REVIEW ARTICLE

Deciphering the hunting strategy of a bacterial wolfpack

James E. Berleman & John R. Kirby

Department of Microbiology, The University of Iowa, Iowa City, IA, USA

Correspondence: John R. Kirby, Department of Microbiology, The University of Iowa, 51 Newton Road, Iowa City, IA 52242, USA. Tel.: +1 319 335 7818; fax: +1 319 335 9006; e-mail: john-kirby@uiowa.edu

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Abstract

Myxococcus xanthus is a common soil bacterium with an intricate multicellular lifestyle that continues to challenge the way in which we conceptualize the capabilities of prokaryotic organisms. *Myxococcus xanthus* is the preferred laboratory representative from the *Myxobacteria*, a family of organisms distinguished by their ability to form highly structured biofilms that include tentacle-like packs of surface-gliding cell groups, synchronized rippling waves of oscillating cells and massive spore-filled aggregates that protrude upwards from the substratum to form fruiting bodies. But most of the *Myxobacteria* are also predators that thrive on the degradation of macromolecules released through the lysis of other microbial cells. The aim of this review is to examine our understanding of the predatory life cycle of *M. xanthus*. We will examine the multicellular structures formed during contact with prey, and the molecular mechanisms utilized by *M. xanthus* to detect and destroy prey cells. We will also examine our understanding of microbial predator–prey relationships and the prospects for how bacterial predation mechanisms can be exploited to generate new antimicrobial technologies.

Microbial signals and antibiotics

Ever since Alexander Fleming's serendipitous observation of the inhibition of *Staphylococcus aureus*' growth by the fungus *Penicillium notatum* and the subsequent purification of the penicillin molecule by Chain and Florey, we have capitalized on the therapeutic benefits of the vast array of unusual chemical structures produced by soil microorganisms (Cheng *et al*., 2003; Rodriguez-Saiz *et al*., 2005). While this work spawned a search for novel producer organisms and a great deal of insight into the chemical synthesis of antibiotic compounds, we understand little of the *in situ* biological function of the secondary metabolites associated with antibiotic activity. In the case of penicillin, it has strong activity against Gram-positive bacteria, but is there some specific Gram-positive organism that *P. notatum* encounters in its native environment that is either a competitor or perhaps a preferred prey species? Is native penicillin produced at or above a minimum inhibitory concentration in natural settings, or was the inhibitory effect observed by Fleming an artifact of laboratory growth conditions?

Indeed, antibiotics may have a concentration-dependent role, where they can act as inhibitors at high concentrations such as those seen in clinical settings, and as intercellular signals at low concentrations likely found in natural environments (Davies *et al*., 2006; Fajardo & Martinez, 2008). Several examples of this phenomenon have been highlighted recently. For example, gene expression in *Salmonella enterica* is influenced by exposure to subinhibitory concentrations of rifampicin [as measured by promoter-*lux* fusions; (Goh *et al*., 2002)] but is independent of known global regulators, and yet is promoter specific (Yim *et al*., 2006). Lantibiotics (Gram-positive, ribosomally synthesized peptides) have also been shown to elicit a quorum-sensing response: mersacidin (an antibiotic active against *S. aureus*) is an autoinducing peptide for the *Bacillus* sp. that produces the molecule. The lantibiotic SapT, produced by *Streptomyces tendae*, can restore aerial hyphae formation and sporulation in developmental mutants of *Streptomyces coelicolor* (Kodani *et al*., 2005; Schmitz *et al*., 2006). Likewise, protein synthesis inhibitors have been shown to differentially affect transcription of heat shock proteins in *Bacillus subtilis* at subinhibitory concentrations: chloramphenicol leads to repression while gentamicin induces expression of heat shock pathways (Lin *et al*., 2005). Lastly, subinhibitory concentrations of some antibiotics have been demonstrated to trigger virulence determinants for *Pseudomonas aerurginosa* (Linares *et al*., 2006). Tobramycin leads to enhanced motility while tetracycline induces expression of the Type III secretion system necessary for production of cytotoxic elements. In a

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natural setting, expression of virulence factors could act as a defense against eukaryotic predators in response to the particular antibiotic being sensed. Thus, secondary metabolite production may constitute part of a complex adaptive response that enables microbial predators and prey alike to respond appropriately to their neighbors.

Microbial predation strategies

Prey cell engulfment (phagocytosis)

Throughout all of the myriad ways that microbial species interact, there are several established interactions that inevitably result in cell death (Jurkevitch, 2007). Many eukaryotic microorganisms, such as *Dictyostelium discoideum*, utilize phagocytosis to engulf and digest prey (see Fig. 1a) (Clarke & Maddera, 2006). Phagocytosis provides exclusive access to nutrients as the prey organism is internalized within the predator cell phagosome. This mechanism also utilizes a fairly simple killing mechanism of acidification combined with a battery of hydrolytic enzymes secreted into the phagosomal vacuole (Krause, 2000). Phagocytosis is limited by the size of prey, as prey must be small enough to fit in the phagosome (Hahn *et al.*, 2000). Thus, one mechanism utilized by potential prey to escape predation by phagocytic cells is through the formation of multicellular structures such as biofilms (Hahn *et al.*, 2000).

Prey cell invasion

Bdellovibrio bacteriovorus is a small deltaproteobacterium that kills other Gram-negative bacteria by burrowing through the outer wall and embedding itself in the periplasmic space (see Fig. 1b) (Jurkevitch *et al.*, 2000; Sockett & Lambert, 2004; Lambert *et al.*, 2006). Again, this mechanism leads to exclusive access to prey cell nutrients, as prey cells are rarely invaded by more than one *B. bacteriovorus* cell. *Bdellovibrio bacteriovorus* grows and divides within the prey cell host, then subsequently lyses the outer wall of the prey host to repeat the predatory cycle. This process is antithetical to phagocytosis and thus requires a prey host cell that is larger than the *B. bacteriovorus* cell. *Bdellovibrio bacteriovorus* cells are therefore small (0.5 μm) and typically obligate predators unable to replicate outside of the host (Lambert & Sockett, 2008).

Diffusible lytic factors

Phagocytosis and prey cell invasion are both predatory mechanisms that require cell contact. In contrast, *Streptomyces* species are well known for their production of diffusible secondary metabolites with antibiotic activity

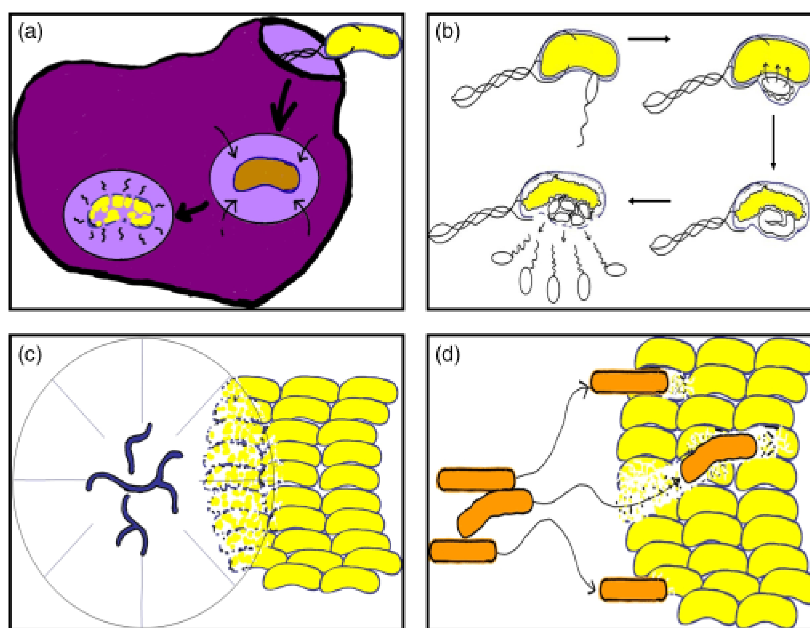

Fig. 1. Microbial predatory mechanisms. (a) Phagocytosis by a eukaryotic cell (purple) utilizes contact recognition of prey bacteria, succeeded by internalization of the prey bacterium (yellow) in a phagosome, where acidification and hydrolytic enzymes lyse and degrade the prey cell, providing a nutrient source. (b) Cell invasion of prey (yellow) by a swimming *Bdellovibrio bacteriovorus* cell (white). After burrowing through the outer wall, *B. bacteriovorus* secretes hydrolytic enzymes to obtain energy for growth and division before lysing the prey cell host to repeat the predatory cycle. (c) Secretion of far-ranging antibiotic secondary metabolites by *Streptomyces coelicolor* (blue, filamentous cells) results in lysis of sensitive bacteria (yellow). *Streptomyces coelicolor* also secretes hydrolytic exoenzymes that could be involved in deriving a nutritional benefit from lysed neighbors. (d) Predation by *Myxococcus xanthus* (orange cells) utilizes prey colony invasion, and an extracellular prey cell killing mechanism. Hydrolytic enzymes and secondary metabolites are secreted, but the specific roles of each for this contact-based killing mechanism are unknown.

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(see Fig. 1c) (Horinouchi, 2007). Under the right conditions, *Streptomyces* spp. will produce and secrete molecules such as streptomycin, resulting in a ring of growth inhibition and/or lysis of sensitive bacteria well beyond the edge of the *Streptomyces* colony (Hu & Ochi, 2001). Secondary metabolite production and secretion is typically dependent on low-nutrient conditions (Geistlich *et al*., 1992; Gehring *et al*., 2004). It is unclear if the release of secondary metabolites by *Streptomyces* is intended to reduce competition or if *Streptomyces* derives some nutritional benefit from the lysis of other microorganisms. Similar mechanisms are also used by nonphagocytic eukaryotes such as fungi, from which the cephalosporin class of antibiotics was first discovered (Balotescu *et al*., 2003; Franco-Hernandez & Dendooven, 2006).

Predatory range

It should be noted that while many predatory bacteria have been identified, most have been only briefly studied, and predation has likely evolved several times, as examples of predatory bacteria are found in the *Proteobacteria*, *Chloroflexi*, *Cytophagaceae*, and Gram-positive lineages (Jurkevitch, 2007). Predation may be facultative or obligate, and predatory ranges and hierarchies among microorganisms are only superficially understood, but are likely to be important in the structure of microbial communities (Casida, 1980; Germida & Casida, 1983). A better understanding of predatory range will be important for future study, as it is tempting to imagine having the capability to restructure microbial communities in a designed manner. Some predators have a narrow range, such as the alphaproteobacterium, *Micavibrio aeruginosavorus*, isolated by the ability to lyse *P. aeruginosa* cells, was unable to lyse any of the 55 other prey species tested (Lambina *et al*., 1983). Other predators, such as *Myxococcus xanthus* discussed in detail below, are capable of lysing a wide range or microbial species.

Myxococcus xanthus predation utilizes a novel strategy

Myxococcus xanthus is a Gram-negative soil bacterium with a complex life cycle including social gliding, fruiting body formation, and predation. The latter behavior is characterized by unusual mechanisms that do not resemble any of the predation mechanisms described above (see Fig. 1d). *Myxococcus xanthus* cells can penetrate prey colonies and lyse nearby cells (Berleman *et al*., 2006; Hillesland *et al*., 2007; 2009). They do not display the expansive range of destruction common to cell killing by diffusible antibiotics such as those observed in *Streptomyces* species, nor do *M. xanthus* cells invade the cell membrane of their prey like *B. bacteriovorus*. Thus, *M. xanthus* predation appears to require close proximity to prey, with prey cell death occurring in the extracellular environment relative to each *M. xanthus* cell. The mechanistic details of how prey cell lysis is achieved by *M. xanthus* is currently unclear. Interestingly, *M. xanthus* harbors a large genome of 9.13 Mb of DNA, which is particularly rich in products dedicated to secondary metabolism and degradative enzymes. One indicator for the production of novel chemical structures is the presence of polyketide synthase (PKS) genes. *Myxococcus xanthus* codes for 36 PKS genes, at the time of this writing; this is second only to *Streptomyces avermitilis* with 37 PKS homologs. By comparison, the *B. bacteriovorus* genome has only one PKS gene. Further research will be required to determine how the number of PKS genes relates to the secondary metabolite profile, but the correlation between PKS indicator genes and predation mechanism may reflect an evolutionary strategy that certain species have developed to handle the difficulty of lysing microorganisms in the extracellular space, without the aid of mechanisms such as phagocytosis or prey cell invasion.

In addition, *M. xanthus* and the *Myxobacteria* in general, are motile organisms, which mark another major difference between the *Myxobacteria* and nonmotile *Streptomyces*. Motility gives *M. xanthus* cells the advantage of being able to (1) actively search for prey and (2) regulate the mechanism of cell killing in a targeted manner such that lytic factors are released in response to prey cell contact, rather than solely in response to nutritional cues. Targeted and regulated secretion would require much lower concentrations of lytic factors than constitutive expression. Thus, study of the *M. xanthus* predation mechanism will require an analysis of the antibiotic metabolites produced, the degradative enzymes secreted, as well as investigations into the cell biology of predator–prey cell contacts and the behavior of *M. xanthus* at both the individual and group levels. A study by Mathew & Dudani (1955) examined the predatory range of two other *Myxococcus* species, *Myxococcus virescens* and *Myxococcus fulvus*, on a variety of human pathogens, including *S. aureus*, *Mycobacterium phlei*, *Shigella dysenteriae*, *Vibrio cholerae*, *Proteus X*, and several *Salmonella* isolates. With the exception of *M. phlei*, all of the examined pathogenic species were completely or partially lysed, indicating that deciphering the predatory mechanism utilized by *Myxobacteria* species is of practical importance to improving our understanding of how to treat bacterial infectious diseases.

Molecular mechanism of cell killing by *M. xanthus*

Evidence for the production of growth-inhibiting factors by *Myxobacteria* dates back to at least 1946, when Oxford & Singh (1946) showed that *S. aureus* growth was inhibited by an extract of *M. virescens*. Several other studies followed up on this phenomenon, examining various species, growth

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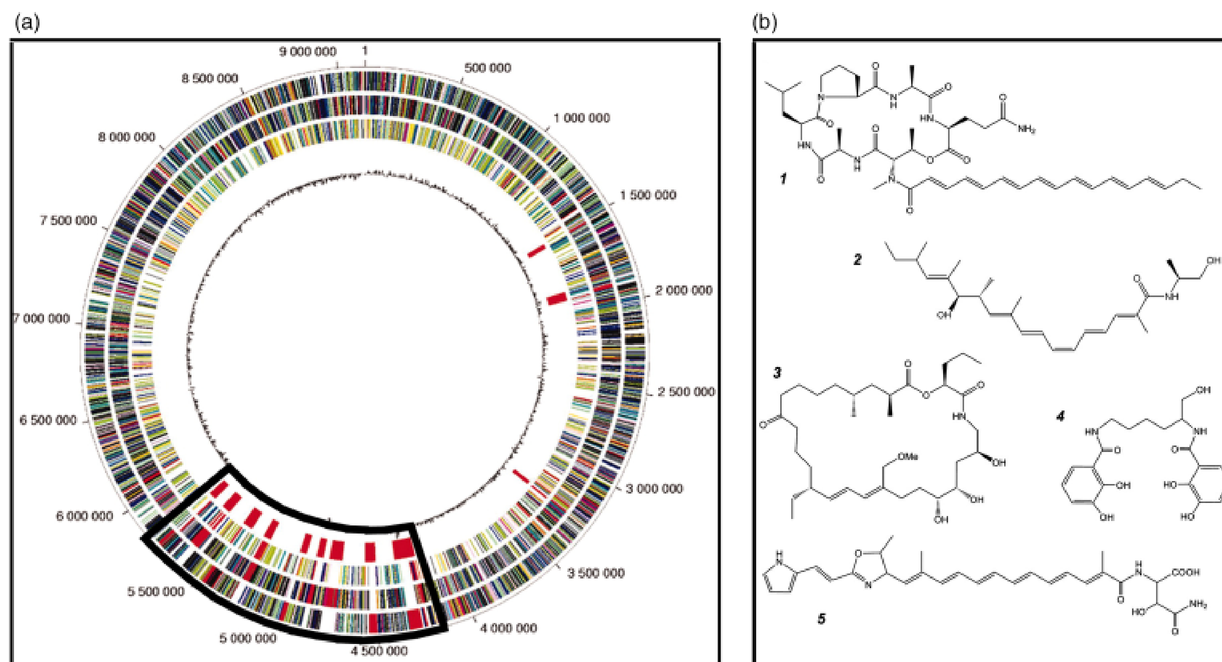

Fig. 2. Secondary metabolism in *Myxococcus xanthus*. (a) Map of the 9.13-Mb *Myxococcus xanthus* genome, with predicted secondary metabolism clusters highlighted in red in the inner circle. The majority of these clusters are located in a 1.5-Mb region of the genome distal to the origin of replication [reproduced from Goldman *et al*. (2006) with permission from the National Academy of Sciences). (b) Novel chemical structures extracted from various strains of *M. xanthus*. 1, myxochromid A; 2, myxalamid A (antifungal activity); 3, myxovirescen A (antibacterial activity); 4, myxochelin A (siderophore activity), and 5, DKxanthene-534 (yellow pigmentation) [reproduced from Krug *et al*. (2008) with permission from the American Society for Microbiology].

conditions and cellular fractions to identify lytic enzymes and chemical lytic factors (Singh, 1947; Kuhlwein, 1955; Ensign & Wolfe, 1965; Wingard *et al*., 1972; Rosenberg *et al*., 1973; Singh & Yadava, 1976). *Myxococcus xanthus* produces both protease(s) and lysozyme(s) that can be purified from culture supernatants (Hart & Zahler, 1966). Enzymes with amidase, glucosaminidase, and endopeptidase activity have also been isolated from culture supernatants (Sudo & Dworkin, 1972). Similar results have been obtained with other members of the *Myxobacteria* family (Ensign & Wolfe, 1965). These experiments were performed before the molecular biology revolution and neither the genes nor the protein coding sequences are known for any of these enzymes. Recent sequencing of the genome indicates that there are >300 genes predicted to code for degradative hydrolytic enzymes (Goldman *et al*., 2006). Therefore, we have only scratched the surface when it comes to discovering the potential of this organism in macromolecule degradation. Future work is needed to identify which of these degradative enzymes are specifically required to break down prey macromolecules, and to determine which of the enzymes, if any, contribute to the lysis of prey cells. It is possible, however, that exoenzymes are only required for macromolecule degradation and that prey cell killing is accomplished through the production of secondary metabolites with antibiotic activity.

The genome sequence of *M. xanthus* indicates tremendous potential for secondary metabolite production (see Fig. 2a) (Goldman *et al*., 2006). Although the *Myxobacteria* have not received the level of attention as the *Actinomycetes* with regard to metabolite production, there are a number of reports characterizing the unusual chemical structures produced by this family of organisms and their activity (see Fig. 2b). Antibiotic TA and sarascen are two molecules that can be extracted from cultures of *Myxobacteria* with demonstrated antibiotic activity (Rosenberg *et al*., 1973; Zafriri *et al*., 1981). However, in neither case do we know the role of the antibiotic molecule and whether it is required *in situ* for prey cell killing. *Myxococcus xanthus* also produces secondary metabolites such as DKxanthene, which produces a yellow pigmentation and may not be involved in predation (Meiser *et al*., 2006). Recent improvements in LC-MS analysis have yielded discovery of even more novel molecules, many of which still need to be structurally characterized (Krug *et al*., 2008).

We consider it very unlikely that *M. xanthus* expresses all of its degradative enzymes and secondary metabolites constitutively. Therefore, one of the most important questions that needs to be addressed in the near future is to understand how the degradative proteome and secondary metabolome are regulated to achieve lysis of prey. In the future, the analysis of predation mutants will reveal whether these

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pathways are specifically triggered depending on the prey species availability or if there is a single broad-range predation mechanism. The predatory mechanism of *M. xanthus* appears at this point to occupy the conceptual middle ground between the cell-invasion mechanism typified by *B. bacteriovorus* and the long-range diffusion of lytic factors by *S. coelicolor*. For this reason, we predict that *M. xanthus* cells detect and respond to the presence of prey similar to *B. bacteriovorus*, yet lyse prey with secreted lytic factors similar to *S. coelicolor*.

Cooperative vs. solitary predation in *M. xanthus*

The predatory mechanism of *M. xanthus* is often compared with a microbial wolfpack (Rosenberg *et al*., 1977; Kaiser, 2004; Hillesland *et al*., 2007). The wolfpack hypothesis proposes that *M. xanthus* cells secrete hydrolytic enzymes, which, at high cell density, pool together in the extracellular milieu generating a shared pool of hydrolytic break-down products that can be imported into individual cells to promote growth. Although this model is admittedly oversimplified, it provides a starting point for examining microbial predation in this species.

A critical aspect to the wolfpack model is that *M. xanthus* cells must work together in order to be successful predators. Like most bacteria, *M. xanthus* is facultatively multicellular. Individual cell traits include the ability to grow and divide in asocial contexts, 'adventurous' gliding motility of isolated cells (A-motility) and rapid sporulation in the presence of cell wall disrupting agents in dispersed liquid culture (Dworkin, 1962; MacNeil *et al*., 1994; O'Connor & Zusman, 1999). However, *M. xanthus* individualism is tempered with group behaviors. Routine liquid culturing of *M. xanthus* results in a distinctive biofilm 'ring' at the air–liquid–solid interface, indicating that even under growth conditions that promote an individual lifestyle, many cells aggregate into a biofilm as well (Dworkin, 1962). At the edge of *M. xanthus* colonies, isolated individual cells can be observed gliding across the surface, but the majority of cells are observed in large tendril-shaped groups utilizing Type IV pilus-mediated social motility (S-motility) (Mauriello & Zusman, 2007). Under starvation conditions, differentiation into spores requires that cells first move into aggregation centers, with cellular differentiation to spores occurring only in the cells present in the fruiting aggregate (Lee *et al*., 2005). These characteristics indicate that both unicellular and multicellular traits are critical features of the *M. xanthus* life cycle.

To determine if high cell density is necessary for predation, Rosenberg *et al*. (1977) showed that growth in liquid culture on the macromolecule casein is dependent on a high cell density of *M. xanthus*. Yet, when cultured with hydrolyzed casein, no significant difference in growth rate was observed in cultures with either high or low *M. xanthus* cell density. The hydrolysis of macromolecules at high cell density was one of the first demonstrations of cooperative behavior in any bacterial species, and supports the hypothesis that a group is required for successful predation. Cell density-regulated processes have since been discovered in a wide range of prokaryotes, and are often due to the production and detection of quorum signals that allow the regulation of gene expression in response to cell density (Bassler, 2004).

Thus, it will be important for future research to establish whether *M. xanthus* shows cell density-dependent gene expression of degradative enzymes. Alternatively, regulation could occur at the level of the secretory apparatus or, as Rosenberg *et al*. (1977) proposed, exoenzyme production may be constitutive, and the difference in growth rate could be attributed to reaching a critical extracellular threshold of hydrolytic enzymes required. Also important will be to establish how protein secretion in a shaking flask culture relates to secretion on solid surfaces where diffusion is more limited, and how hydrolysis of the casein protein compares with the lysis of prey.

The work by Rosenberg and colleagues supports the importance of cooperation during predation; however, evidence for successful individual predation was observed in cocultures of a closely related species, *Myxobacter* strain FP-1, with a *Cyanobacteria* prey species (see Fig. 3a) (Shilo, 1970). Later work by McBride & Zusman (1996) showed that individual cells of *M. xanthus* are capable hunters as well. By analyzing single cells in the presence of *Escherichia coli* microcolonies consisting of *c*. 20 cells, the authors observed that individual *M. xanthus* cells would move back and forth within the microcolony until all of the available prey cells had been lysed. *frz* mutants defective in regulating cell movement were still able to lyse prey cells that they made direct contact with, but then exited the microcolony, leaving several prey cells intact. Interestingly, the *frz* genes encode for products similar to chemotaxis proteins in enteric bacteria suggesting that chemotaxis-like mechanisms regulate predation, which is discussed in more detail below in Tactic mechanisms: chemotaxis and Tactic mechanisms: predataxis. Also important is the fact that prey cells that are not contacted by *M. xanthus* are not lysed, suggesting that cell contact may be required for transmission of *M. xanthus* lytic factors. This study supports the idea that lytic factors produced by *M. xanthus* may lose their potency with diffusion, but contradicts the notion that a cooperative 'wolfpack' is essential for predation. Thus, even if an *M. xanthus* pack provides predatory benefits, individual cells are capable predators. This study raises several questions. How do *M. xanthus* cells sense a prey colony in order to methodically lyse all of the available cells, as close proximity is required for lysis? Is exoenzyme secretion

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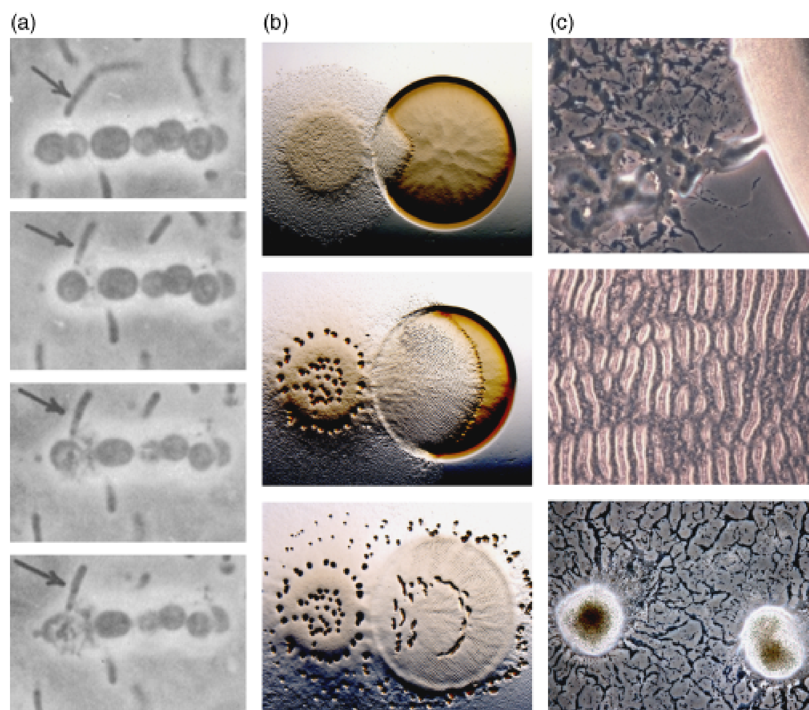

Fig. 3. Group and solitary-based predation by *Myxococcus xanthus*. (a) Time course of solitary predation by myxobacter FP-1 on cyanobacteria cells. The cyanobacterial species can be observed as a chain of large, spherical cells. The arrow points to a rod-shaped myxobacter cell in the process of lysing two cyanobacteria cells [reproduced from Shilo (1970) with permission from the American Society for Microbiology]. (b) Time course of *M. xanthus* invading and lysing a colony of *Escherichia coli* prey bacteria. (c) These images show the three major morphological traits of group-mediated predation: colony invasion (top), rippling wave structures (middle), and fruiting bodies (bottom). Reproduced from Berleman *et al*. (2006) with permission from the American Society for Microbiology.

constitutive and prey cell death limited by lytic factors rapidly losing their efficacy as they diffuse away from an *M. xanthus* cell? Is secretion of lytic factors triggered by cell–cell contact with prey, and delivered in a directed manner such that noncontacted cells escape predation? More research will be required to understand how the combination of cooperative and individualized predation takes place.

Group behavior and multicellular organization during predation

Another prediction that stems from the wolfpack hypothesis is that if *M. xanthus* cells hunt prey cooperatively, then cells should display organized, coordinated behavior indicative of cooperation during predation. Because individual cells can alter their behavior after contact with prey, then what is the organization of a pack of *M. xanthus* cells? A common misconception is that *M. xanthus* cells surround their prey (Goldman *et al*., 2006). Unlike a wolfpack, *M. xanthus* cells have not been observed to surround their prey (Berleman *et al*., 2006). Rather, the early steps of predation involve motile *M. xanthus* cells gradually penetrating prey colonies (Fig. 3b and c) (McBride & Zusman, 1996; Berleman *et al*., 2006). In addition, *M. xanthus* cells have not been observed to swarm over prey, rather *M. xanthus* cells tend to maintain contact with the agar surface, such that predation occurs at the interface between the prey cells and the agar. Within a few hours after entry into the prey colony is achieved, prey cell lysis is observed, and after 16–20 h *M. xanthus* cells display a striking change in colony morphology as ripples appear on the colony surface (Berleman *et al*., 2006). To the naked eye, ripples appear as fixed structures, but through time-lapse microscopy ripples are shown to be moving structures (Reichenbach, 1966; Shimkets & Kaiser, 1982). Rippling occurs for several days during predation and, interestingly, is only observed within the area originally covered by the prey colony (Berleman *et al*., 2006). After expanding beyond the prey colony, the *M. xanthus* swarm quickly returns to the tangled appearance observed in monoculture conditions.

Rippling was first reported by Reichenbach in 1966, and was noted to occur in several myxobacterial species in both the presence and the absence of prey. A detailed analysis of rippling induction by Shimkets & Kaiser (1982) showed that in both monoculture and coculture conditions rippling was induced by the presence of extracellular peptidoglycan. Berleman *et al*. (2006) observed similar results in the presence of proteins and chromosomal DNA. Polysaccharide macromolecules such as starch and glycogen were tested, but with no consistent induction of rippling observed (unpublished data). Rippling was observed during incubation with diverse prey substrates such as P1 phage, *E. coli*, *B. subtilis*, and *S. cerevisiae* all eliciting a similar rippling response. These data support the hypothesis that rippling is a predatory behavior induced by macromolecular growth substrates. Shimkets and Kaiser also observed rippling in the presence of peptidoglycan monomers Ala, NAG, and NAM, but rippling was not observed by Berleman and colleagues

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with any monomeric substrate (Shimkets & Kaiser, 1982; Berleman *et al*., 2006). This could be due to differences in assay conditions, or as discussed by Shimkets and Kaiser, the presence of peptidoglycan monomers can sometimes stimulate the release of larger peptidoglycan fragments from cell walls.

Rippling also occurs sporadically in starved monocultures of *Myxobacteria* before, during, and after fruiting body formation (Gronewold & Kaiser, 2001). For this reason, it has been proposed that rippling is an intermediate, organizational stage during fruiting body formation (Kaiser, 2004). There are two possibilities: one is that rippling is utilized by *M. xanthus* cells for two different purposes; the other is that starvation and predation conditions share a common signal. In support of the latter hypothesis, there is evidence that mutants defective in fruiting body development are also defective in predation (Pham *et al*., 2005). Because cell lysis is often observed in *M. xanthus* cultures under stringent growth conditions, it is possible that the release of macromolecules by lysing *M. xanthus* cells provides a predatory stimulus and rippling is utilized during both conditions (O'Connor & Zusman, 1988; Nariya & Inouye, 2008). Dead *M. xanthus* cells stimulate rippling similar to live *E. coli* cells (Berleman *et al*., 2006). Thus, under stringent conditions that involve high levels of cell lysis, the occasional observation of rippling before fruiting body formation may be indicative of a predatory phase, in which *M. xanthus* cells cannibalize their sisters. Also in agreement with the idea that rippling is a predatory behavior even during fruiting body formation is the fact that in strains with a very low level of autolysis, such as the wild-type strain DZ2, rippling is rarely observed in monoculture conditions, but is consistently observed during predation (Berleman *et al*., 2006).

Mutants lacking proteins important for motility, such as PilA, the major subunit of Type IV pili, and regulation of reversal frequency, such as the methyltransferase of the Frz chemosensory system, FrzF, display no rippling behavior (see Fig. 4a and b). Nevertheless, *pilA* and *frzF* cells are still capable of penetrating prey colonies and lysing prey cells, indicating that rippling is not essential for predation (Berleman & Kirby, 2007; Berleman *et al*., 2008). While not strictly essential, rippling is required for efficient predation of *E. coli* colonies with the strains tested. In fact, a *pilA* strain shows a reduced rate of prey cell lysis and a *frzF* mutant shows reduced swarm expansion when moving through a prey colony. Interestingly, a hyper-rippling *frzG* mutant (the methylesterase of the Frz pathway) is also defective at migrating through a prey colony (Berleman *et al*., 2008). This indicates that formation of a multicellular rippling structure alone is not enough to provide a significant benefit during predation, and that fine control of cell behavior during rippling is required for the behavior to elicit a positive effect on predation efficiency.

Behavior of individual cells during predation

How do individual *M. xanthus* cells regulate movement to produce rippling structures? A preliminary hypothesis was that *M. xanthus* cells form an agglutinated wave that travels unidirectionally across a prey colony. This assertion was shown to be incorrect by (Sager & Kaiser 1994) through examination of a minority population of green fluorescent protein (GFP)-labeled cells mixed with a majority population of unlabeled cells. The authors showed that *M. xanthus* cells reverse direction frequently during rippling, such that the illusion of a traveling wave comes from colliding waves of cells reflecting off each other in a repetitive manner. The authors also observed that an approximately equal number of cells were moving in each direction and proposed that rippling behavior results in no net cell movement of individuals. Although there are no long-distance-traveling waves, do *M. xanthus* cells form cohesive groups during rippling? Closer examination of *M. xanthus* cells during rippling showed that cells moving in opposite directions tend to interpenetrate one cell length before reversal is triggered (Sliusarenko *et al*., 2006). It has also been observed that during wave collision reversal does not always occur (Igoshin *et al*., 2001). Similarly, some cells have been observed to reverse in the low-density troughs that arise during rippling behavior, indicating that individualistic decisions are made that do not necessarily correspond to the actions of the nearest neighbors (Igoshin *et al*., 2001).

How is predatory rippling behavior controlled at the molecular level? One hypothesis is that *M. xanthus* cells produce a signal that allows them to respond to head-to-head collisions with other *M. xanthus* cells during rippling behavior (Sager & Kaiser, 1994; Igoshin *et al*., 2001). The *csgA* gene is required for rippling behavior and has been proposed to signal a collision between cells moving in opposite directions. The 25-kDa CsgA protein is secreted through an unknown mechanism and during starvation conditions is cleaved by the PopC protease to produce a 17-kDa form (Rolbetzki *et al*., 2008). CsgA has been shown to decorate the extracellular matrix around the entire cell (Shimkets & Rafiee, 1990). Thus, CsgA from neighboring cells could be detected and the signal transduced across the cell envelope to elicit a reversal. Unfortunately, without a bona fide C-signal receptor, it is difficult to conclusively characterize the CsgA protein as a bona fide cell–cell signal. It is possible that the proteolysis event by itself signals a collision, such that PopC proteolysis activity of CsgA on a neighboring cell triggers the cell expressing PopC to reverse. This idea is supported by the fact that that *popC* mutants cannot be complemented extracellularly by mixing with PopC+cells (Rolbetzki *et al*., 2008). In this scenario, PopC would act as both protease and signal transducer. Because

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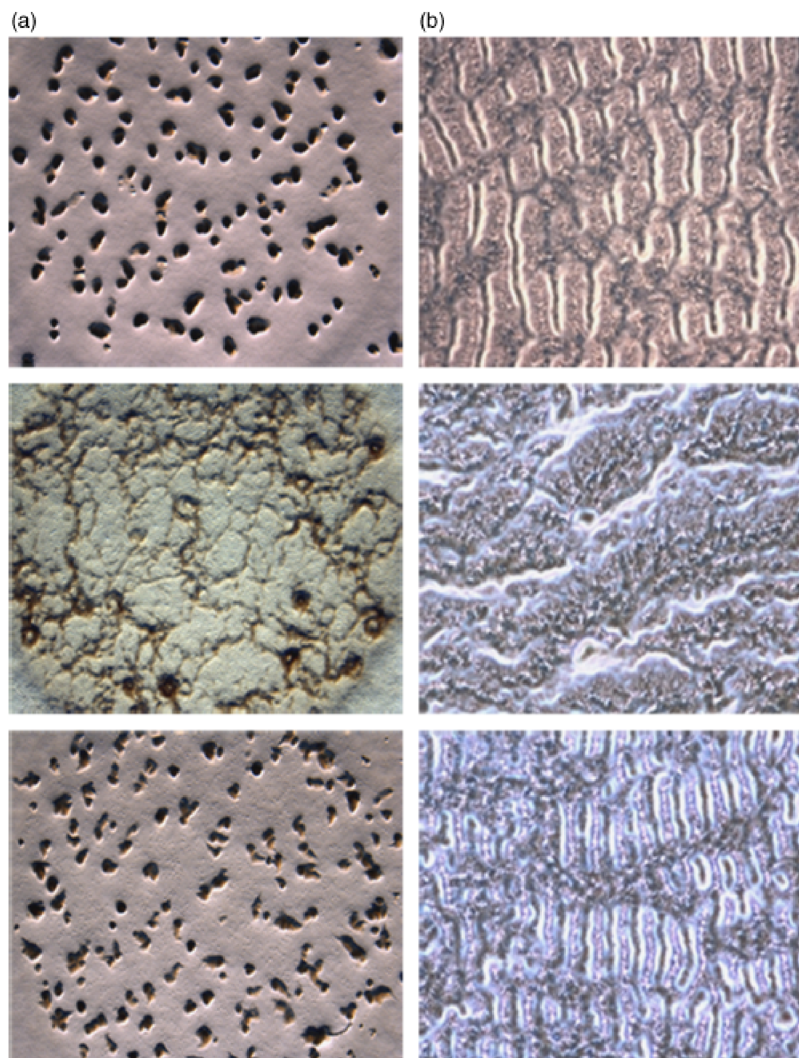

Fig. 4. Regulation of *Myxococcus xanthus* cell behavior by the Frz pathway. (a) Fruiting body formation assay with wild-type DZ2 (top), Δ*frzF* (middle), and Δ*frzG* (bottom). (b) Rippling behavior of the same strains in the presence of *Escherichia coli* prey. Δ*frzF* is unable to construct ripple structures, whereas Δ*frzG* forms tightly packed ripples.

PopC is also extracellular there is still the problem of transducing the signal into the cell. It is not known if the 17-kDa form of CsgA is generated during predation, but it is not detected during vegetative growth with high-nutrient broth (Sogaard-Andersen *et al*., 1996).

Another possibility is that *M. xanthus* cells do not directly signal to each other during the rippling behavior. Instead, the signal that drives rippling behavior may come solely from prey macromolecules (Berleman *et al*., 2008). In this model, each *M. xanthus* cell responds to the presence of prey autonomously and the ripple structures that arise are a consequence of the shifting movements of individuals reaching a tenuous state of equilibrium. As the local density of *M. xanthus* cells increases, each cell will be more likely to trigger a reversal as it becomes surrounded by inedible sister cells. A reversal under this circumstance has the potential to move a cell away from an area crowded with predators and back toward an area with more prey contacts available. Both of these possibilities rely on the signal (whether self-generated or prey-generated) to be transduced to the motility organelles through the Che-like Frz pathway (McBride *et al*., 1989; Igoshin *et al*., 2004).

Tactic mechanisms: Chemotaxis

Myxococcus xanthus cells are motile on solid surfaces through a mechanism termed gliding motility. Gliding motility is a ubiquitous trait in the *Myxobacteria*, and is observed in a number of other bacteria as well (Jarrell & McBride, 2008). All gliding bacteria move on solid surfaces in the direction of the long axis of the cell, and in *M. xanthus*, the leading cell pole is observed to switch periodically. In *M. xanthus*, gliding is powered by two synergistic systems: one system powered by retracting Type IV pili localized at the leading pole of the cell (Sun *et al*., 2000) and the other utilizing focal adhesion sites that are initiated at the leading cell pole and then distributed along the entire cell body (Mignot *et al*., 2007; Sliusarenko *et al*.,

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2007). Other extracellular components also play a critical role in gliding motility such as fibrils, composed of protein–exopolysaccharide mixtures, lipopolysaccharide and extrusion of polysaccharide slime trails (Behmlander & Dworkin, 1991; Lu *et al*., 2005; Yu & Kaiser, 2007). Both motility systems are regulated by the Frz pathway, which consists of a cytoplasmic receptor, FrzCD, that senses an unidentified signal and transduces this information to the FrzE–FrzZ two component system (Inclan *et al*., 2007, 2008). Because *M. xanthus* cells are motile and capable of changing direction, one of the initial hypotheses of the wolfpack model is that *M. xanthus* cells use chemotaxis to aggregate toward a susceptible prey colony.

Cellular reversals in *M. xanthus* are often compared with how swimming *E. coli* cells switch the rotational direction of the flagella in response to chemical diffusible signals. (Dworkin, 1983). If reversals were indicative of a chemotactic response, then chemicals released from a prey colony might serve as chemoattractants to draw *M. xanthus* cells toward suitable prey. Several investigations into chemotaxis by *M. xanthus* toward typical chemoattractants such as amino acids and sugars revealed no bias (Dworkin, 1983). *Myxococcus xanthus* cells move slowly at $0.02\,\mu m\,s^{-1}$ compared with $50\,\mu m\,s^{-1}$ for a swimming *E. coli* cell and $2–4\,\mu m\,s^{-1}$ for gliding cells of *Flavobacterium johnsoniae*. Also, while reversal of the flagellar motors in *E. coli* results in an immediate random reorientation of the cell in a three-dimensional space, a reversal by *M. xanthus* results in a very predictable change because on a two-dimensional surface, the change in direction after a reversal is predictably the exact linear opposite of the previous direction of movement. *Myxococcus xanthus* rods are flexible and nonlinear changes in direction tend to occur as cells gradually move forward. It is also important to consider that the energetics of switching the rotation state of the flagellum are likely to be much less demanding than the requirements for changing the leading gliding pole in *M. xanthus*. A change of the leading pole requires translocation of some motility proteins across the entire length of the cell, such as RomR and FrzS, and duplicate expression of the remaining motility proteins at both cell poles, which must be periodically activated or inactivated (Mignot *et al*., 2005; Nudleman *et al*., 2005).

Attempts at observing changes in *M. xanthus* reversal frequency in response to chemicals have yielded some interesting results. Shi and Zusman showed that very steep chemical gradients in which the concentration changes 10-fold in 1 mm could yield positive and negative chemotactic results (Shi *et al*., 1993, 1994; Shi & Zusman, 1994a, b). Interestingly, although positive directed movement was observed with *M. xanthus* colonies toward casitone–yeast extract mixtures, directed movement of individuals toward these mixtures was not observed. Similar results were obtained by Taylor & Welch (2008), who concluded that positive chemotaxis was an emergent property of an *M. xanthus* collective of cells working together . A change in the behavior of individual cells was also observed upon incubation with chemorepellents such as isoamyl alcohol (IAA) (Shi & Zusman, 1994b; Shi *et al*., 1994). In the presence of IAA, *M. xanthus* cells show a dramatic increase in cellular reversals. A deeper analysis of the negative chemotactic response showed that the IAA assay may be revealing an inhibition of cell migration in cells closest to high concentrations of IAA, combined with a lack of inhibition in cells that are further away from the IAA, giving the appearance of an overall negative tactic response (Xu *et al*., 2007). Thus, while IAA elicits a change in behavior that requires a functioning Frz pathway, the altered behavior does not appear to confer the ability to move away from the IAA stimulus. This idea is supported by the fact that certain mutations in the FrzCD receptor lead to a hyper-reversal phenotype and these hyper-reversing mutants also show little to no net movement at either the cellular or colony level (Blackhart & Zusman, 1985).

Lipid extracts from *M. xanthus* solubilized in chloroform also generate a positive chemotactic response at the colony level (Kearns & Shimkets, 1998). Analysis of *M. xanthus* cells in the presence of specific, slowly diffusing fatty acid substrates has revealed three derivatives of phosphatidyl ethanolamine (PE) that inhibit reversals in individual cells, 12:0, 18:1w9, and 16:1w5c (Blackhart & Zusman, 1985; Kearns *et al*., 2001). Of these, 16:1w5c has the strongest

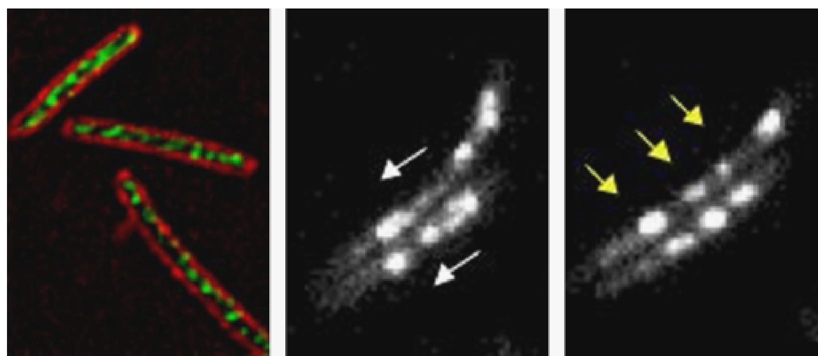

Fig. 5. Cell contact sensing by *Myxococcus xanthus*. Immunofluorescence staining of the FrzCD receptor reveals a helical arrangement of clusters throughout the entire cell length (left). FrzCD-GFP clusters show dynamic localization patterns, with discrete clusters observed to align at times during cell–cell contacts between *M. xanthus* cells (middle 0 s, right 30 s) (courtesy of E. Mauriello and D. Zusman).

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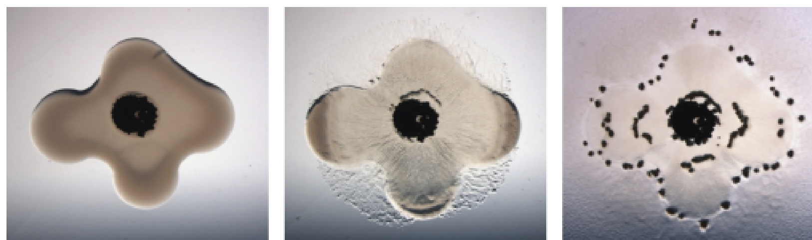

Fig. 6. Multicellular development during predation in *Myxococcus xanthus*. Time course of predation on an irregular shaped prey colony, in which fruiting bodies can be observed to form preferentially along the prey colony edge [reproduced from Berleman & Kirby (2007) with permission from the American Society for Microbiology].

effect, eliciting a response at *c*. 2 ng, whereas 12:0 and 18:1w9 inhibit reversals at *c*. 2 μg, 1000-fold higher than the 16:1w5c threshold (Kearns *et al*., 2001). In addition, prolonged incubation in the presence of 12:0 has been shown to inhibit cell movement (Bonner *et al*., 2005). 16:1w5c is an uncommon fatty acid in natural samples but is abundant in *M. xanthus* membranes at *c*. 10–15% of the fatty acid pool (Curtis *et al*., 2006). Inhibition of reversals stimulated by decorated PE does not appear to require the Frz pathway, as a *frzCD* mutant was shown to have a similar fourfold reduction in reversal frequency in the presence of 12:0 PE (Kearns & Shimkets, 1998). There is evidence that the Dif chemosensory pathway also contributes to the PE response, but this is complicated by the fact that Dif mutants have severe defects in exopolysaccharide biosynthesis that have an epistatic effect on cell movement (Yang *et al*., 2000). Further examination is needed to determine how the inhibition of reversals in the presence of PE affects the net cell displacement of *M. xanthus* cells. It also remains unclear what natural circumstances elicit release and solubilization of PE substrates such that they could be used by *M. xanthus* as a chemotactic signal to locate prey.

Tactic mechanisms: Predataxis

Recently, predatory rippling behavior was examined for chemotactic-like changes in cell behavior (Berleman *et al*., 2008). Because rippling occurs during contact with prey, it is not expected to be utilized to locate prey at a distance, rather it is hypothesized that rippling may occur as a result of directed movement when *M. xanthus* cells directly contact prey macromolecules. Analysis of GFP-labeled *M. xanthus* cells within swarming groups in the presence and absence of prey indicates that movement in the absence of prey is random with infrequent cellular reversals, gradual changes in direction through cell bending and little net movement of the entire population. In the presence of prey, *M. xanthus* movement is characterized by frequent changes in gliding direction through cellular reversals, inhibition of changes in direction through cell bending and a net movement of the population of cells in the direction of increasing quantities of prey. Observations of this 'predataxis' behavior are in stark contrast to the predictions based on previous chemotaxis experiments, and may be indicative of a regulatory behavior that is mechanistically distinct from the *E. coli* paradigm.

Rippling behavior has been shown to change over time such that the space between the ripple crests, or the wavelength, increases over time (Berleman *et al*., 2008). Although prey cells are immobilized in this assay such that there is no spatial gradient, prey-derived macromolecular growth substrates are expected to decrease over time as they are consumed. If true, then this would create a temporal gradient in which *M. xanthus* cells should detect a significant decrease in resource availability without a significant change in position. Thus, even though *M. xanthus* cells move extremely slowly, detection of temporal changes in nutrient availability may be critical to maintaining close proximity to prey and benefit later decisions such as fruiting body formation and sporulation.

Chemosensory pathways in bacteria have the interesting feature of being temporal in nature rather than spatial such as in chemotactic eukaryote cells. To determine if predataxis is necessary in a spatial assay, Berleman *et al*. (2008), utilized long strips of prey to exaggerate the spatial component of predataxis behavior. During predataxis, the rate of swarm expansion increases relative to swarm expansion in the absence of prey. This increase is dependent on a functional Frz pathway as both *frzG* and *frzF* mutants are unable to increase swarm expansion through prey colonies. This indicates that predatactic behavior requires regulation of cell reversals through the Frz pathway, analogous to the *E. coli* chemotaxis signal transduction paradigm. However, the input signal from sensation of prey and the output response through the gliding motility system are different from what has been observed in *E. coli*. Recent analysis of FrzCD receptor localization indicates that the protein is distributed in a helical pattern of clusters across the entire cell (see Fig. 5) (Mauriello *et al*., 2009). This is in stark contrast to *E. coli*, which has a single polar receptor cluster (Banno *et al*., 2004). FrzCD localization was also observed to change upon cell–cell contact with other *M. xanthus* cells. We propose that this mechanism could also be used to track

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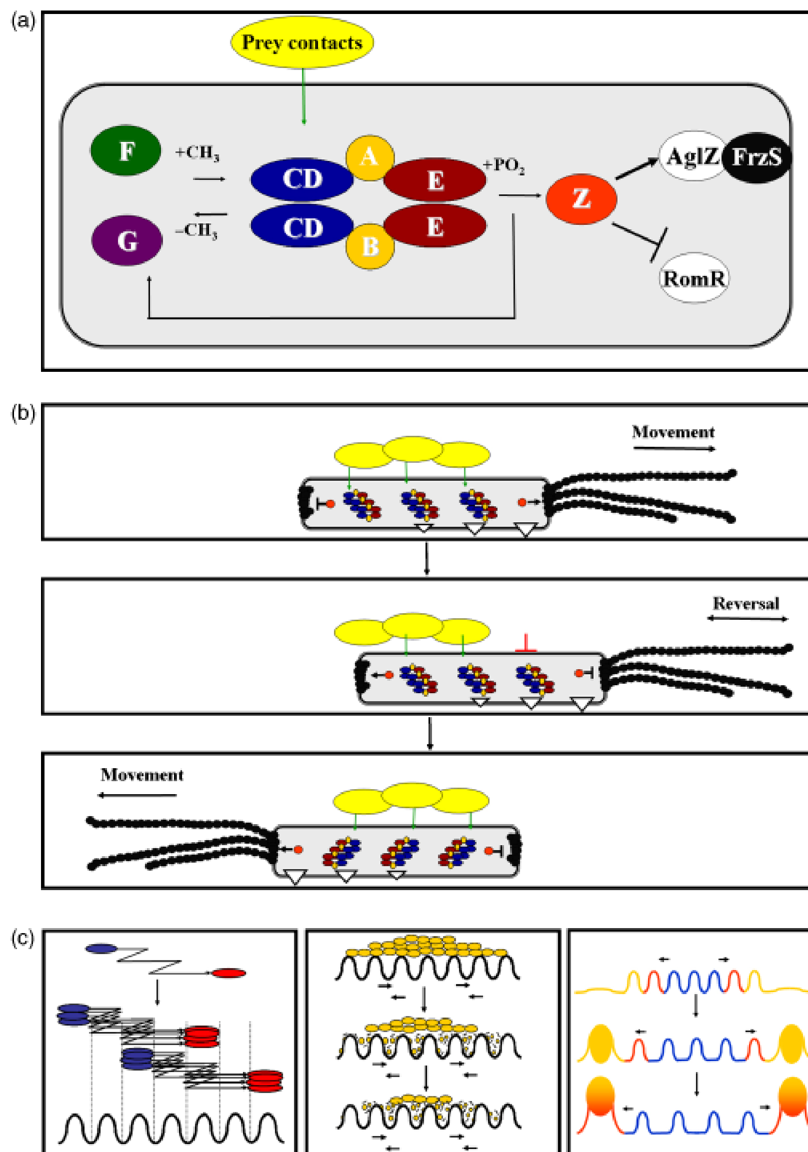
contact with prey, through distributed clusters along the cell body (see Fig. 6). Movement beyond the prey cell colony, or movement into a crowd of *M. xanthus* cells could both result in a loss of prey contact, triggering a cell reversal.

Fruiting body function

Fruiting bodies also form during predation (see Fig. 7). This is enigmatic because fruiting body formation has long been known to be inhibited by growth on nutrient-rich media and induced when exposed to low or no nutrients (Dworkin, 1962). The ability to construct large multicellular structures from populations of essentially independent individuals, has been a major focus of study on the *M. xanthus* model system (Shimkets, 1999). Fruiting bodies are aggregates that typically consist of *c.* 10^6 cells, and in most species of *Myxobacteria* mature fruiting bodies contain cells that have differentiated into metabolically dormant spores (Lee *et al.*, 2005). In some species, spore-filled fruiting bodies are embedded within the biofilm matrix; in other species, the fruiting bodies protrude up from the surface in a complex morphology consisting of stalks and appendages (Shimkets, 1999). But a common trend is the separation of cell type that is demarked by the boundaries of the fruiting body structure. Cells within the fruiting body differentiate into spores, while cells outside of the fruiting body remain in the vegetative state.

In the laboratory, fruiting body formation can be rapidly induced through plating cells with a combination of high cell density and low nutrient availability. This shift in



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nutrient conditions results in typically randomly distributed fruiting aggregates where cells rapidly differentiate into spores. During predation, however, fruiting bodies form in predictable patterns around the edges of a prey colony, such that fruiting bodies can be observed at the boundary between predatory rippling populations and nonrippling populations (see Fig. 7) (Berleman & Kirby, 2007). Nonrandom fruiting body formation was also observed during monoculture analysis of rippling behavior (Welch & Kaiser, 2001). In this case, fruiting bodies were also observed to form in a circle surrounding the rippling region. If rippling behavior is a result of predatory feeding on macromolecules from prey or lysed *M. xanthus* sister cells, then detection of a sudden loss in macromolecule availability could stimulate a change in behavior that results in fruiting body aggregation. In support of this, fruiting bodies were induced by a step-down in prey availability, but not by a corresponding step-up (Berleman & Kirby, 2007). This pattern was observed across a wide range of prey cell densities and basal nutrient levels, indicating that the decision to aggregate into fruiting bodies during predation results from relative changes in nutrient availability rather than a single, absolute starvation threshold. Additionally, *relA* and *asgD* mutants, while unable to aggregate into fruiting bodies in monoculture conditions (Singer & Kaiser, 1995; Harris *et al*., 1998; Cho & Zusman, 1999), were shown to form fruiting bodies when coincubated with prey. *relA* and *asgD* code for proteins essential for producing intracellular ppGpp and extracellular A-signal, respectively. These signals are the earliest known required steps of fruiting body formation and sporulation in monoculture conditions. Although fruiting body structures were formed, neither the *relA* nor *asgD* strain showed any significant differentiation into spores. Thus, during predation the multicellular fruiting structure can be stimulated by changes in extracellular nutrient availability, but the final conversion to spores requires sensation of an absolute starvation threshold and production of the appropriate cellular signals. Interestingly, while starvation has often been thought of as inducing a program of fruiting body formation and subsequent sporulation, it may be that in natural settings, induction of fruiting aggregates occurs in response to a relative decrease in prey and/or nutrient availability, followed later by an absolute starvation threshold that induces sporulation of cells within the aggregate. This could explain why *relA* and *asgD* mutants remain competent for fruiting body formation in the presence of prey, yet are unable to sporulate.

The identification of rippling as a form of multicellular development utilized during predation leads to some exciting possibilities. There are several hypotheses that are worth considering. The first model to consider is that the rippling pattern observed provides no significant group benefit; it only arises through the repetitive behavioral pattern elicited as each individual moves tactically in response to a similar prey stimulus (see Fig. 6c, The autonomous behavior model). Synchronization of cells could result from a combination of factors including proximity to prey for nutrients, access to oxygen for respiration, and contact with the agar surface for movement. Integration of all these factors could lead to the emergence of a multicellular pattern, without providing any additional benefit to the group. Another model to consider is that the synchronized multicellular

Fig. 7. Modeling predatory behavior in *Myxococcus xanthus*. (a) Model of the Frz signal transduction pathway. FrzF and FrzG add and remove methyl groups to modulate the signaling state of the cytoplasmic receptor, FrzCD. FrzCD detects prey through an unknown mechanism and transduces this signal through the coupling proteins FrzA/FrzB to the histidine kinase, FrzE. FrzE autophosphorylates and transfers phosphoryl groups to the response regulator, FrzZ. At the leading cell pole, the Frz pathway is predicted to activate motility proteins such as AglZ and FrzS while inhibiting RomR. The Frz pathway should have an opposite regulatory effect on the lagging cell pole. The specific interactions between Frz pathway components and motility organelle proteins are unknown and may occur directly through FrzZ, or indirectly through other protein partners. (b) Prediction for how receptor clusters could be involved in sensing prey. In chemotaxing enteric bacteria, there is typically only a single cluster of receptor proteins that regulate the motility behavior of the cell. In *M. xanthus*, contact with prey cells (colored yellow) could be monitored by the presence of multiple signaling clusters along the entire length of the cell, such that when an *M. xanthus* cell moves beyond the available prey, or is crowded with other *M. xanthus* cells, loss of prey signal (green arrows) at one Frz receptor cluster triggers a reversal of the leading cell pole, resulting in a change in the direction of cell movement that would inhibit the cell from leaving prey behind. (c) There are several models that can be used to explain multicellular development during predation. *The autonomous behavior model* (left): The precise, repetitive movements of individual cells detecting prey leads coincidentally to a multicellular structure, with no definable benefits derived from the multicellular pattern observed. The zig-zag arrows represent an idealized pattern of directed movement of *M. xanthus* cells from point A (blue) to point B (red) during predation. The wave structures form as a result of cell accumulations from the highly repetitive behavior of individuals. *The grinder model* (middle): The movement of the wave structures of *M. xanthus* cells during rippling causes a physical disruption of the prey colony that no single cell could elicit individually, providing increased access to insoluble macromolecular growth substrates. The arrows indicate lateral motion of the rippling waves, which typically form in between the prey colony and the agar surface. Colonies or biofilms of prey cells (yellow) are predicted to be physically disturbed by the rippling motion. *The population control model* (right): Accumulation of cells into waves creates the greatest surface area possible for direct contacts between predator and prey cells. The ripple wavelength correlates with the prey cell availability, and as prey are consumed over time the increased wave spacing pushes excess predator cells (colored yellow and orange) to the edges of the rippling area. On this outer edge, a build-up of *M. xanthus* predator cells leads to the formation of fruiting body structures, where starving vegetative cells differentiate to spores. This model predicts that multiple *M. xanthus* cell types exist within closely associated microenvironments, and that specialization into active predatory cells and dormant spores benefits the population as a whole.

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movement of *M. xanthus* cells may result in a physical disruption of prey biofilms that could not be accomplished by uncoordinated individuals (see Fig. 6c, The grinder model). If true, then nonrippling mutants should be inefficient predators, particularly in situations where prey are embedded in a sturdy biofilm.

A third model to consider is that *M. xanthus* cells utilize rippling to regulate cell density and cellular differentiation (see Fig. 6c, The population control model). It is important to consider that direct contact with prey macromolecules may be required in order to utilize this nutrient source. In other words, there might not be a substantial pool of diffusible nutrients as proposed by the original wolfpack model, but rather a limited number of nutrient-rich prey macromolecule access sites. If contact with prey is essential for nutrient access, then the rippling pattern may allow for a greater number of direct contacts with prey as a wave creates a greater surface area than a flat plane. As prey are consumed and the number of prey contact sites diminishes, individual cells may have to range farther for sufficient prey cell access. Although this change in range may be in the order of a few microns of extra movement between reversals, this can be observed at the population level as the distance between rippling waves of *M. xanthus* cells increases over time during predation. This change in ripple spacing effectively decreases the local cell density of *M. xanthus* and forces the excess *M. xanthus* cells out and away from the remaining available prey. Cells that are forced out of macromolecule-rich areas aggregate into fruiting bodies, where prolonged nutrient depletion will result in sporulation. Thus, the population is segregated into rippling cells that maximize growth, and aggregating cells that maximize survival by differentiating to spores, rather than a single population that promotes growth unchecked until nutrient exhaustion.

As with any social process, it is possible that *M. xanthus* predation is susceptible to the presence of cheater subpopulations that reap nutrients without a corresponding cooperative contribution to the group (Velicer, 2009). This could occur, for instance, in mutants deficient in exoenzyme production, which expend less energy but benefit from the exoenzymes of neighboring cells. It will be interesting to see if the complex behavior and development of *M. xanthus* cells during predation provides a mechanism for insulating the population from cheater phenotypes. Further experiments will be required to distinguish between group and individualistic tendencies in predatory *M. xanthus* populations.

Concluding remarks

The 21st century is likely to be defined by how our global society comes to terms with the reality of limited resources. One example of this is the use of antibiotics because their commercial introduction 80 years ago. The first century of antibiotic usage was marked by the assumption that there will always be another antibiotic available. Yet, as molecule after molecule loses its effectiveness in the wake of emerging multi-drug-resistant pathogens, it becomes ever more clear that this approach is not likely to be sustainable over the next 80 years. Among the many cultural changes necessary to improve health in our society, one is simply to gain a better understanding of the organisms that produce antibiotic molecules, and learn how it is that they have managed to remain successful at killing other microorganisms over the past few millions of years – particularly the small fraction of microorganisms that have the capacity to evade or subvert the human immune system. Indeed, many microorganisms in nature appear to be better at killing pathogens than we are. The challenge is to understand how they do it.

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