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HORIZONS

Explaining the causes of cell death in cyanobacteria: what role for asymmetric division?

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Cyanobacteria contribute a significant fraction of global primary production and are therefore of great ecological significance. An individual cyanobacteria cell has four potential fates: to divide, perhaps after a dormant period, to be eaten, to undergo viral lysis or to undergo cell death. In some studies, cyanobacteria cell death has been classified as programmed cell death, borrowing a concept more widely known in metazoan cells, and there are various biochemical parallels to support such a categorization. However, at the same time, there is a growing awareness of asymmetric division as a fundamental process in bacterial division which can result in non-equal daughter cells with differing fitness. Thanks to recent theoretical and experimental advances, it is now possible to explore cyanobacteria cell death in the light of asymmetric division and to test hypotheses on the ultimate causes of cyanobacterial cell death. Assessing the degree of protein damage within individual cells during population growth is a sensible initial research target as is the application of techniques which allow the tracking of cell lineages. The existence of asymmetric division in cyanobacteria is likely given its suggested ubiquity across the bacterial

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domain of life. It will be technically difficult to test the interaction of asymmetric division with environmental variability, and how that leads to individual cell death via differing susceptibilities to environmental stress. However, testing such ideas could confirm asymmetric division as the ultimate cause of cell death in cyanobacteria and thereby allow a better understanding of the patterns of cell death in natural populations.

KEYWORDS: cyanobacteria; asymmetric division; irradiance; oxidative damage; PCD

How phytoplankton die largely determines how other marine organisms live (Kirchman 1999).

INTRODUCTION

Since the ubiquity of cyanobacteria in marine ecosystems was recognized about 30 years ago the vast, and increasing, contribution of oceanic forms such as *Prochlorococcus* and *Synechococcus* to global primary production has become clear (Polovina *et al.*, 2008; Flombaum *et al.*, 2013). Given this, it is important to know why and how individual cyanobacteria die since this process will profoundly influence carbon flow within the food web and thus the overall structure of the food web. While cyanobacterial cells may be eaten by a diverse selection of zooplankton grazers (e.g. Hirose *et al.*, 2008), or undergo viral lysis (Munn, 2011), a third potential non-dividing fate, cell death, is possible though poorly appreciated, perhaps stemming from the fact that bacteria are often generally assumed to be functionally immortal. Cell death in cyanobacteria will lead to cell lysis and the release of dissolved organic carbon, an outcome similar to that of viral infection and lysis. Although bacterial cell death is, in general terms, difficult to recognize and measure (Davey, 2011) understanding it better will be essential in forming a more accurate picture of carbon flow. For a long time, bulk measures of bacterioplankton (heterotrophic bacteria) growth have indicated large numbers of inactive cells within populations which has led to the conclusion that more effort should be devoted to resolving the demographic structure of bacterioplankton assemblages (Kirchman, 2000). Such a conclusion would also hold true for populations of autotrophic cyanobacteria; forms such as *Synechococcus* can enter a resting state (Sauer *et al.*, 2001; Schwarz and Forchhammer, 2005) in response to nutrient limitation and so high variability in *Synechococcus* activity rates are possible. However, where environmental stress exceeds cell tolerances cell death is observed and in certain situations this has been categorized as programmed cell death (PCD). Occasionally, an adaptive role has been suggested for cyanobacteria PCD whereby the death of some individuals optimizes the probability of population persistence (e.g. Berman-Frank *et al.*, 2004). The diagnosis of PCD in studies of cyanobacteria ecology, like phytoplankton in general (Franklin *et al.*, 2006), is made with variable amounts of evidence, but relies mainly upon a demonstration of increased caspase (protease) activity during cell death, as caspases are thought to be a metabolic signature of PCD. Many commercial kits are now available for testing caspase activity and since caspase activity is thought to lead to DNA fragmentation, the detection of DNA fragments (via the TUNEL assay) is a further diagnostic feature of programmed cell death. In this article the proposed causes of cyanobacteria cell death are explored and it is argued that one fundamental process, asymmetric division, could explain the forms of cell death observed in diverse settings.

IS PROGRAMMED CELL DEATH (PCD) AN IMPORTANT PART OF BACTERIAL AND CYANOBACTERIAL POPULATION ECOLOGY?

The existence of PCD in cyanobacteria is uncertain. In those forms where differentiation within a colony occurs in order to facilitate dispersal, or produce heterocysts, cell death can appear to have a programmed dimension (e.g. Reddy *et al.*, 1987; Adamec *et al.*, 2005) and has been further diagnosed, via caspase and DNA fragmentation assays, as "autocatalytic" in dying cells (e.g. in *Trichodesmium*; Berman-Frank *et al.*, 2004). Differentiation within a cyanobacteria colony in order to optimize overall colony fitness is a situation where PCD would appear to make evolutionary sense for bacteria.

However, in another colonial form, *Microcystis aeruginosa*, the significance of DNA fragmentation (and therefore PCD) is less clear as fragmentation occurs seemingly randomly, in terms of colony position and cell size, during periods of population decline (Sigee *et al.*, 2007). Whilst the existence of, and a role for, PCD in colony dynamics may sometimes be inferred, for solitary cyanobacteria the evolutionary basis for PCD would be much less clear. In heterotrophic bacteria, "programmed death" diagnosed mainly on the basis of caspase (protease) activities is thought to be important in the various differentiation processes that occur during colony or biofilm existence (Lewis, 2000). In *Microcystis aeruginosa*, PCD has also been diagnosed through experiments which expose cells to oxidants (Ross *et al.*, 2006; Ding *et al.*, 2012; Mikula *et al.*, 2012); as cells are poisoned with exogenous oxidants dying cells exhibit markers of PCD such as increased caspase activity. By extension, environmental factors which would induce intracellular oxidant formation are thought to lead to PCD (Ross *et al.*, 2006; Guo *et al.*, 2012; Moon *et al.*, 2012). While cyanobacteria do have metacaspase-like proteases (Choi and Berges, 2012) the degree to which caspase expression, activity and specificity can be associated with dying cells varies considerably depending upon the sophistication of the methods used. Therefore, while PCD has been diagnosed in cyanobacteria the field of study is young and the burden of proof is quite variable. Across the studies which diagnose PCD as a cause of cyanobacteria cell death, there are differing interpretations of why such a pathway might have evolved. For some authors, PCD amounts to little more than a morphological description of dead or dying cells. In this interpretation, cells have simply been killed when their metabolic apparatus is pushed beyond its limits and PCD markers have little specificity. However, being killed by environmental stress is not the only interpretation of what is happening during cyanobacteria population declines. In *Trichodesmium* colonies, some fraction of the cells (about 30%) survive during Fe-stress-induced mass cell death, which can be diagnosed as PCD on the basis of caspase activities and metacaspase expression (Bar-Zeev *et al.*, 2013), and it is speculated that intercellular signalling may control the onset of such mass cell death. Such an interpretation may be inconsistent with an alternative view, namely that when growth becomes uncoupled from metabolism (i.e. though nutrient limitation), the generation of intracellular oxidants kills the cells (Sakamoto *et al.*, 1998; Suginaka *et al.*, 1999), if one accepts that cyanobacteria populations have within them a "persister" fraction which is more resistant to environmental stress than the majority of the population. Although the "persister" concept is used in the wider bacteriological literature as a mechanism which facilitates population differentiation through PCD (Lewis, 2000), its use as an explanatory concept in cyanobacteria ecology has been limited.

In conclusion, although the PCD concept has recently been applied to explain cyanobacteria cell death its validity is not always clear. Given the role of PCD in metazoan development, it is tempting to seek an adaptive role for PCD in bacteria and in those instances where differentiation is important ecologically there would appear to be sound reasons for PCD, presumably controlled by intercellular signalling. In general, such signalling likely controls the widespread differentiation that occurs within populations of genetically identical bacteria (Muller and Davey, 2009). However, beyond PCD, and possibly with a more fundamental role in shaping patterns of cell death, exists the process of asymmetric division.

EVIDENCE FOR ASYMMETRIC DIVISION, AND CELL AGING, IN BACTERIA AND THEREFORE CYANOBACTERIA

Morphological and physiological differences between daughter cyanobacterial cells have been known for some time (Carr, 1995), for example in the chain-forming *Anabaena catenula*, division within a colony always results in one large and one smaller cell. The smaller cell subsequently takes longer to divide and may further differentiate into a heterocyst (Mitchison and Wilcox, 1972). In this example, asymmetric division has a clear role in colony differentiation. More fundamentally though, asymmetric division may lead to the routine generation of daughter cells with differing fitness. In *Escherichia coli*, the two daughter cells that are produced during mitosis are functionally asymmetric; one daughter cell is aged, in the sense that its lineage will show reduced fitness (an increased probability of death) relative to the other daughter cell (Stewart *et al.*, 2005). Consequently, *E. coli* is not functionally immortal. Using one daughter cell to act as a repository for parental metabolic waste, leading to a rejuvenation or increased fitness of the other daughter, may be an optimal, and therefore ubiquitous, evolutionary strategy in bacteria as it can help to maintain a higher overall growth rate (Watve *et al.*, 2006; Ackermann *et al.*, 2007). Further modelling work has shown that a bacterial mother cell that allocates more non-genetic (oxidative damage) in one daughter cell has a higher evolutionary fitness (Chao, 2010; Rang *et al.*, 2011). Non-colonial cyanobacteria, such as *Prochlorococcus* and *Synechococcus* are known for their high growth rates (Vaulot *et al.*, 1995; Shalapyonok *et al.*, 1998) which

enable the formation of vast oceanic populations despite substantial grazing pressures (Boyd *et al.*, 2010). The idea that bacterial cell division may routinely produce dead cells as a by-product of division is not new but is perhaps poorly appreciated by ecologists. Older observations support the idea: in batch cultures, so long as the death rate is less than the growth rate, the viable population can be observed to grow exponentially and in chemostat culture, a viability index (the probability that a newly formed cell is viable) can be calculated in order to assess the extent of errors during "autosynthesis"' leading to the production of non-viable cells whose fate is "autolysis"' under different conditions (Pirt, 1975). It would seem reasonable to suppose that bacterial cells can be stillborn because those individuals represent the culmination of a process which distributes metabolic damage in one daughter cell over the other, as has now been demonstrated in *E. coli*. It is an open question as to how this process operates in cyanobacteria though it is a powerful concept which could have the potential to unify the mixed explanations of the causes of cyanobacteria cell death.

CYANOBACTERIA CELL GROWTH AND CELL DEATH IN THE OCEAN

In the ocean, cyanobacteria production is dominated by *Prochlorococcus* and *Synechococcus*. Together these two groups contribute up to 50% of total fixed carbon in low latitudes (Partensky *et al.*, 1999). In *Prochlorococcus* populations peak abundance ($\sim 150 \times 10^3$ cells mL^{-1}) occurs in the top 50 m of the water column and cells undergo a phased division close to the maximum potential growth rate (Vaulot *et al.*, 1995) with a division rate of >1 doubling day^{-1} possible (Shalapyonok *et al.*, 1998). Irradiance has long been known to exert a major control on cyanobacteria proliferation; excessive light can cause cell death (referred to as "photo-oxidative" cell death;, e.g. Eloff *et al.*, 1976, Vonshak *et al.*, 1996) and *Prochlorococcus* and *Synechococcus* have evolved slightly different strategies to minimize the light-induced oxidative stress which can be lethal (Mella-Flores *et al.*, 2012). Nevertheless, a substantial proportion of cyanobacteria undergo photo-oxidative stress and cell death in surface waters (Agusti, 2004); the proportion of *Prochlorococcus* and *Synechococcus* cells that are non-viable increases over the day and peaks in the late afternoon (Llabres *et al.*, 2011). Such high variability in the proportion of non-viable cyanobacteria cells clearly indicates intense population dynamics (Llabres *et al.*, 2011) which can have major ramifications for the heterotrophic food web (Gasol *et al.*, 1998). Cyanobacteria mortality in this case is closely linked with the cell division cycle and results in substantial losses from the population. Although the association of mortality with both division and irradiance is clear the mechanistic basis for cell death in these individuals is more difficult to decipher. One explanation is that cells perish due to an accumulation of irradiance-induced DNA damage which prevents transition past the G1 phase of the cell cycle (Llabres *et al.*, 2011). Such observations also raise the question: are the individuals who die in these situations individuals which have accumulated metabolic damage via asymmetric division? In *Prochlorococcus*, a process of extensive genomic streamlining (Partensky and Garczarek, 2010) has resulted in cells which have become metabolically dependent on the surrounding bacterial assemblage in a way that is only just becoming clear (Scanlan *et al.*, 2009; Morris *et al.*, 2011). Such a view of cyanobacteria biology indicates the minimalist strategy which forms such as *Prochlorococcus* have adopted, but is not necessarily inconsistent with asymmetric division influencing the fitness of individual cells. The previously mentioned studies concerning the general characteristics of bacterial growth in culture and asymmetric division in *E. coli* suggest that asymmetric division could also be an underlying and fundamental process in cyanobacteria. Experimental work to test these ideas is now becoming possible thanks to several experimental and technical advances.

HOW TO ASSESS ASYMMETRIC DIVISION, AND ITS CONSEQUENCES, IN CYANOBACTERIA; HYPOTHESES TO TEST

Protein damage is the mechanistic basis for declining fitness in cells that are produced via asymmetric division and this process operates across all domains of life. In eukaryote cells, the unequal accumulation of damaged and misfolded proteins during somatic cell mitosis is linked with periods when the capacity of cells to degrade such proteins is exceeded (Rujano *et al.*, 2006). In the diatom *Ditylum brightwelli*, the unequal inheritance of oxidative damage in daughter cells is also thought to lead to differing fitness (Laney *et al.*, 2012). Protein damage can be assessed by measuring protein carbonylation (which results from oxidative damage) and budding yeast mother cells retain oxidative damage in a form of division asymmetry (Aguilaniu *et al.*, 2003). Similarly, in bacteria (*E. coli*), damaged protein is aggregated into inclusion bodies which tend to an unequal allocation between

daughter cells (Lindner *et al.*, 2008). Given the importance of irradiance in cyanobacteria ecology (Scanlan *et al.*, 2009), protein carbonylation could be a useful signature of sublethal protein damage which might be asymmetrically allocated during subsequent division. There are currently two commercially available methods for assessing protein carbonylation; a "bulk" method involving homogenization of the entire population (e.g. the OxyBlot approach; Higo *et al.*, 2008) and a cell-specific (flow cytometric) method (e.g. Millipore FlowCellect). This second type of method has not yet been applied and validated for use in cyanobacteria. If cells with an elevated oxidized protein content were detectable with flow cytometry, then it would be possible to see if the phenotype of the cell (shape and size) was affected by the burden of oxidized protein. Such a relationship might be expected given that the development of cell morphology is dependent upon the coordinated activity of a number of proteins. An initial hypothesis would be that protein carbonylation increases over the cell cycle (carbonyl groups in G2 cells > G1 cells), and that this effect is more significant in cells which have recently been subject to an increase in irradiance as photoacclimation would be incomplete. Following on from this, the hypothesis that damaged protein can become aggregated into inclusion bodies which are asymmetrically allocated could be tested. Several types of inclusion bodies exist in cyanobacteria cells (Jensen, 1984) with the term inclusion body perhaps most frequently being used to refer to the structures handling the short-term storage of metabolic products, which can be a significant process in cyanobacteria ecology (e.g. Welsh *et al.*, 2008). It is not clear if the damaged protein which cannot be degraded any further is aggregated into inclusion bodies in cyanobacteria as it is in *E. coli* (Desplanq *et al.*, 2005). Therefore, molecular work is needed to test the hypothesis that cyanobacteria cells aggregate abnormal proteins into inclusion bodies, using the fluorescent reporter techniques similar to that developed for *E. coli* (Lindner *et al.*, 2008). If aggregation does occur then the next challenge would be to track the distribution of inclusion bodies during division, and to follow the fate of cells which receive a greater burden of inclusion bodies. Although this has been achieved in *E. coli* through a combination of advanced microscopy and image analysis techniques (Stewart *et al.*, 2005), it would be substantially more difficult to achieve in coccoid cyanobacteria as it would be less clear how the division poles are orientated within the cell. While lineage tracking would ultimately be necessary to test the role of asymmetric division in determining cell fate, assessing protein aggregation within cells would provide useful initial evidence. If protein aggregation and the accumulation of oxidized protein is found to be a significant process in cyanobacterial division, then exploring the possible interaction between oxidized protein content and phage infection, in addition to its effects on fitness, may also be possible.

CONCLUSIONS

In summary it is proposed that ultimately, there might be one major cause of cyanobacteria cell death; asymmetric division, leading to an accumulation of metabolic damage and therefore increased susceptibility to environmental stress. All other currently recognized causes could be a manifestation of this process; accumulated metabolic damage might eventually result in a set of morphological characteristics in dead or dying cells which is diagnosable as PCD and similarly, photo-oxidative cell death could result from metabolic failure brought about by lowered fitness. Photo-oxidative cell death may be frequently inferred, since it has a proximate and relatively easy to measure environmental cause. However, the ultimate driver of cell death could be asymmetric division leading to a differential burden of metabolic damage between cells at the outset of their existence. In recent years, various ideas have emerged in bacteriology which could help unify asymmetric division with cell aging as well as PCD. The challenge now is to examine the mechanistic basis for asymmetric division in cyanobacteria and to then examine its role in cyanobacteria population dynamics.

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