stable over long times. We used a theoretical analysis to investigate this.

There is a comprehensive mathematical framework for describing processes in populations that consist of individuals of different ages, and where reproduction (and possibly survival) of individuals changes with age (7). One general result is that such populations typically will converge to a stable age distribution; once populations reach this stage, the fractions of individuals of different ages will remain constant over time (7). Also, populations at stable age distributions will then grow exponentially at a constant rate, since they consist of constant proportions of individuals with different rates of reproduction and survival (7).
In *M. extorquens*, the properties of an individual – its size and cell division timing – does not only depend on its own pole age; it also depends on the pole age of its mother (Fig. 3D-F). This makes the situation more complicated than traditional models of age-structured populations, and the results derived from these models are not directly applicable. It is therefore not clear whether such dependencies of cell size on pole ages lead to populations that are stable over long time scales. To investigate this, we constructed an individual-based numerical model to analyze the dynamics of different age classes. In this model, each individual was characterized by its pole age, and by the pole age that its mother had when the individual was produced. These two quantities determined the time to division and the size of each cell (the parameters in the model were based on the experimental data of Fig. 3). We initiated these individual-based models with a single individual, and let the population grow exponentially until it reached a certain population size, which we then maintained by removing individuals at random from the population as new individuals were born (similar to the situation in a chemostat).

The main result of this model is that these simulated populations of *M. extorquens* rapidly reach an equilibrium where the different types of individuals co-exist in stable frequencies; 26.0% of all individuals have pole age one and a mother with pole age one. 12.5% have pole age one and a mother with pole age two. 11.0% have pole age two and a mother with pole age one.
age one, and so on (Fig. 4). If, for comparison, we model a population where all classes have the same time to division (i.e., a situation where the pole age of an individual, and the pole age of its mother, have no influence on the time to division), then we find that these frequencies are 25% for individuals with pole age one and a mother with pole age one, 12.5% for individuals with pole age one and a mother with pole age two, 12.4% for individuals with pole age two and a mother with pole age one, and so on (i.e., the frequency of individuals with pole age n and a mother of pole age m is about $2^{-(m+n)}$; details not shown). The simulated *M. extorquens* populations differ from these simple pattern because individuals in some classes take longer until they divide and thus move to the next class; these classes are thus more numerous than other classes in which individuals reach the next division more quickly. The differences in age-distributions between the two models are statistically significant at $p<0.001$, except for the number of individuals with pole age one and a mother with pole age two, which is very similar. The standard errors of the means are smaller than 0.05% for all the estimated frequencies reported above, based on ten different runs of the numerical model.

That different types of individuals reach stable frequencies has an important consequence: the distribution of cell size and the population growth rate also reach equilibrium. At this equilibrium, *M. extorquens* populations consist of a mixture of small, intermediate, and large cells – but the proportion of cells in different size classes does not change over time. As an example, Fig. 4B shows
the theoretical stable distribution of cell size just after division. These results show that, despite the dependence of an individual’s size on its pole age and the pole age of its mother, cells do not continuously become smaller, but reach a stable age distribution.

In conclusion, our analysis shows that a substantial fraction of the variation in cell size and the timing of division in clonal populations of *M. extorquens* AM1 result from differences in pole age between individuals, and from differences in the pole age of the cells that produced these individuals. This result is interesting in the light of a series of recent studies reporting phenotypic variation between genetically identical bacteria that live in homogeneous environments (4, 9, 10, 22, 24, 26-28). These differences include cell size, interdivision intervals, gene expression, and behavior. Usually, this variation is attributed to stochastic aspects of biology – the fact that many proteins, mRNA species, and other biological molecules occur in small numbers (9, 18). As a consequence, the concentration of these molecules varies over time, and between cells, and can thus lead to variation between genetically identical individuals. Our results suggest that, at least in some systems, not all variation between cells needs to be stochastic in nature; differences between individuals in the age of their cell poles is a regular, deterministic processes that can also affect biological properties, and lead to differences between individuals. It will be interesting to see whether the distribution of pole ages explains some of the phenotypic variation that has been observed in other
systems. However, it should also be noted that not all variation in cell size and interdivision in *M. extorquens* is based on differences in pole age: previous studies that followed single cells over several generations have shown substantial variation in these traits that cannot be attributed to pole age effects (27).

While our discussion so far has focused on the molecular and cellular processes that lead to phenotypic heterogeneity in clonal populations, a second important aspect of heterogeneity is its biological significance, and possible benefits that it can confer to clonal populations. There is evidence that phenotypic variation in clonal populations can be adaptive, by allowing bacteria to cope with fluctuating environments (1, 5) or by promoting interactions between different phenotypes (4, 17). For the variation in cell size and interdivision intervals reported in this study, the physiological significance and possible benefits for the clonal populations are not clear, and would need to be addressed by future studies.

We see two main questions emerging from these results. The first question is about possible functional consequences of pole age differences between individuals. Does pole age only affect cell size and the timing of cell division, or does it also influence other biological traits of a cell – gene expression, metabolism, and behavior?. In the alpha-proteobacterium *C. crescentus*, cells with old and young cell poles show functional differences and different
behavior (14, 21); do other alpha-proteobacteria show similar, although maybe more subtle, effects? And how does pole age affect biological traits in other types of prokaryotes, outside of the alpha-proteobacteria? Pole age has been shown to affect rates of growth (25, 30) and survival (32), and the localization of proteins (20) and protein aggregates (16, 33); it will be interesting to look for other effects of pole age on the single-cell gene level, for example effects on gene expression.

A second question is about the coupling of cell size and cell division. We found that *M. extorquens* cells do not divide at a constant size; cells with young poles are smaller when they divide than cells with older poles. They also do not divide after a constant time interval; cells with young poles take longer until they divide than cells with older poles. This suggests that cell division is controlled by a rule that integrates size and time, or, alternatively, by a rule that changes with pole age. Analyzing cell division control in *M. extorquens* and in other alpha-proteobacteria might thus lead to interesting new insights into the rules that control cell division, and that guarantee cell size homoestasis.

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References


Figure Captions

Figure 1: Representative lineage trees of *M. extorquens* (top) and *E. coli* (bottom). Each lineage tree is based on a time lapse microscopy experiment: a single cell (represented at the bottom of each lineage tree) was placed on a pad of nutrient medium under the microscope, and grown into a clonal population. Images were recorded every 20 minutes for *M. extorquens*, and every 4 minutes for *E. coli*, and the lineage tree of the clonal population was reconstructed from these images. The lineage trees are ordered according to pole age. At each cell division (represented by branching events in the lineage tree), the branch that represents the cell with the new cell pole extends to the left; the branch that represents the cell with the older pole extends to the right (for the first cell division, at the bottom of each lineage tree, the old and new pole cannot be distinguished; these branches are thus dashed). (A) Old- and new-pole cells of *M. extorquens* differ in the inter-division intervals; branches that extend to the left tend to be longer than branches that extend to the right, indicating that new-pole cells take more time than old-pole cells until they initiate the next division. (B) In order to illustrate the pole age nomenclature, a section (section a1; framed by the dashed line) in the *M. extorquens* lineage tree is magnified. Each color in the magnified section denotes one individual (A, A’ and A’’ is the same individual at increasing age; B is the daughter of A; see the main text for our definition of ‘individual’ and ‘daughter’). Individuals at different ages are represented by rounded rectangles; the number on the right side in each rounded rectangle denotes the...
age of the older (age-defining) pole of this individual; the number on the left
side (in grey) denotes the age of the other pole, which is zero for all cells. (C)
Magnification of section a2 illustrates cell pole age during cell division. Cells
are labeled as in panels (A) and (B). Individual B is flipped horizontally
compared to panel (B), in order to show the orientation of the cell poles as a
cell emerges from division. (D) In *E. coli*, no systematic differences between
old- and new-pole cells manifest in the lineage tree.

Figure 2: Cell size, and the timing of cell division, as a function of the age of a
cell’s pole. Information from eight independent lineage trees for *M.
*extorquens* (left; based on eight independent microcolonies with a total of 1619
cell divisions) and three independent lineage trees for *E. coli* (right; based on
three independent microcolonies with a total of 842 cell divisions) was
separated according to the pole age of each cell. Each line depicts information
for cells with poles of a particular age. The left end of each line depicts the
moment when cells of a particular pole-age are formed by division. The y-
position of this end is the cell size after division, the x-position is the time
since division, which is zero. The right end depicts the moment just before a
cell divides again. The y-position is the size prior to division, the x-position is
the time since the last division (i.e., the inter-division interval). The
numbered arrows mark the following events: (1) birth of a cell with a cell pole
of age one; (2) division of a cell with a cell pole of age one; (3) formation of a
cell with a cell pole of age two. Error bars are standard error of the mean, with lineage trees as units of replication. In *M. extorquens*, an individual’s pole age affects its cell size, and the interval between divisions. In *E. coli*, no such effects are discernible.

Figure 3: Detailed analysis of the size after and before division, and of the interdivisional interval, for *M. extorquens*. To account for possible (pole-age-unrelated) changes of cell size and interdivison intervals during the growth of a microcolony, we calculated for each cell how much these traits differ from all other cells that were present at the same stage of microcolony growth. These differences are ‘residuals’, and the corrected traits are ‘residual cell size after division’, ‘residual cell size before next division’, and ‘residual time to division’. The patterns that we observed in Fig. 2 are robust: cell size after division (A) and just before the next division (B) increases with increasing age of a cell’s pole, and the interdivision interval decreases (C). These patterns are statistically significant: the letters (above each column) label homogeneous subsets, based on a Ducan post-hoc test, with lineage trees as units of replication. Columns with different letters are statistically significantly different. Panels D-F focus on cells with a new cell pole (of age one), and ask whether the cell pole age of these cell’s mothers have an effect on their properties. Cells that are produced by mothers with young poles are born smaller (D), and they take longer until they divide again (F); there are no consistent effects on their size prior to the next division (E).
Figure 4: Stable age distribution in *M. extorquens* populations, as predicted by a theoretical model. A population of 10’000 individuals of *M. extorquens* was simulated in an individual-based model. Within a time-span corresponding to about 50 hours, the fraction of individuals in different age-classes reaches equilibrium. (A) The stable distribution of individuals in different classes, where each class is characterized by the pole age of cells (first horizontal axis), and by the pole age of the mothers of these cells, at the moment the cells were produced (second horizontal axis). Most individuals have pole age one and a mother of pole age one. (B) The distribution of cell size after division in a population in stable age equilibrium. According to the results of the theoretical model, the fraction of cells in different size classes remains constant once a population reaches the age equilibrium.

Figure S1: Single cell growth patterns of cells with different pole ages. We analyzed growth patterns of 514 individual cells from five lineages, based on time-lapse experiments with three frames per hour. For each cell, we log-transformed the cell length at each time point (each 20 minutes). To allow for comparisons of growth patterns between cells, we standardized the log-transformed maximal cell length and the interdivision interval to 1. The six panels show the growth patterns of cells of pole ages one to six (15.5 % of the cells with a coefficient of determination for a quadratic regression $R^2<0.98$ were excluded). We calculated the average quadratic term of the quadratic