How Myxobacteria Cooperate

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Abstract

Prokaryotes often reside in groups where a high degree of relatedness has allowed the evolution of cooperative behaviors. However, very few bacteria or archaea have made the successful transition from unicellular to obligate multicellular life. A notable exception is the myxobacteria, in which cells cooperate to perform group functions highlighted by fruiting body development, an obligate multicellular function. Like all multicellular organisms, myxobacteria face challenges in how to organize and maintain multicellularity. These challenges include maintaining population homeostasis, carrying out tissue repair and regulating the behavior of non-cooperators. Here, we describe the major cooperative behaviors that myxobacteria use: motility, predation and development. In addition, this review emphasizes recent discoveries in the social behavior of outer membrane exchange, wherein kin share outer membrane contents. Finally, we review evidence that outer membrane exchange may be involved in regulating population homeostasis, thus serving as a social tool for myxobacteria to make the cyclic transitions from unicellular to multicellular states.

Introduction

Transitions from unicellular to multicellular life are classified as major evolutionary events [1]. Within the three kingdoms of life, it has been estimated that unicellular organisms have made successful multicellular transitions at least 25 times, with eukaryotes constituting the most complex and spectacular examples [2]. In contrast, for reasons that are unclear, bacteria and archaea have made only limited forays into multicellularity. However, among these latter kingdoms, the myxobacteria have arguably made the most sophisticated transition into multicellularity. In so doing, the myxobacteria have also retained a unicellular life stage. Strikingly, the myxobacteria life cycle is functionally analogous to that of social eukaryotic slime molds (amoebae), in particular, the model organism *Dictyostelium discoideum.* Both of these organisms can exist as single cells or small groups of cells during vegetative growth that transition into obligate multicellular fruiting bodies in response to starvation. It is also striking that these are the only known groups of organisms to share this strategy of building multicellular structures by gathering cells from the environment, which in both cases results in fruiting body formation. Given their evolutionary success [3], the ability to transition from unicellular to multicellular life (fruiting bodies) on a temporal basis offers important fitness advantages for these organisms.

For multicellular organisms to succeed, the individual cells within the collective must cooperate. Such cells need to communicate and coordinate their behaviors to create a functional unit—a tissue. Challenges in this evolutionary transition include the development of (i) biochemical mechanisms for self-recognition; (ii) a means to communicate, organize and synchronize cell behaviors and (iii) a means for the population to reach homeostasis. These "bioengineering" steps represent significant evolutionary hurdles and likely account, at least in part, for why there have been relatively few successful transitions toward multicellularity in the tree of life. In addition to the bioengineering challenges, there are counter-productive Darwinian forces at play [1]. The multicellular environment, in which cells share their resources or "public goods", provides a breeding ground in which cells can

mutate and exploit their clonal environment with selfish and detrimental outcomes. In animals, these Darwinian forces manifest in the relentless development of cancerous cells. To counter this pervasive threat, animals have developed complex immune systems to recognize and remove detrimental cancerous cells, as well as foreign cells, from the body. Multicellular or cooperative microbial communities are also threatened by such exploiter cells. If the population has no mechanism to counteract exploiters, then the demise of the population will likely ensue by a tragedy of the commonsmechanism [4]. In this scenario, the population loses its cooperative fitness advantage. As described below, cooperative cells have evolved mechanisms to regulate selfish behaviors.

The transition to multicellularity requires cooperation among individual cells. In the last quarter century, there has been a greater appreciation for bacterial cooperation within and even across species. Within the realm of biofilms, diverse mechanisms of cell–cell adhesion, public commodity sharing and communication have shown how groups of bacteria can work together [5]. This is not surprising when one considers the advantages inherent in cooperation over individuality [6]. It is plausible that communication and cooperation among related bacteria is the rule rather than the exception. However, very few bacteria are obligate cooperatorsin which cell autonomy has been lost in a commitment toward multicellularity. In contrast to biofilms or colonies, obligate cooperators function exclusively as a multicelled unit, as found in plants and animal species. In bacteria, a rare example is filamentous cyanobacteria. Here, vegetative and heterocyst cells must function together as a unit, since the growth of one cell type is dependent on the fixed carbon or nitrogen provided by the other type of cell [7].

Myxobacteria have been studied for over half a century for their ability to coordinate cells as a unit during the processes of social motility, predation and the formation of elaborate developmental structures

(Fig. 1). As individual cells, myxobacteria are thought to struggle to survive [8], but as a collective, they are very successful and even dominant species [3], as evident by their great abundance in a wide range of soil and water habitats [8–10]. Their two gliding motility engines and molecular arsenal of exoenzymes and secondary metabolites make them mobile predators. When nutrients are exhausted, a myxobacterial swarm will develop into fruiting bodies that contain environmentally resistant spores (Fig. 1) [11]. Curiously, many cells lyse during development, while others help form the fruiting body structure, leaving only a fraction of the original population as viable spores [12]. Whether this cell lysis is a product of programmed cell death and altruism or intra-swarm competition, or both, is still inconclusive [13]. Fruiting body formation provides the benefit of survival and dispersal at the expense of the majority of the cells that initiated the process.

As outlined by Hamilton's theory of kin selection (inclusive fitness) [14,15], the evolution and maintenance of cooperation are possible only in groups of closely related individuals. This process requires (i) a mechanism to discriminate kin from non-kin and (ii) the means to keep those kin in close proximity as a viscous or dense population. Myxobacteria use both strategies to ensure that cooperative behaviors will most likely benefit cells that are highly related. Herein we will review these strategies.

The success of myxobacteria as a functional collective is governed by cell–cell communication, coordination and cooperation among individual cells. A fundamental challenge then is how to create a large, functionally homogeneous collective from a diverse group of cells. That is, in spite of mechanisms to maintain genetic relatedness within a group, cell diversity nevertheless exists within myxobacterial populations [16]. Moreover, within some microenvironments, genetic diversity can vary and be extremely high [3,17], as spores are stable to environmental stresses and readily disperse by wind and water, creating a global microbial
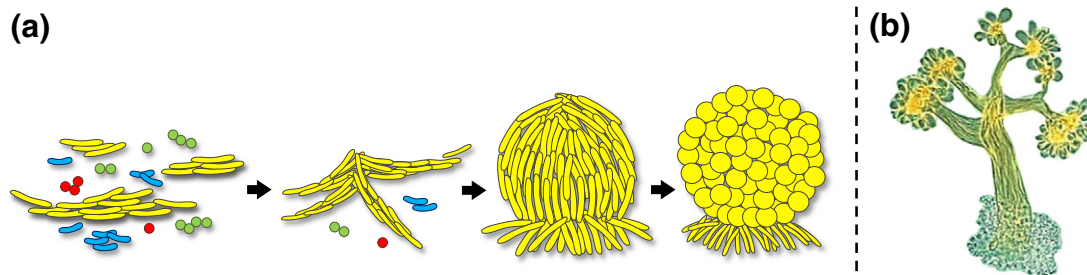
Fig. 1. Cell recognition and fruiting body development in myxobacteria. (a) A schematic model of fruiting body formation by *M. xanthus* cells (yellow). The natural soil environment of myxobacteria contains diverse microbial communities (depicted by cells of different shapes and colors, left). Upon starvation, *M. xanthus* cells recognize kin and migrate into aggregates. The aggregates increase in size and form a haystack-shaped fruiting body, within which cells sporulate (depicted by circles). (b) Micrograph of *C. crocatus* fruiting body [20] (courtesy of Hans Reichenbach).

melting pot of species and sub-species. In other cases, heterogeneous nutrient availability in soil limits clonal expansion, and thus, islands of separate populations can develop and change, and groups that are compatible may eventually merge (Fig. 1) [17]. Of course, mutations within populations will constantly be introduced, leading to mutants that might be defective cooperators. In other cases, cells might be genetically identical but physiologically distinct due to aging, starvation or cellular wounds. Therefore, the collective functions of a swarm can be threatened by a multitude of factors including non-cooperators, cheaters, incompatible strains and damaged cells. The ability to maintain coordinated behavior in spite of these challenges is likely crucial to the success of an obligate cooperator.

Much research into bacterial cooperation has focused on the use of public goods. For instance, quorum sensing is a communication strategy in which chemical signals are produced by a community. When the density of the signal reaches the minimum threshold concentration, it elicits a response from surrounding cells, which coordinate their gene expression in response to the local concentration of the signal (reviewed in Ref. [18]). In another example, siderophores are secreted to sequester iron for the producer and its kin [19]. In myxobacteria, one cooperative behavior involves membrane fusion and the exchange of large amounts of outer membrane (OM) components between cells (reviewed in Ref. [20]). Therefore, in this system, OM components including lipids, lipoproteins and lipopolysaccharide (LPS) can be viewed as public goods. However, unlike the conventional sense of a public good, this resource is guarded from non-kin cells—the homophilic cell surface receptor TraA both identifies kin and catalyzes the membrane fusion event (Fig. 2) [21]. Therefore, outer membrane exchange (OME) is a unique platform for the sharing of cell contents that we propose contributes to the cooperative behaviors of myxobacteria. In the following sections, we discuss these behaviors and expand on the mentioned concepts.

Cooperative Behaviors Used by Myxobacteria

Social movement

The intricate myxobacterial lifestyle involves a number of multicellular behaviors, one of which is coordinated group movement. *Myxococcus xanthus* cells typically travel within dynamic multicellular
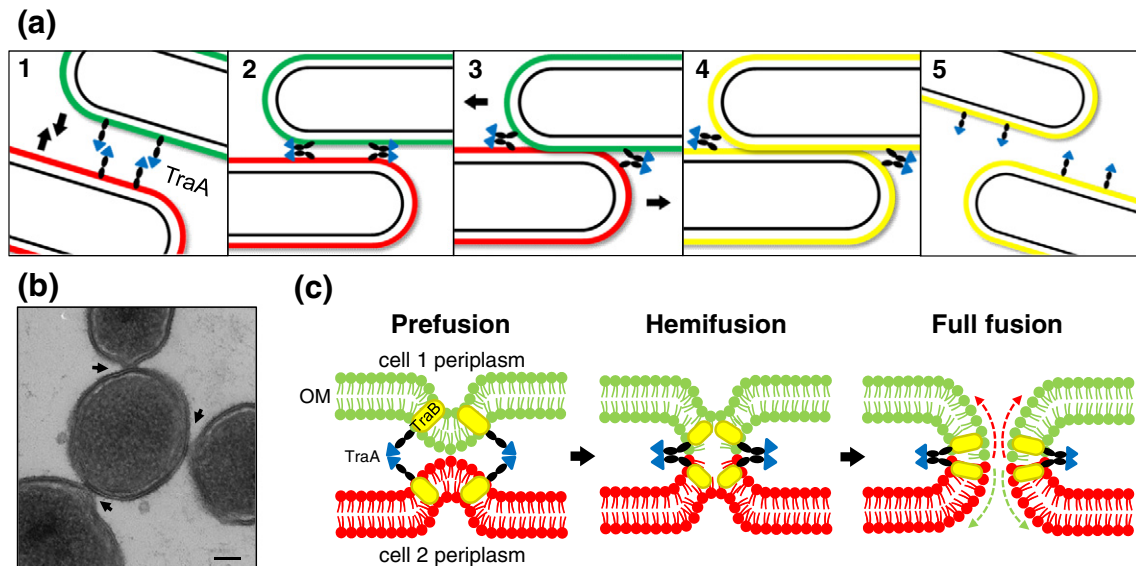
Fig. 2. Model for OME in myxobacteria. (a) (1) TraA–TraA recognition occurs between neighboring cells. Green and red symbolize different fluorescent markers in the OM. (2) Cells are brought into close proximity upon recognition and motility. (3) Prefusion junctions form and cells continue to move. (4) Motility helps trigger fusion to facilitate OME. (5) Cells separate after OME. (b) TraAB overexpression leads to cell–cell prefusion junctions (arrows) as seen by cryo-electron microscopy. Cross-section shows center cell interacting with three neighboring cells. Bar, 100 nm [85]. (c) Schematic model of OM fusion dynamics (based on eukaryotic models) during OME. Upon TraA–TraA interaction, the OMs of two aligned cells come into close contact and form a prefusion junction. The outer leaflets of the two OMs then fuse creating a hemifusion complex. Subsequently, full fusion of the OM (both outer and inner leaflets) is presumed to occur, though soluble periplasmic proteins have not been found to transfer. Broken arrows indicate lateral diffusion of OM components between cells.

structures called swarms. Although single cells moving at the edges of *M. xanthus* swarms are frequently observed, the majority of cells on an agar plate are found within the swarm, where they use cooperative social (S) motility to facilitate swarm expansion [22]. S-motility requires type IV pili (TFP) and exopolysaccharides (EPSs). TFP are assembled at the leading cell pole and bind to EPSs present on neighboring cells or on the gliding surface. The retraction of TFP generates a force to propel the cell forward [23–25]. Moreover, S-motility is a cell-contact-dependent behavior that requires a large number of cells in close proximity, and a maximum swarm expansion rate is achieved at high cell density [22,26]. One advantage for individuals living within the high-density swarms might be that those cells are able to share the pool of secreted EPSs (public good) contributed by the group, and therefore, the burden of EPS production on each individual is reduced. A recent study showed that *M. xanthus* EPSs not only help mediate surface adhesion but also exhibit lubricating properties to facilitate cooperative movement [27]. This finding provides an explanation for the "stick-slip" movement observed in S-motility, where cells move in bursts followed by pauses. Additionally, experimentally evolved cooperative motility has been described in myxobacteria [28]. Here, in the absence of functional TFP, mutants that develop a *de novo* cooperative swarming phenotype are selected. Mechanistically, this occurs by enhanced production of an extracellular fibril matrix that co-opts the *M. xanthus* adventurous (A) motility system to function socially by bundling cells together [28]. Despite the cost of fibril overproduction, the resulting cell–cell attachment provides the benefit of achieving mobility on a specific medium (semi-solid agar) and results in a new pathway to achieve multicellular behavior.

M. xanthus cells constantly interact with each other to coordinate their movements in a swarm. Interestingly, cells within a swarm often pause and reverse directions [29,30], and this periodic reversal of gliding direction helps to coordinate swarm expansion [31]. Reversals are controlled by the Frz chemosensory pathway, which drives a small G-protein switch (MglA) to reverse the gliding direction [32–36]. Mutations in *frz* genes can cause non-optimal reversal frequencies, which lead to defects in swarming, lipid chemotaxis and developmental aggregation [29,31,32,35,37]. A relatively recent study suggested that FrzCD, a methyl-accepting chemotaxis protein, might play a role in cell–cell interactions [38]. FrzCD protein clusters transiently realign between adjacent cells upon side-by-side contact to trigger cell reversals [38]. These interactions between side-by-side cells may help the population to coordinate and synchronize cell movements [39]. Thus, *M. xanthus* uses a variety of cell–cell interactions to organize the macro-scale movement of the swarm unit.

Predation

When prey or other nutrients are present in the environment, *M. xanthus* swarms will feed upon them collectively. Unlike other predation strategies (e.g., phagocytosis [40], prey cell invasion [41], far-ranging diffusible antibiotic secretion [42]), myxobacteria can penetrate prey colonies and secrete extracellular enzymes and antibiotics to kill and consume prey cells (reviewed in Ref. [43]). During predation, populations of *M. xanthus* cells ripple, a phenomenon where cells form self-organized and rhythmically traveling high-density waves [44]. Rippling is a cooperative behavior involving cell–cell alignment and periodic reversals [35,45]. The opposite-moving waves of cells appear to collide and subsequently reverse direction after contact so that each wave appears to oscillate back and forth [46]. By allowing more frequent prey–predator contacts, this judicious oscillation behavior is thought to help enhance predation efficiency [43,46]. Rippling has also been observed during starvation and fruiting body formation [44,47]. Initial studies regarding this behavior primarily focused on developmental rippling, whereas predatory rippling was rarely mentioned [46]. However, it was recently suggested that rippling in *M. xanthus* is solely a predatory behavior and that developmental rippling results from nutrient scavenging of lysed cells [46]. As for the physiological role of rippling behavior, both computational simulations and experimental data suggest that ripples cause faster penetration of predators into prey colonies via a signaling mechanism that is dependent on side-by-side contact between *M. xanthus* cells [38,48]. Also, rippling helps the predators remain on the prey region for a longer time, which is likely due to organized cell–cell alignments and periodic cell reversals that prevent cells from drifting randomly [48].

In addition to rippling, predation efficiency is also enhanced by a high cell density [43]. *M. xanthus* predation is often compared to a "wolf-pack" model [43,49,50], suggesting that cells living in a high density work cooperatively as an efficient predatory unit [43]. Generally, the high-density predatory swarms secrete increased concentrations of antibiotics and hydrolytic enzymes (public goods) to their local environment [43,49]. Individual predators thus benefit from this group effort by sharing the pool of hydrolyzed products from the prey cells to promote growth. Recent studies also suggested that *M. xanthus* produces OM vesicles, containing multiple antibacterial molecules and enzymes, to assist with predation [51,52]. The packaging of multiple secondary metabolites and enzymes within one vesicle might be delivered as a lethal cocktail to help ensure that predation occurs efficiently [52].

Development

Perhaps the most striking example of cooperation in myxobacteria is fruiting body development (Fig. 1).

Upon starvation, *M. xanthus* enters a developmental program and forms multicellular fruiting bodies, within which the cells differentiate into spores [53]. Fruiting body morphology ranges from relatively simple mounds found in *M. xanthus* to elaborate tree-like structures of *Chondromyces crocatus* (Fig. 1) [54]. Following starvation, individual cells of *M. xanthus* arrange themselves into simple aggregates that develop into dome-shaped mounds containing ∼100,000 cooperating cells [55]. In addition to mound formation, rippling behaviors can also be observed in some areas during the early developmental stage (referred to as the aggregation phase) [44,56]. It has been suggested that these traveling waves carry piles of cells that collide with each other and form "traffic jams" [56,57]. These masses of cells enter the mound from different directions and are thought to get caught within the jams, which leads to aggregate formation [56,57]. After the aggregation phase, sporulation proceeds and a small fraction of the cells differentiate into spherical dormant spores [12,55]. The remaining cells in the population differentiate into peripheral rods (persister-like cells) or lyse [12,58,59]. Development is also a cell-density-dependent behavior in *M. xanthus* [60]. Cells sporulate less efficiently, or not at all, if their density is below a threshold concentration [61,62].

Coordinated cell aggregation into multicellular fruiting bodies is regulated by cell–cell signaling. During the initiation of development, *M. xanthus* cells accumulate elevated levels of (p)ppGpp in response to starvation and produce A-signal, an extracellular quorum-like signal that is thought to monitor local cell density [63,64]. Once the extracellular A-signal exceeds the minimum threshold, ensuring that a minimum population (a quorum) of starving cells exists, the expression of development-specific genes regulated by A-signal is initiated [63,65]. Cooperative aggregation ensues and further morphogenesis is dependent on another signal, the C-signal, which is a morphogen that requires cell–cell contact for its activity [11,66,67]. The nature of the C-signal is not clear, although it has been suggested that the CsgA protein may act as a morphogen itself or as an enzyme to generate the C-signal [68,69]. The transmission of the C-signal seems to rely on end-to-end contacts between cells [70,71], and the frequent cell–cell interactions that occur within high-density aggregates increase the level of C-signaling [66]. One model suggests that C-signal transmission provides a positional clue to regulate directional cell movement [69]. Here, during the aggregation stage, C-signaling helps recruit cells into chains via pole-to-pole interactions [69]. According to this model, cells from disordered fields are organized into streams and move toward the aggregation centers with a higher speed and a lower reversal frequency, compared with cells that are not recruited [66]. The increased cell density within the aggregates in turn provides positive feedback that helps raise the level of C-signaling until it reaches the threshold required for spore differentiation [72,73]. However, a more recent study proposed a different model, suggesting that the reduction in cell velocity, rather than the suppression of cell reversal, is responsible for aggregate formation [74]. Here, cells were found to slow down and orient themselves in parallel within high-density aggregates. It has also been suggested that different experimental conditions might account for some of the different observations found during development [35,74].

Outer membrane exchange

Previously, we described a novel process whereby OM proteins and lipids from *M. xanthus* are exchanged between individual cells [75,76]. Studies relating to OME can be traced back to 1977 when extracellular complementation (stimulation) was first discovered for motility mutants in *M. xanthus* [77]. In those studies, a small subset of nonmotile mutants was found to be rescued after transient contact with mutants from a different complementation group. Later studies revealed the mechanism of stimulation, in which the functional motility proteins are transferred from a donor strain to restore the motility defect of the mutant recipient [78,79]. Stimulation involves only transient phenotypic changes upon cell–cell contact, and the "rescued" mutant becomes nonmotile over time if it is physically separated from the donor cells, suggesting that only proteins, instead of DNA, are transferred [20,21,63]. By this genetic approach, six OM proteins (CglB/C/D/E/F and Tgl) have been identified as substrates for transfer by OME [20,80]. One important feature of these stimulatable proteins is that they contain either type I or type II signal sequence peptides (SS) [20,81–83]. Based on these findings, a heterologous reporter (SS$_{OM}$-mCherry) was constructed and shown to transfer in live cells. Here, the type II SS fusion directs the mCherry reporter to the OM, where it then becomes a competent substrate for cell–cell transfer [76]. Lipids were also found to be transferred via OME by tracking transfer among live cells labeled with lipophilic fluorescent dyes [75]. These observations, combined with the fact that an inner-membrane-localized mCherry reporter (SS$_{IM}$-mCherry) is not transferred [76], suggested that OME involves the transient fusion of the OMs between cells. Strikingly, both endogenous and heterologous cargo transfer experiments found that the donor and recipient cells equally share the amount of exchangeable OM materials within a relatively short period of time, indicating that transfer is an efficient process [75,76,78].

OME is a mutual or cooperative behavior, as it requires two or more cells to make direct contact and

because transfer is bidirectional (Fig. 2). Physically, the cells need to be on a hard surface, as exchange does not occur in liquid medium or on semi-solid agar [76,84]. Motility (either A-motility or S-motility), which helps to align and facilitate cell contacts, also plays a critical role in OME [76,84]. Among nonmotile cells, transfer is barely detected; instead, for efficient transfer, at least one partner (either donor or recipient), or even a third-party cell, needs to be motile [20]. OM lipoproteins, proteins, lipids [75], LPS [85] and a putative signal have been shown to be transferred to date [86].

Two host genetic determinants, called *traA* and *traB*, are required for OME [75]. OME requires that the TraA and TraB proteins be present in both engaged cells, as disruption of either of the two proteins blocks OME [75]. Additional analyses found that TraA functions as a cell surface receptor, governing the cell–cell interactions involved in OME [21,75]. The TraA protein contains a distant PA14-like domain, Cys-rich tandem repeats and a putative protein sorting tag called MYXO-CTERM thought to facilitate the localization of TraA to the cell surface [75].

Interestingly, TraA has a domain architecture and sequence that are similar to those of the FLO1 cell surface receptor, which is required for flocculation in *Saccharomyces cerevisiae* [87]. The FLO1 sequence varies across environmental isolates, and it plays a key role in self-recognition and has been described as a "greenbeard" gene that directs cooperative interactions toward other cells that bear the same allele [87]. We have reported that TraA has an analogous role to FLO1 in myxobacteria. That is, TraA acts as a molecular specificity determinant and exhibits allele-specific interactions during OME between different environmental isolates [21]. In this case, OME is a cooperative behavior that involves the sharing of public goods among individuals carrying identical or very similar *traA* alleles. Thus, the TraA receptor represents a perceptible trait that is used to distinguish kin from non-kin and governs OME among related individuals. In this regard, TraA can be classified as a rare greenbeard gene and, to our knowledge, is the first greenbeard gene in bacteria known to be involved in beneficial or altruistic-like behaviors [21].

A further requirement for a greenbeard classification is that the gene must be polymorphic to allow specificity during recognition; that is, the greenbeard function allows select individuals in a diverse population to benefit from interactions, whereas others are excluded. Importantly, TraA is polymorphic within the PA14-like domain, and sequence conservation within this domain correlates with cell–cell recognition [21]. Based on these and other experiments, we propose that the polymorphic domain serves as the molecular recognition determinant through homotypic interactions between

TraA receptors. In support of this model, we have shown that cell–cell recognition can be reprogrammed by swapping *traA* alleles from different strains [21]. The C-terminal portion of TraA contains Cys-rich repeats that likely form disulfide bonds, and we hypothesize that this region functions as a rigid stalk to display the polymorphic binding domain on the cell surface, which appears to also function in membrane fusion (Fig. 2).

The role of TraB in OME is not well understood. It contains an OmpA-like domain at its C-terminus that is predicted to bind the cell wall [75]. Sequence analysis of *traB* alleles suggests the N-terminal region contains a β-barrel domain. Therefore, as a putative β-barrel protein in the OM, one possible function of TraB would be to facilitate TraA transport to the cell surface. TraB might also interact with TraA to form a functional complex for OM fusion.

The OME mechanism is proposed to involve the fusion of the OM (Fig. 2) [63]. Although OM-enclosed tubes OMTs are produced in large quantities by myxobacteria [88], we do not think that OMTs are required for OME [89]. Under certain conditions, however, OMTs might be produced as a byproduct of OME and motility [90]. According to our current model, *M. xanthus* cells physically interact with each other, and TraA–TraA interactions then force the opposing membranes to make contact, effectively displacing water between them, to catalyze OM fusion (Fig. 2b) [91,92]. Fusion is followed by lateral diffusion and exchange of OM contents between aligned cells (Fig. 2a). Supporting the concept of OM fusion, we discovered that TraA/B functions as a cell–cell adhesin. Specifically, TraA/B overexpression leads to the formation of tight cell–cell adhesion zones, which based on cryo-electron microscopy suggest that they form "prefusion junctions" [85] (Fig. 2b). However, for OM fusion to occur, cells need to be placed on a hard surface, and, we presume, the prefusion junctions are stressed through cell movements, which in turn helps to destabilize the OM and trigger fusion as outlined in Fig. 2.

How OME benefits the individual and group

TraA is highly conserved across most *Myxococcales*, and its function has apparently been maintained despite significant phylogenetic divergence within the order. In addition, there are no TraA/B orthologs found in sequenced genomes outside of this order. Given these findings, what then is the driving force to maintain conservation of TraA/B and why is it unique to the myxobacteria? In other words, what is the function of OME and how is it important to the myxobacterial lifestyle? Though some information has recently come to light [85], the answer may not be straightforward. Here, we discuss what is known about the utility of OME, including how this
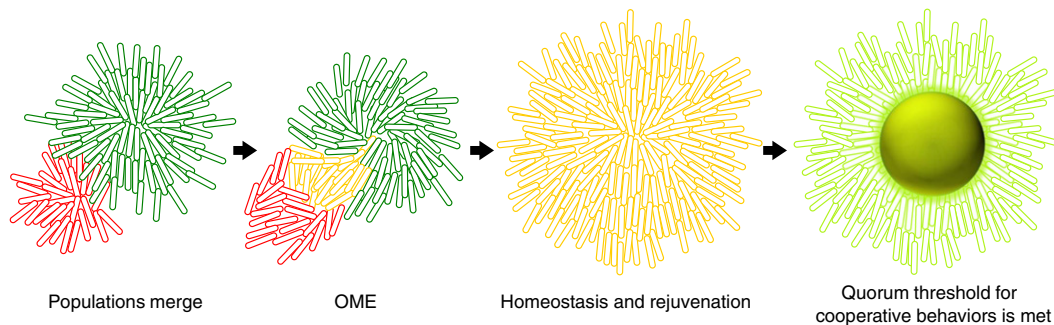
social behavior can benefit the individual cell and the group, as well as the other ways it may contribute to the behavior of myxobacteria.

The Allee effect [93], or the idea that fitness is positively correlated with population density, figures prominently in the literature on myxobacteria [61]. As described above, the importance of population density has been observed during growth [49], social motility [22], feeding [49] and development [62,65] and may be important for competitive interactions between isolates. However, population homogeneity is not a given. For instance, the prevalence of myxobacteria in small-scale soil patches indicates that encounters among motile populations may occur frequently [94,95]. Even when stochastic encounters occur among compatible environmental strains, they may not be genotypically identical or may have adapted phenotypic differences because of environmental circumstances. Given these plausible scenarios, how can a heterogeneous collection of cells become a coherent functional unit?

One answer to the above mentioned dilemma may lie with OME. Recently, we used a genetic model of membrane damage in which affected cells have deleterious or even lethal changes to their LPS and showed that damaged cells were repaired by OME with healthy LPS donors [85]. More specifically, LPS mutants with impaired motility and abolished sporulation were rescued when mixed with healthy *traA*+ cells, but not with Δ*traA* cells. Furthermore, mutants unable to synthesize lipid A by a conditionally lethal mutation were maintained in a population of healthy cells when OME could occur but perished in the absence of OME. Therefore, during the merging of healthy cells and cells with a damaged OM in nature, OME can enable membrane homeostasis to be reached, which can also rejuvenate or heal wounds on ailing cells (Fig. 3).

When we simulated the merging of membrane-damaged population (in this case, sporulation deficient) and healthy wild-type population, we found that an increase in cell density could also lead to a significant increase in sporulation outcomes during development as compared with healthy cells incubated alone [85]. This increase far surpassed what can be explained by the restoration of sporulation to the damaged cell population, indicating that an increase in cell density above a threshold level along with cell rejuvenation had synergistically increased sporulation efficacies. However, when the healthy population was incapable of OME (Δ*traA*), the increase in cell density did not improve the sporulation outcome. Put simply, whereas the Δ*traA* strain has no obvious developmental phenotype in a pure culture [75], it has a clear developmental phenotype in heterogeneous populations. Therefore, during the merging of damaged and healthy colonies, an increase in cell density (above a threshold level) is beneficial only when the damaged cells can be healed and contribute to group behaviors. In this manner, there is a benefit for healthy cells to temporarily sacrifice their fitness by merging with damaged cells. In this scenario, both the donor and recipient cells benefit; this can explain how OME is evolutionarily maintained by mutual benefit to donor and recipient cells that bear compatible TraA alleles.

In total, motility, development, antibiotic resistance and even lethal biosynthetic defects can be phenotypically complemented by OME in myxobacteria [85]. From an evolutionary standpoint, this finding is curious because it suggests that deleterious mutations to some OM proteins or OM biosynthesis proteins could be maintained in the population by phenotypic complementation. Could this mean that all cells that contribute to a population are important

Populations merge   OME   Homeostasis and rejuvenation   Quorum threshold for cooperative behaviors is met

Fig. 3. A model for the utility of OME in a bacterial community. Two distinct but compatible populations meet (green and red, left), and OME catalyzes OM mixing (yellow cells contain components from red and green cells). The mixed population then transitions toward membrane homeostasis (middle right), in which membrane content from both populations is equally distributed among cells. This integrated population is now larger, more homogenous and better equipped for cooperative behaviors (fruiting body, right). Damage that has occurred to some cells can be repaired by dilution followed by active repair (yellow-to-light green transition).

regardless of the functional properties of their OMs, and if so, how long will these defective cells be maintained? During development, the majority of cells die, apparently to support the sporulation of the minority population [96]. Perhaps this strategy promotes intra-swarm competition to eliminate less-fit cells from the spore or "germ" pool [13]. Alternatively, perhaps the maintenance of diverse OM phenotypes within the spore pool contributes to the phenotypic diversity of strains found within and between populations [3,16,97,98]. Last, it cannot be forgotten that myxobacteria transition between single and multicellular life stages and, consequently, selection against deleterious mutations in a haploid genome is strong when solitary cells cannot be complemented by OME. Future research is required to resolve these possibilities.

Although the benefit of OME to heterogeneous populations is reasonably clear, there may be other benefits to the "shared membrane concept". As cell-to-cell interactions are guarded from exploitation by non-kin cells, OME could provide a platform to communicate intra-swarm signals. Indeed, we have observed that some motility mutants inhibit the motility and development of their wild-type kin in a Tra-dependent manner (see below) [75,86]. In addition, OME allows phenotypic plasticity, in which the OM phenotype of a single cell is not completely dictated by its genotype. For instance, the LPS of some myxobacteria are modified during development [99] and perhaps those modified molecules are transferable. In addition, it has long been known that bacteria can modify their OM in response to antimicrobial compounds or stress [100]. Because myxobacteria transfer lipids, proteins and LPS, it is likely that phenotypic changes can be relayed to cells that have not yet encountered the forthcoming stimuli in their environment. In this and other scenarios, scout cells might relay information in the form of an adapted OM proteome and lipidome to the central group to quickly respond to stimuli at the periphery of the swarm.

Finally, the TraA cell surface adhesin in its own right could contribute to cooperation in myxobacteria. Population viscosity, which involves limited dispersal of related individuals, is one strategy for maintaining a high degree of relatedness between nearby cells, which is critical for cooperation to evolve [1]. As an adhesin that discriminates kin from non-kin [21], TraA could contribute to population viscosity in myxobacteria. Indeed, as mentioned above, when *traAB* is overexpressed, cells specifically adhere to one another (Fig. 2). Perhaps when surrounded by both kin and non-kin, TraA can help parse the populations.

Cheating

Cooperation benefits individual members of a population and also increases the fitness of the population as a whole [101]. However, cooperation can be exploited. Although the production of public goods benefits the population at a sustainable cost to its actor [102], some individuals can exploit this situation by using public goods without contributing toward their production. Such individuals have a fitness advantage, as they do not devote their own resources to the behavior yet they enjoy the benefits. This leads to an increase in the number of these social cheaters in the population. As exploiters, their population outcompetes cells that cooperate, and eventually the entire social structure will collapse as the critical public good will not be available at sufficient levels. This phenomenon is referred to as the tragedy of the commons, in which the exploitative nature of a few individuals can lead to the destruction of a cooperative population [103]. For example, increased virulence of a few phages in a population of phage with low virulence can lead to the abolishment of the host and, consequently, the destruction of the phage population [104]. In another example, the mixing of myxobacteria cheaters with cooperators results in a scenario where the whole population is destroyed by the exploitative nature of the cheaters [105].

Policing

Cooperation is subject to exploitation. One strategy to help prevent exploitation involves kin recognition: cooperative behavior is directed toward related individuals and excluded from non-kin. In fact, such behavior is found in *D. discoideum* and *M. xanthus* natural fruiting bodies, in which individuals are highly related to each other [16,106]. The evolution of "policing" functions is another way to help protect the integrity of cooperating communities. The concept of punishment as a form of policing is found in eukaryotes [107–109]. Thus, both cooperation and policing are important in maintaining a healthy population [110]. For instance, cell competition and culling occur to allow eukaryotic tissues to become more homogenous and fit [111]. Additionally, higher eukaryotes have immune systems that provide surveillance and protection against foreign and cancerous cells across tissues. In soil, there is a large diversity of myxobacteria, based on the 16S rRNA sequence analysis [97], and thus, cell–cell surveillance and culling may be an important step to build functional and healthy communities from diverse populations. Indeed, in the laboratory, myxobacteria can evolve policing capabilities to control cheating associated with a *csgA* mutant [112]. Here, continuous exploitation by cheaters of multicellular development and sporulation leads to the development of an evolved strain from a cooperator strain that can police or control the *csgA* cheaters. In this case, the mechanism of policing is unknown but could involve the production

of an inhibitory agent. Other bacteria police behaviors, such as *Pseudomonas aeruginosa* can police cheaters through quorum sensing, in which cooperators use a regulatory cascade to catalyze cyanide synthesis and cheaters are unable to synthesize the enzyme that degrades cyanide, thus becoming susceptible to killing [113].

As noted above, OME involves kin recognition and is highly selective. The polymorphic domain in TraA acts as a molecular identity card to recognize individuals that bear identical or very similar TraA receptors [21]. Because OME involves the bulk transfer of proteins and lipids, and does so efficiently, cells would be at a major disadvantage to exchange with non-cooperators. Thus, *a priori*, one might predict that there is a mechanism to prevent exploitation. Indeed, this appears to be the case. The fact that both partnering cells must express compatible TraA proteins helps to ensure that public goods are guarded: OME occurs among kin and transfer is bidirectional, meaning one cell cannot unilaterally siphon goods. An advantage of this system is that the goods are exchanged only after the involved parties are verified in a cell-contact-dependent manner. This system is in contrast to secreted public goods that are poorly controlled once they leave the cell boundary. As outlined below, we are investing whether there is another level of policing function involved in OME.

Swarm inhibition

OME can regulate the behavior of mixed cell populations. In this phenomenon, motile *M. xanthus* cells are inhibited from swarming when mixed with nonmotile cells [75]. Inhibition is Tra-dependent, as a *tra* mutation in either the motile or the nonmotile strain relieves swarm inhibition. Using swarm inhibition as an assay, we devised a forward genetic screen to investigate OME in more detail [86]. The two questions we sought to address were (i) whether other proteins in addition to TraA/B are directly involved in OME and (ii) what downstream pathways control swarm inhibition. Importantly, from the screen, no other players, besides TraA/B, were found by this criteria to be directly involved in OME, as all mutants isolated ($>$50) mapped to the *traAB* locus [86]. We did, however, isolate a new mutant class that is not defective in OME yet can be relieved from swarm inhibition. The mutant was named OmrA for OME response. OmrA contains a single domain that is homologous to the lipid flippase domain found in the *Staphylococcus aureus* protein MprF [86]. In *S. aureus*, MprF controls cytoplasmic membrane homeostasis by flipping modified phospholipid components from the inner leaflet to the outer leaflet [114]. MprF is a bifunctional enzyme in which a second domain catalyzes the synthesis of lysyl-phosphatidyl-glycerol from lysyl-tRNA and phosphatidylglycerol

precursors. Thus, once the aminoacylated phosphatidylglycerol is synthesized, MprF flips it to the outer leaflet [114].

In *S. aureus*, gain-of-function mutations in *mprF* confer resistance to cationic antibiotics, such as daptomycin, by modifying the surface charge on the cytoplasmic membrane [114]. We found a second gene with homology to the synthase domain of MprF in the *M. xanthus* genome and constructed a mutant and tested it for a swarm relief phenotype. As predicted, this mutant, *omrB*, exhibits a swarm relief phenotype (partial) in *M. xanthus* [86]. From these results, we hypothesized that OmrA/B are involved in modifying and regulating inner membrane homeostasis and that inactivating these genes perturbs the inner membrane in such a way that the motile responder cells are no longer regulated by "signals" transferred from nonmotile cells [86].

To understand what causes swarm inhibition and to gain insight into OME, we labeled motile and nonmotile strains with distinguishable fluorescent proteins to track their fates in a mixed colony. Interestingly, the motile cells disappear by 72 h when mixed with nonmotile cells, and their disappearance is Tra dependent (Dey *et al.*). Additionally, the motile cells become filamentous after a 24-h incubation in co-culture. From these results, we concluded that the nonmotile cells are killing the motile cells by a Tra-dependent mechanism. One possible mechanism for this is that the nonmotile cells exclusively express a toxin/antitoxin pair. OME is then presumed to deliver the toxin to the susceptible motile strain. Consistent with our abovementioned findings, when the motile strain contains an *omrA* mutation, killing is blocked (Dey *et al.*). From these results, we concluded that, among certain closely related strains, there is a sibling killing mechanism. Although it remains unclear why there is killing between related myxobacteria strains, we suspect that it is a form of social surveillance or a policing behavior. Thus, in contrast to cooperative and beneficial functions described for OME, there are also adversarial functions.

Conclusion

Surviving in hostile natural environments is a constant struggle. Like many eukaryotes, myxobacteria have evolved multicellularity, whereby unity of numbers provides advantages over individuality [6]. These multicellular-related functions require communication, coordination and cooperation among related cells in a diverse population. Because of these properties, myxobacteria represent ideal organisms for the study of complex multicellular behaviors that are experimentally tractable. We hypothesize that OME provides a mechanism that helps individual cells come together to form a multicellular unit that has tissue-like qualities, involving resource sharing and

maintaining homeostasis. Myxobacteria may also use OME to police social interactions and our future work will explore these possibilities.

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