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Cumulative cell division time asymmetry in *Staphyloccus aureus*

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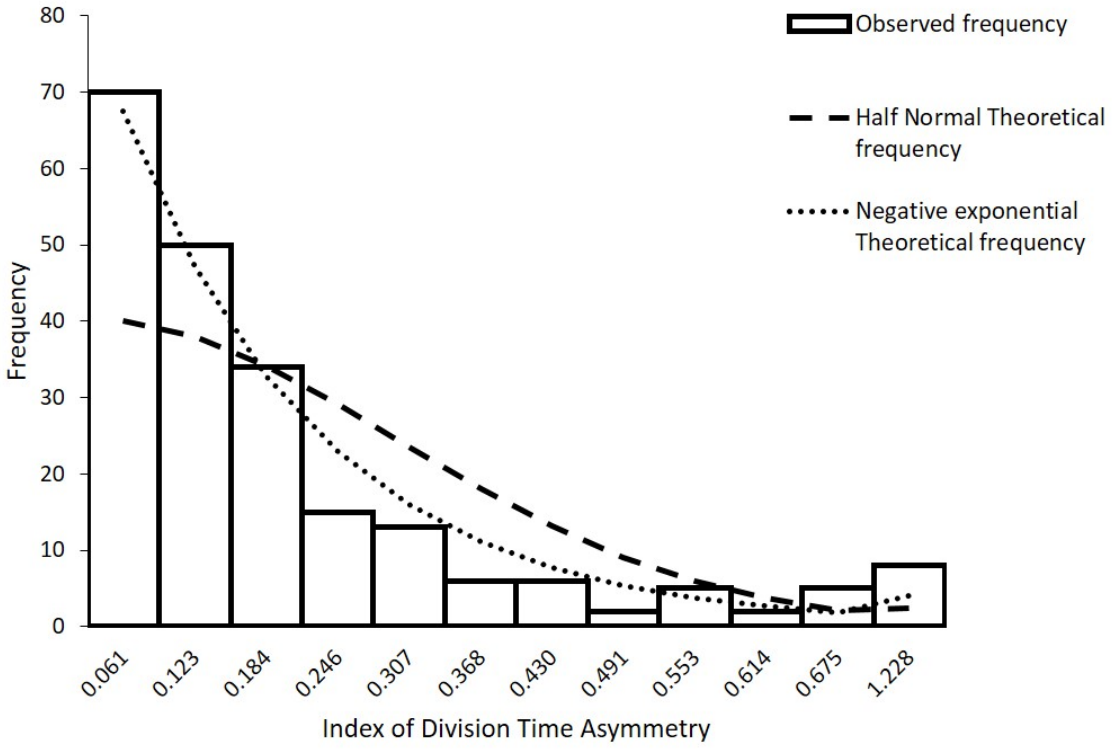
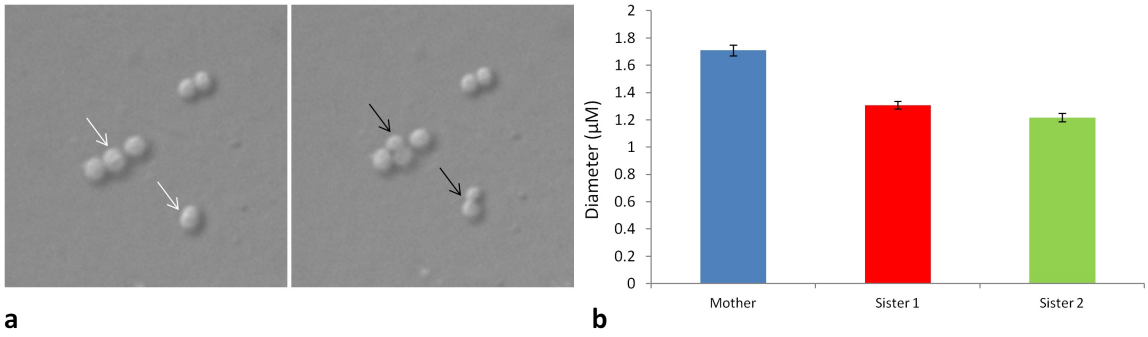
Abstract
Asymmetry of cell division involving asymmetric damage segregation is shown to be linked to cellular senescence in bacteria. According to the current mainstream thinking, the asymmetry is observed along the old and new pole cells, wherein old pole cells accumulate damage and eventually succumb to senescence, while giving rise to fresh new pole cells at every division. If the old pole-new pole axis is central to cell division asymmetry, and thereby aging, it would be interesting to see whether cumulative cell division asymmetry is seen in spherical organisms such as Staphylococci that change the plane of division at every cycle and therefore may not have polarity. We show here that in growing microcolonies of *Staphylococuus aureus*, two daughter cells produced by one cell division show difference in the time taken for further division. This asymmetry is cumulative, giving rise to a frequency distribution of asymmetry which is significantly different from the distribution expected by stochastic asymmetry. Demonstration of cumulative cell division asymmetry in *S. aureus* suggests that functional asymmetry in cell division can exist independent of the old pole-new pole axis.

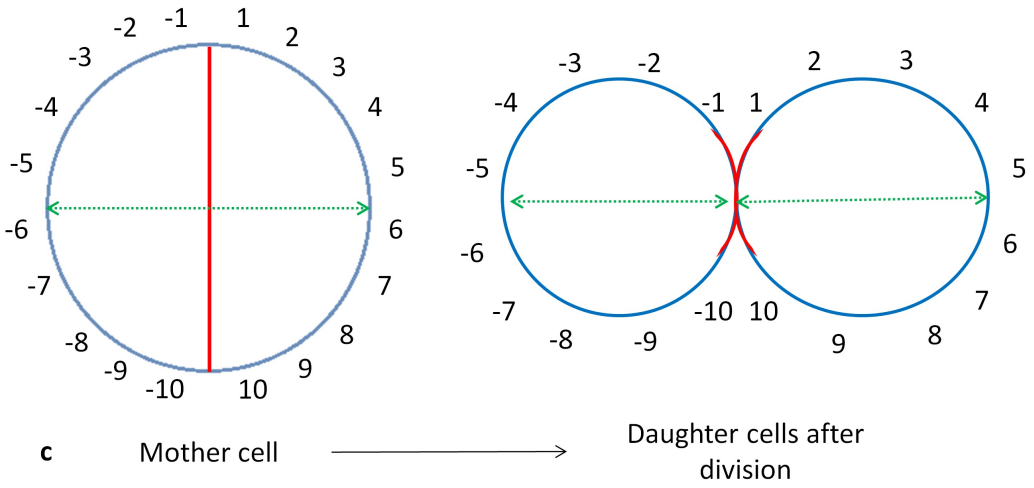
Introduction
Aging was first demonstrated in prosthecate bacteria like *Caulobacter* sp that have a morphological and functional asymmetry in division [1]. Experiments in morphologically symmetric rod-shaped *Escherichia coli* showed that the cell inheriting old pole suffered from aging and exhibited a decreased growth rate, retarded division cycle, and an increased probability of death [2] [3] [4] [5] [6]. *Bacillus subtilis* showed similar asymmetry with endospores being preferably formed at the old pole end [7] [8]. The asymmetry in cell division is assumed to be due to asymmetric damage segregation, a major component of which is aggregated proteins [9] [3]. Protein aggregates are observed to occupy polar positions very frequently, though not invariably [6]. Experiments using a microfluidic device raised an apparent challenge to the theory of bacterial aging, showing that the growth rates of the mother cell (old pole) remained consistent over a long time contrary to expectations, but the probability of cell death increased [10]. On a different note, Turke (2008) raised the possibility that if the old pole-new pole axis (OPNPA) is central and critical to asymmetric division and aging in bacteria, then spherical organisms that change their plane of division and thereby do not have a fixed OPNPA could be immune to aging [11]. Testing this possibility empirically will help understanding the relationship between cell polarity and aging more clearly.

Objective
To explore functional asymmetry in cell division of spherical cells of *Staphyclococcus aureus.*


Observed frequency
Half Normal Theoretical frequency
Negative exponential Theoretical frequency
Frequency
80
70
60
50
40
30
20
10
0
0.061
0.123
0.184
0.246
0.307
0.368
0.430
0.491
0.553
0.614
0.675
1.228
Index of Division Time Asymmetry

a


a
b
Diameter (µM)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Mother
Sister 1
Sister 2


c
Mother cell
Daughter cells after division

b

Figure Legend

Figure 1.

The distribution of division time asymmetry indices (n= 223, Number of frequency classes= 12). The bars represent the observed frequencies. The lines are best fit expected distributions for half normal (Dashed line) (X_2= 63.044, p <0.05) and negative exponential (Round dotted line) (X_2= 17.722, p >0.05). Chi square goodness of fit rejects a half normal distribution and fits a negative exponential distribution which is expected by the cumulative asymmetry dynamics.

Figure 2. Sizes of *S. aureus* cells before and after division.

(A) Differential interference contrast images of *S. aureus* during early growth on agar medium. White arrows indicate cells before division and black arrows indicate daughter cells immediately after their division. Note that the daughter cells are smaller in diameter than the mother cell and there is size difference between sister cells as well.

(B) Bar diagram showing mean maximum diameter of cells parallel to the plane of division. Blue, red and green bars represent diameter of the cells before division (mother cell), larger and smaller one of the daughter cells respectively. The mean diameter of daughter cells was 20% smaller than the mother cell. Paired t-test; n= 20, p <0.0001.

(C) Change in the physical location of cell envelop positions owing to change in diameter after division. In the daughter cells red portions represent newly formed cell wall. Values -1 to 10 represent equidistant positions on cell envelop before and after division. Note, the inevitable change in envelop positions relative to the plane of division accompanying change in cell size. Since there is no fixed envelop position with respect to plane of division, even if we assume strictly orthogonal planes of division, there cannot be any consistent envelop position comparable to a pole in rod-shaped organisms.

Microscopy and cell division observations

The wild-type strain of *Staphylococcus aureus* 2121, obtained from National Collection of Industrial Microorganisms (NCIM), Pune, was used for the study. Photomicrography was used to observe cell division in developing micro-colonies. A nutrient broth culture in its mid-exponential phase was poured on a sterile slide coated with nutrient agar medium (HiMedia). The slides were incubated at 37°C for up to 1 h prior to imaging. Imaging was done on an automated X–Y stage microscope (EVOS® FL Auto Cell Imaging System) equipped with EVOS® Onstage Incubator for controlled temperature and humidity, using Differential Interference Contrast (DIC) microscopy with an objective of 100X OEL (1.25NA). The software used for image analysis was EVOS® FL Cell Imaging System.

Index of cell division time asymmetry

For quantifying functional symmetry in cell division an index of cell division time asymmetry defined by Lele et al (2011) was used [4]. The method is based on the assumption that if the damage segregation was asymmetric, the daughter cell receiving damaged components will take longer to complete the next cell division cycle. Therefore an index of asymmetry was defined as the ratio of the difference in division time (sign ignored) of two sister cells as to the average division time of the two. The index returns a value of zero if both sister cells complete their next division cycle at the same time. If the division of the slower of the two cells takes twice as much as the faster one the index takes a value of 2/3 and so on. The index is independent of the absolute time of division and does not reflect absolute growth rate.

Results & Discussion

Cell division time asymmetry in *S. aureus*

We observed microscopically cell divisions of *S. aureus* on agar surface to follow the fate of two daughter cells until their next division. The index of division time asymmetry used for *E. coli* was used for *S. aureus* division. The time of next division of two daughter cells was observed to be substantially different in *S. aureus* with a mean difference of 8.3 minutes which is 17% of the mean doubling time observed. This indicates that there is substantial division time asymmetry between two daughter cells in *S. aureus*. However, owing to the absence of obvious poles and a different pattern of microcolony

development, the statistical inference of *S. aureus* data needs to be treated differently than that of *E coli.*

In *E. coli* the development of a microcolony takes place on a single plane for at least a few generations and therefore it is possible to keep a track of the clone for at least 5–6 generations. This enables keeping a track of old and new pole cells and showing that the division time of old pole cells differs significantly from that of new pole cells. In *S. aureus,* since the planes of division are shifting in three dimensions, cells start overlapping quickly [12], making it difficult to follow individual cells in the clones for many consecutive generations. In order to answer whether *S. aureus* shows cumulative asymmetry in cell division, it is necessary to differentiate stochastic differences in cell division times from differences arising from asymmetric damage segregation. Differences arising from asymmetric damage segregation are cumulative, that is, the cell receiving damaged components is expected to go on becoming slower and slower over generations, thus cumulatively increasing the difference in the cell division times of two daughter cells. On the other hand, stochastic asymmetries are bound to arise in cell division. Even in the absence of asymmetric segregation, if the cell division is slightly off-centered, the larger of the daughter cells may grow and divide faster than the smaller one. This should be taken as a null model for any experiment on functional asymmetry related to aging. Since we assume the mean plane of division to be the centere of the cell and errors distributed around it, the asymmetry can be assumed to be normally distributed. However from the way we defined the index of cell division time asymmetry, the mean should be zero and only the positive half of the distribution will be recorded. Therefore according to the null model, the observed distribution should be "half- normal" or "mod-normal". On the other hand, if the asymmetry systematically accumulates as in the aging model, following the modified Leslie Matrix model of Watve et al (2006) we expect a negative exponential distribution of age classes and, therefore, a negative exponential distribution of cell division time asymmetry index [13]. We tested these two alternative models on the distributions of cell division time asymmetry indices and used the chi square goodness of fits using the software XLSTAT pro. Figure 1 shows that the indices of division time asymmetry followed a highly skewed distribution. The chi square goodness of fit rejected a half normal distribution and fitted a negative exponential distribution satisfactorily. Therefore evidently *S. aureus* shows cumulative cell division asymmetry. Cell division in *S. aureus* is jerky, violent and three-dimensional, affecting the relative positions of neighboring cells too and, as a result, it is difficult to follow cell lineages longitudinally. Nevertheless, in 14 cases where history of two subsequent generations was reliably traced, the hypothesis could be tested longitudinally. By the cumulative asymmetry hypothesis, one of the daughter cells should take longer to divide than its mother cell. On the other hand, the other daughter cell should take less or comparable time with the mother cell. Since the observed cells were freshly plated on nutrient agar bed for observations, the mean division time is expected to change rapidly in the first few generations that represent a transition to exponential phase. Therefore, instead of comparing absolute division times, we compared the asymmetry indices with which the mother cell was born with asymmetry indices of the further division of the two daughter cells. In the 14 triads compared, one of the daughter cells had an index greater than the mother in all cases. The other daughter cell had an index lower than the mother in 8 out of 14 cases. The former difference was statistically significant (Mann-Whitney U test; Median for mother cells= 0.07415; Median for the slower of the daughter cells= 0.24705; p= 0.003) whereas the latter was not significant (Mann-Whitney U test; Median for mother cells= 0.07415; Median for the other daughter cells= 0.05295; p= 0.241). Although the longitudinal data are limited, the results comply with the expectations of cumulative asymmetry very well.

Is there polarity in *S. aureus*?

The results can either be interpreted to mean either that the old pole-new pole axis (OPNPA) is not necessary for cell division asymmetry or that *S. aureus* also has functional OPNPA which needs to be demonstrated if present.

Cocci may have mechanisms of asymmetric damage segregation in the absence of a morphological pole [14]. Unlike rod-shaped bacteria, many cocci exhibit no obvious morphological polarity, but they can have functional poles if the plane of division is

constant. This is likely to be true for Streptococci. If the plane of division is strictly orthogonal, we expect a regular three-dimensional crystal-like lattice which is typical of organisms such as *Micrococcus luteus*. *S aureus* appear as clusters of cells without an obvious geometric arrangement. One possible interpretation of this arrangement is that the plane of division is randomly decided at every cycle leading to a three-dimensional irregular cluster. A more commonly held alternative view is that the planes of division are regular orthogonal [15] [16] [12] [17] and the growth of the new wall is at the plane of division [18] [19] [20]. This should lead to a crystal-like lattice, but the activity of lytic enzyme, that is responsible for the splitting of the division septum, appears to cause a post-fission movement of the cells [16]. The apparently random co-attachment of cells after the movement, rather than randomized planes of division, leads to the formation of irregular clusters [20]. If the planes of division are predictable and consistent in relation to certain positions along the cell envelope, then it is possible that even in the absence of a constant plane of division, certain positions along the cell envelop hold a constant geometric relationship with the division planes and they can be considered analogous to polar positions.

However there are two reasons to doubt the presence of such pole analogues in *S. aureus*. One is the observation that the diameter of cells parallel to the plane of division becomes smaller after division (Fig. 2A & 2B). Also often, if not always, there is a difference in the diameters of two sister cells immediately after division. A change in size necessitates a shift in the relative positions along the cell envelop as shown in figure 2C. A change in size implies that the envelop gets stretched or pulled during and after division which makes it difficult that some envelop positions have a constant spatial relationship with the orthogonal planes of division. The other problem is that the evidence for orthogonal planes of division comes from study of the peptidoglycan layer. The protein aggregates or other intracellular damaged components are more likely to have an association, if any, with the fluid cell membrane rather than the cell wall. Therefore demonstration of orthogonal planes of division at the peptidoglycan layer is not a convincing evidence for any fixed functional pole in *S. aureus*.

Conclusions

Currently, although there is evidence for orthogonal planes of division in *S. aureus*, there is no demonstration of OPNPA. In principle, OPNPA is not necessary for asymmetric damage segregation, and thereby cell senescence. If the damaged proteins or any other components have a tendency to aggregate, the aggregate would be located in one of the daughter cells by default. Whether it is inherited by the old pole or new pole cell may not have a central role in the evolution of division asymmetry. It is likely that in rod-shaped bacteria like *E. coli,* the association of aging with old pole is incidental, as damaged components are more frequently associated with the old pole. But there is no need to assume that OPNPA is essential for asymmetric damage segregation and aging in bacterial cells. Cellular senescence by asymmetric damage segregation thus appears to be a more fundamental process than cell polarity. This also raises the possibility that there is "pole flipping" with some probability, that is, the old components may drift to the morphological new pole randomly. Even if this probability is small, the mandatory association of morphological old pole with aging can break. This resolves the paradox raised by Wang et al (2010) [10].

Additional Information

Methods

Microscopy and cell division observations

The wild-type strain of *Staphylococcus aureus* 2121, obtained from National Collection of Industrial Microorganisms (NCIM), Pune, was used for the study. Photomicrography was used to observe cell division in developing micro-colonies. A nutrient broth culture in its mid-exponential phase was poured on a sterile slide coated with nutrient agar medium (HiMedia). The slides were incubated at 37℃ for up to 1 h prior to imaging. Imaging was done on an automated X–Y stage microscope (EVOS® FL Auto Cell

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Supplementary Material

Please see https://sciencematters.io/articles/201603000022.

Ethics Statement

Not applicable.

Citations

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