Multicellular behavior in bacteria: communication, cooperation, competition and cheating

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Summary

The sociobiology of bacteria, largely unappreciated and ignored by the microbiology research community two decades ago is now a major research area, catalyzed to a significant degree by studies of communication and cooperative behavior among the myxobacteria and in quorum sensing (QS) and biofilm formation by pseudomonads and other microbes. Recently, the topic of multicellular cooperative behaviors among bacteria has been increasingly considered in the context of evolutionary biology. Here we discuss the significance of two recent studies[1,2] of the phenomenon of ''cheating'' mutants and their exploitation of cooperating microbial populations of *Pseudomonas aeruginosa*. *BioEssays* 30:296–298, 2008. © 2008 Wiley Periodicals, Inc.

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The cumulative efforts of numerous microbiologists during the past 20 years provide a compelling argument for the role of communication and cooperative behavior of bacteria in activities ranging from production of antibiotics, to symbiotic interactions with vertebrate hosts, production of plant and animal diseases or developmental processes such as sporulation.[3] It is clear that extracellular signal molecules produced by microbial cells can be detected by other cells, and regulate expression of genes. In many cases, these genes encode products, e.g. degradative enzymes, the enzymes of light production, production of biofilm extracellular matrix, that might not be consequential when produced by a single cell growing in isolation, but would comprise valuable ''community goods'' to a population of cells of sufficient proximity and density to benefit from a coordinated behavior. The accumulated body of studies of microbial multicellularity (initially compiled by Shapiro and Dworkin[4]) spawned the coining of phrases such as ''Quorum Sensing'' (QS)[5] and ''Sociomicrobiology'',[6] which have become prevalent in the current Microbiology research literature. Although the molecular structures of the signals, the composition of the sensing machinery and the functional output of the signaling process show remarkable diversity among different microbes, the biological relatedness of these processes is incontrovertible.

One microbial activity in which cell–cell signaling is believed to be a major player is the formation of biofilms. In particular, the role of cell–cell signaling in biofilm formation by *Pseudomonas aeruginosa* has received sufficient attention of investigators to become a major experimental paradigm. This organism is a popular research subject due to its metabolic versatility, its ability to thrive in many different environments, and its important role as an opportunistic pathogen in life-threatening infections of wounds and the lungs of cystic fibrosis (CF) patients. Seminal studies demonstrating the role of the quorum-sensing machinery of this organism, previously shown to control expression of toxins and other virulence factors,[7] in biofilm development,[8] and the discovery that CF infections involved organisms growing in a biofilm state[9] served to further accelerate this area of study. A potential application of this research is in development of small molecule inhibitors of quorum sensing as antimicrobial agents.[10]

However, the importance of quorum sensing in the pathogenesis of CF lung infections has not been universally accepted. A significant number of clinical isolates from such infections contain mutations in the QS genes and, thus, the suggestion has been made that QS might not be an important determinant of colonization and pathogenicity in this context.[11] An alternative explanation for the isolation of QS mutants among CF clinical isolates has come from consideration of QS and other forms of cooperative multicellular microbial behavior in light of evolutionary biology theory,[12] which predicts the emergence and proliferation of ''cheating'' variants among populations of individuals engaged in cooperative behavior. Such cheaters have been described for the myxobacterium, *Myxococcus xanthus*[13] and recently for *Pseudomonas aeruginosa*[1,2] The cheaters reap the benefits of the cooperating population without contributing ''public goods'' or costly resources that benefit the community as a whole. In the short term, the cheaters can become predominant in the community since they do not invest the energy required to produce their share of public goods and therefore have a higher level of competitive fitness relative to

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the wild-type cooperating individuals. Eventually however, the entire population may crash because the fraction of individuals contributing the public goods required for stable growth and maintenance becomes too low. Thus, evolutionary biologists have devoted attention to the effects of cheating in terms of long-term survival of the cooperative behavior in the population.[12] In terms of CF lung infections, the emergence of non-QS cheaters and their presumed growth advantage requires an explanation for the stability and persistence of such QS populations.Two interesting recent papers have reported on the dynamics and interactions between QS and non-QS (cheater) populations of *P. aeruginosa*.[1,2] While Diggle et al.[1] studied competition between defined QS mutants during mixed growth with QS-proficient wild-type cells, Sandoz et al.[2] examined QS mutants that had emerged spontaneously during growth of the QS wild type. The essential findings of both studies confirmed many evolutionary predictions and can be summarized as follows:

1. Prolonged cultivation of wild-type *P. aeruginosa*, in medium where QS-controlled protease production is necessary for growth, resulted in the spontaneous emergence of QS mutants (cheaters).
2. While such cheaters in pure culture were at a growth disadvantage under conditions requiring QS-controlled protease production, in co-culture with the QS wild type they grew more rapidly than the wild type.
3. Under these conditions, the proportion of cheaters in the population increased.
4. When the proportion of cheaters in the population increased beyond a critical point, the overall growth rate of the co-culture declined.

The maintenance of the QS property in microbial populations—in a sense, the analog of the persistence of altruists in populations of more-complex organisms—demands an explanation. In higher organisms, the hypothesis of kin selection has been advanced and largely accepted by evolutionary biologists.[12,14] Kin selection theory is a theory that relates to altruistic cooperation between relatives. If an individual can help a close relative to reproduce, then that individual can indirectly pass its own genes to the next generation. The basis of this theory is called Hamilton's rule,[14] which posits that altruism is favored when $rb-c>0$; where c is the fitness cost to the altruist, b is the fitness benefit to the beneficiary and r is their genetic relatedness. This predicts that greater levels of altruistic cooperation are expected when relatedness or the fitness benefit is high, and when cost to the altruist is low.

To determine whether or not kin selection was operating in their experiments, Diggle et al.[1] examined the influence of relatedness on the relative fitness of QS^+ wild-type and QS^- cheater clones using an experimental evolution approach. The percentage of QS^+ cells during repeated rounds of growth increased in the "high relatedness" populations but decreased during growth of the "low relatedness" populations. The authors concluded from this experiment that kin selection operated to prevent the takeover of the population by the QS^- cheaters of *P. aeruginosa*.

A close examination of the experimental protocol suggests that alternative interpretations are possible. In the low-relatedness cultures, an initial round of 1:1 mixed cultivation of the wild-type and QS^- mutant strain was done, and subsequent replicate cultures were initiated from mixed strain pools. After multiple passages, the proportion of QS^- cheats (40–60% of the population) approximated that proportion observed in a previous experiment (Fig. 3A of Ref. 1) demonstrating that cheaters inoculated at low density can increase during mixed growth. It is more likely that this ratio simply represents the stable equilibrium ratio for the strains in growth medium requiring QS-controlled protease production after culture to stationary growth phase, i.e. the optimal number of cheaters that can be supported by the QS^+ cooperators under the specified growth conditions.

In the high-relatedness cultures, after the initial round of 1:1 mixed cultivation of the wild-type and QS^- mutant strain, subsequent cultures were initiated with essentially clonal populations of either strain, since isolated colonies (which were selected at random) were used to inoculate the replicate cultures for the next round of growth. Since it was previously established that, in the medium used for these studies, the QS^+ wild-type strain grows to higher cell densities than the mutant strain, the wild-type colonies would outnumber the mutant colonies when the pooled replicate cultures were plated (assuming no differences in plating efficiencies). Thus, if colonies were selected at random from the pool plates, there was a higher probability that the colony selected for the next round of replicate cultures was the wild-type strain, rather than the mutant. With multiple rounds of independent culture followed by random selection of colonies from pooled replicates, the ratio of wild-type to mutant colonies would continue to increase, along with the likelihood of choosing a wild-type colony by random selection from the pool plates. According to this line of reasoning, it is no surprise that the wild-type (QS^+) strain predominated in the high-relatedness cultures given the experimental design. Thus it is not clear that this experiment directly tested the role of kin selection in the persistence of the QS trait in the face of emergence of cheaters.

Interpreting the role of kin selection (as defined in mainstream evolutionary biology) in the evolution of microbial QS is further complicated by the results of Sandoz et al.,[2] who observed the frequent emergence of secondary compensatory mutations in cheater populations; these could suppress the phenotypic effects of the original cheater mutations. Diggle et al.[1] used a luciferase reporter gene

(whose insertion into the QS locus actually generated the QS mutation) to enumerate the wild-type and cheater populations in their studies. However, the QS-controlled phenotype being analyzed in terms of selection was the production of proteases (required to digest proteins in the culture medium and thus liberate amino acids required for growth). Thus it is conceivable that undetected point mutations in the protease genes could render a QS-proficient clone protease-negative, or that secondary mutations outside the QS loci could lead to QS-independent protease production in the QS-negative strains. Thus, the expression of the luciferase reporter gene in a colony might not accurately indicate a lack of protease production by QS^- clone and vice versa.

The importance of secondary mutations suppressing cheater phenotypes is also underscored by the landmark studies of cheating among myxobacteria during iterative cycles of vegetative and developmental (sporulation) growth.[13] These investigators actually determined the complete genome sequences of wild-type, cheater mutant (spontaneous mutants that can develop only in mixed culture and at the expense of the wild type), and a "Phoenix" revertant in which a single base change in non-protein coding region of the genome resulted in both restoration of cooperative behavior and in immunity to exploitation by the cheater strain. Clearly, the three types of myxobacterial strains analyzed by Fiegna et al.[13] all show an extraordinary level of genotypic relatedness across their genomes, yet they exhibit huge differences in the cooperative behavioral traits that they display during sporulation. In this sort of situation, the case could be made that selection is acting at the level of a single gene (or gene cluster encoding the trait of interest) rather than the bacterial cell that serves as the host of this genetic material. In a previous review,[12] the same authors as the present paper,[1] acknowledged this situation and suggested an adjustment in the working definition of genetic relatedness for bacterial studies that focused on the single trait (in this case QS) rather than the entire organism. However, we feel that the use of the term "kin selection" for bacteria is unfortunate, implying as it does a familial structure and a vertical, sexual transmission of properties that is inappropriate in bacteria.

Finally, we note that microbes can acquire genes via horizontal gene transfer and internally rearrange their own genomes extremely rapidly;[15] one important practical consequence of this is that regulatory circuits and signaling systems that may have originally evolved to control one function can quickly be co-opted for other purposes, making it potentially difficult to elucidate the selective forces that drove the evolution of any given regulatory system. Thus, on an evolutionary time scale, maintenance of QS behavior in *Pseudomonas* may have been affected by selection pressures completely unrelated to those operating in the CF lung. We believe that analysis of multicellular cooperative behavior of microbes in the light of evolutionary biology needs careful consideration of the unique aspects of the genetics and ecology of microbes. Ultimately this will enhance cooperative interdisciplinary research between microbiologists and evolutionary biologists, to the great benefit of both fields.

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