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By

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ABSTRACT

EVOLUTIONARY ECOLOGY OF PREDATION BY THE SOIL BACTERIUM, *MYXOCOCCUS XANTHUS*

By

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The ability of a predator to kill prey is partially determined by features of the predatory environment. This relationship may be modified by the evolution of traits involved in searching for prey or handling (capturing, killing, and consuming) prey once they have been found. The course of such predatory evolution may depend on the same ecological variables that affect prey-killing ability. I have sought to better understand the relationships between ecological variables, predatory performance, and evolution in the soil bacterium *Myxococcus xanthus* by designing predation arenas that consisted of square petri dishes filled with buffered agar that had patches of prey bacteria (*Escherichia coli* or *Micrococcus luteus*) distributed in a grid on top of the agar. I used these predation arenas to test the effects of several ecological variables on predatory performance and on the evolution of predatory traits.

Assays with these arenas showed that predatory performance of *M. xanthus* is influenced by the prey species that is available, surface hardness, and food availability. In general, *M. xanthus* swarms expanded over a greater area such that they could attack more prey when resources were common compared to when they were scarce, regardless of whether the resources were prey patches or homogeneous distributions of synthetic nutrients. Resource level also modified the response of *M. xanthus* swarms to surface hardness. On low-nutrient surfaces *M. xanthus* swarmed faster on hard compared to soft agar. This ranking was reversed if nutrients were distributed at high concentrations.

Examination of the swarming rate of motility mutants across a range of casitone and agar concentrations indicated that this result was caused by elevated swarming by the social gliding motility system at high nutrient concentrations and was facilitated by extracellular structures called fibrils.

I also used the predation arenas to test whether there was a trade-off between adapting to a prey-free environment and being a good predator. Eight populations that evolved in a liquid, prey-free environment for 1000 generations were all worse than the ancestor at encountering prey patches and killing prey in shaking liquid, indicating that adaptation to this environment generally involved loss of predatory ability.

Finally, predation arenas were used to test whether prey density affects the evolution of searching and handling of prey, and if the effects depend on the relative impact of these traits on the rate of prey consumption. As predicted, evolution of eight populations in a low patch-density environment for ~100 generations consistently led to an increase in the rate at which patches were encountered by the swarm and a 7-fold overall increase in the rate of swarming across the surface between patches (searching). The degree of searching improvement of eight populations that evolved in a high patch-density environment was less pronounced (~2-fold). Handling of prey patches improved slightly overall, but the extent of improvement was not affected by patch density, as had been predicted. These results show that searching improvements have a greater effect on fitness in the low-density environment where more searching is required for consumption of each patch.

This dissertation is dedicated to my husband Jason and my father David, who continue to challenge me to expand my knowledge and view the world from new perspectives - and also to my mother, Linnea, who showed me by example how to dedicate myself to achieving ambitious goals.

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CHAPTER 1

MYXOCOCCUS XANTHUS AND THE EVOLUTIONARY ECOLOGY OF PREDATION

Predators can be found in every community and many major taxonomic groups, including insects, plants, mammals, protists, prokaryotes and viruses. These organisms affect not only the populations that they prey upon, but also the structure of entire food webs (Mittelbach et al. 1995; Estes et al. 1998; Bohannan and Lenski 2000). If we can predict how ecological relationships and evolutionary forces affect the interactions between predators and prey, we will be better able to manage the communities that affect human health and agriculture, including endangered and genetically modified species in natural environments. Moreover, by researching the interactions of predators with their environment, we learn more about the forces that shape the diversity of life.

To advance our knowledge of the relationships among ecology, evolution, and the impact of predators on communities, I have chosen to study the evolutionary ecology of predation in the microbial predator *Myxococcus xanthus*. Below I describe how ecological and evolutionary factors may affect components of predatory fitness.

I outline the advantages of combining experimental evolution with foraging theory to investigate the causes of predatory evolution and further explain why it is important to study microbial predators. I then review current knowledge about the biology of *M. xanthus* predation and conclude with a brief overview of the research presented in this dissertation.

Predatory ecology

Predators can have a variety of effects on community structure. By keeping the density of prey populations in check, predators can influence the abundance of other species within the food chain (Mittelbach et al. 1995; Estes et al. 1998; Relyea and Yurewicz 2002). Depending on their abundance, and on whether they consume competitively dominant or inferior species, predators and other consumers can increase or decrease the diversity of communities (Lubchenco 1978; Spiller and Schoener 1998; Bohannan and Lenski 2000). In addition to affecting the size of prey populations, predators can affect the morphology (Brönmark and Miner 1992; Hahn et al. 2000; Johansson et al. 2004) and behavior of prey (Schmitz 1998; Relyea and Yurewicz 2002). These non-lethal effects can, in turn, influence the structure of food webs (Schmitz 1998; Relyea and Yurewicz 2002).

The effect of a predator on a community depends on both which prey populations it interacts with and how effectively the predator kills its prey (Lubchenco 1978; Mittelbach et al. 1995). Predation generally occurs in two phases (Holling 1959). First, prey must be discovered by the predator during the 'searching' phase. Once a prey item is found, the predator must capture, kill, and consume it. This process is referred to as 'handling' and the rate at which it occurs depends on the

capabilities of the predator and qualities of the prey. The time required to search for prey, on the other hand, varies with prey density because it depends on both the distance between prey units and the predator's own searching capabilities. The overall rate of prey consumption is at least partially a function of prey density whenever searching is required. If prey are so abundant that search time is negligible, then the predation rate is determined entirely by how quickly prey can be handled in succession and it would not increase beyond the maximum handling rate with further increases in prey density. At densities below this level of saturation, prey consumption rates increase with increasing prey density because searching takes less time at high densities. This general relationship between prey density and consumption, referred to as the functional response, has been demonstrated in many predators (Holling 1959).

Environmental variables may also influence the shape of the functional response and the effect of predators on prey populations. Abiotic variables, such as temperature, may influence the predator's searching or handling capabilities. For example, the rate of killing of snowshoe hare by Canadian Lynx varies geographically with climatic conditions. Climate determines whether the snow is typically hard or soft. If the snow is soft, it is more difficult for lynx to run because they sink in the soft snow (Stenseth et al. 2004). Complex physical environments may also change the functional response by causing aggregation of prey or increasing the number of dimensions that a predator must search through for prey (Pitt and Ritchie 2002; Hoddle 2003). The functional response of a given predator may also vary between prey species if one is more difficult to handle than others (Stephens and Krebs 1986; Hoddle 2003).

In addition to altering the functional response directly, environmental variables may also indirectly affect prey consumption rate by influencing how the predator invests its time and energy in foraging. Predators are often presented simultaneously with several important tasks, each of which requires a different response (Stephens and Krebs 1986). For example, effective predation may require searching broadly across open spaces, but this may also put the predator in danger from its own enemies. When searching for prey, a predator may simultaneously encounter two different prey species, but it can only pursue one (Stephens and Krebs 1986). How the predator responds behaviorally to these and other conflicting options can influence the dynamics of the interaction of predators with their prey (Abrams 1992).

In order to predict how predators will respond to these situations, ecologists have developed a series of models termed 'foraging theory'. These models describe how a predator should direct its foraging effort such that it will maximize its energy intake given a particular distribution, abundance, and diversity of prey or enemies (Pyke 1984; Stephens and Krebs 1986). Foraging theories have qualitatively predicted the conditions that would influence some spiders to build webs cooperatively (Gillespie and Caraco 1987), the searching strategy of terrestrial isopods (rolly pollies) in various resource distributions (Tuck and Hassall 2004), the timing of phage lysis under different host densities (Abedon et al. 2003), diet breadth of sunfish (Werner and Hall 1974), and many other predatory behaviors (Pyke 1984; Stephens and Krebs 1986).

The dynamics of prey consumption can also be affected by evolution, which can alter the prey and predator's capabilities. Significant evolutionary changes can occur on what some would consider 'ecological' time scales (Grant and Grant 1995;

Rainey and Travisano 1998; Thompson 1998; Hairston et al. 1999; Bohannan and Lenski 2000; Huey et al. 2000; Palumbi 2001; Yoshida et al. 2003). This rapid evolutionary change can affect both ecological relationships and human economic concerns (Palumbi 2001). The population dynamics and stability of predator-prey interactions can be shaped by rapid evolution of predators or prey (Abrams 1992; Abrams 2000; Johnson and Agrawal 2003; Yoshida et al. 2003). If predators become more or less specialized to prey, or otherwise change prey preferences, then the connections within food webs can be affected (Thompson 1998). Therefore, when identifying the causal basis for the interaction of predators with their communities, it is important to consider ecological variables that might affect the performance, behavior, and evolution of the predator.

Investigating the causes of predatory evolution

The course of evolution in a particular organism is determined both by its selective environment and the details of its genetic system. Together these variables determine which phenotypes are possible and their effect on fitness (Lenski and Levin 1985; Remold and Lenski 2001; Bull et al. 2004). Some phenotypic traits might often provide the same fitness benefit under specific conditions regardless of most peculiarities of the organism that carries the trait. For example, diverse prey species might each evolve faster escape abilities when there are high densities of predators. Similarly, diverse organisms may have analogous genetic constraints, or trade-offs, that limit adaptation. While it is not possible to predict the evolution of all aspects of an organism's physiology (Travisano et al. 1995) and it is difficult to accurately forecast long-term evolutionary outcomes (Grant and Grant 2002), it would be useful

to identify genetic constraints or environmental variables that have similar effects on phenotypic evolution in a variety of species. Knowledge of such patterns will help satisfy our curiosity about the adaptive significance of diverse predatory phenotypes and provide testable hypotheses about the cause of change in specific communities. It will also generate reasonable predictions about the outcome of human induced environmental change.

In order to identify relationships between particular ecological parameters and the evolution of particular phenotypes, it is necessary to find examples of natural selection acting on a predatory trait and demonstrate the ecological basis for selection. One approach is to test models derived from foraging theories in a variety of organisms. Foraging theories define a set of 'rules' that should govern a predator's 'decisions' if it behaves optimally relative to variables in the environment, such as the distribution of prey that vary in profitability (Stephens and Krebs 1986). It is assumed in these models that predatory behavior has been optimized by natural selection to provide a maximum rate of energy intake (MacArthur and Pianka 1966; Stephens and Krebs 1986). Researchers have typically used this theory to understand the mechanistic basis for predatory behavior by comparing variation in observed behavior between environments to predictive models. When the predator's behavior fits the predictions of the model, this is taken as evidence that the decision rules in the model accurately describe the mechanistic basis for the behavior (Stephens and Krebs 1986). For behaviors that are known to be favored by natural selection, foraging models may help the researcher to understand why they were favored. However, this approach is ineffective for proving that a behavior is adaptive (Gould and Lewontin 1979; Perry and Pianka 1997). This limitation arises when the predator's behavior does not fit the model, because it is unclear if the decision rules are wrong or if the

behavior is simply not adaptive (Pyke 1984; Endler 1986; Perry and Pianka 1997). Thus, the typical foraging theory approach may be useful but it must be augmented with other approaches.

Another approach to identifying general relationships between ecological variables and phenotypic change is to observe natural selection across many predator species and look for patterns. Natural selection can be detected in wild populations by measuring the relationship between fitness and heritable phenotypic traits (Endler 1986; Wade and Kalisz 1990; Grant and Grant 1995). The environmental source of selection can be deduced from the covariation of these phenotype-fitness relationships with particular environmental parameters (Wade and Kalisz 1990), preferably supplemented with additional experimental manipulations (Endler 1986; Wade and Kalisz 1990; Reznick et al. 1997). This approach has been used successfully to demonstrate natural selection and its ecological causes in a variety of species (Endler 1986; Reznick et al. 1997; Thompson 1998; Reznick and Ghalambor 2001), including some predators (Grant and Grant 1995; Benkman 1999; Geffeney et al. 2002).

There are some drawbacks, however, to relying entirely on nature for examples of natural selection. For example, the procedure and requisite follow-up experiments to identify causation may be laborious (Endler 1986). Therefore, it may take much longer to generate enough examples to propose a reasonable general theory. In addition, it may be advantageous to see how an organism evolves in an environment that is different from what it encounters in its natural range. This approach would allow scientists to explore which phenotypes are genetically possible and expand the test of a particular hypothesis to more species than would be possible if restricted entirely by natural conditions (Conner 2003).

These issues can be addressed by using experimental evolution to test predictions of foraging theory. There are several advantages to using experimental evolution that are amplified by using microbial systems (Elena and Lenski 2003). First, if the environment is controlled and manipulated, it is easier to deduce what aspect caused the evolutionary change, especially if there are multiple evolution treatments that differ in only one variable. Second, multiple populations of microorganisms that all derive from a single clonal ancestor can be allowed to evolve independently. This feature makes it easier to assess which evolutionary changes occurred by chance (e.g., random genetic drift) and which may be due to natural selection. Third, experimental evolution allows the researcher to better control environmental conditions that could best test a hypothesis in a particular organism, rather than relying on complex and uncontrolled natural habitats. Finally, many microorganisms grow rapidly enough that many generations of evolution can be observed in a relatively short time interval.

Microbial systems have been used successfully to test hypotheses about the ecology and evolution of predator-prey interactions (Bohannan and Lenski 2000; Abedon et al. 2003). Conducting evolution experiments with microbial systems to test aspects of foraging theory provides the opportunity to significantly advance our knowledge about the causes of predator evolution. The advantage of using foraging theory as a source of hypotheses is that the researcher can choose among many well-developed theories and a wealth of testable predictions about which predatory phenotypes may be favored in particular environments. For example, there are several predictions about how predators should evolve in high versus low resource conditions. In high resource conditions, predators should evolve to leave prey patches before they have been fully exploited, but not in low density conditions (Stephens and

Krebs 1986). Also, when food is scarce, predators should be less choosy about their prey compared to when food is abundant (MacArthur and Pianka 1966; Stephens and Krebs 1986). In addition, predators should be more likely to search in groups and invest time in hiding from predators under high resource conditions compared to when food is scarce (Giraldeau and Caraco 2000).

Importance of studying microbial predators including M. xanthus

Microorganisms are often used by researchers because they are easy to manipulate and have short generation times, but there are two reasons why I think microbial predators especially should more frequently be the focus of evolutionary ecology studies. One reason to study microbial predators is to improve our understanding of the diversity of predatory organisms. Most biological diversity is found in the microbial world, yet we know much more about non-microbial predators than about microbial predators, especially bacterial predators (Woese 1998; Martin 2002). Numerous prokaryotic predators have been observed in soils and aquatic systems (Lambina et al. 1983; Lambina et al. 1985; Sillman and Casida 1986; Martin 2002) and they exhibit a variety of mechanisms to kill prey. Some burrow into the prey cell and lyse the prey from inside the cytoplasm or periplasm (Martin 2002). Others secrete enzymes that lyse the prey extracellularly with diffusible enzymes, or through passage of enzymes to an attached host cell (Lambina et al. 1983; Martin 2002). An interesting feature of some bacterial predators, including Myxobacteria species, is that predation is not obligatory, as they are able to grow in synthetic media (Liu and Casida 1983; Zeph and Casida 1986; Casida 1988).

Another reason to study the ecology and evolution of microbial predators is to understand the basis of microbial community structure and how it might be affected by environmental changes. Protozoa and phage affect the structure of soil (Alexander 1981; Pantastico-caldas et al. 1992; Rønn et al. 2002) and aquatic (Jurgens and Matz 2002; Martin 2002; Simek et al. 2002) communities, the growth rate of rhizosphere organisms (Jjemba 2001) and affect carbon utilization (Frey et al. 2001). Prokaryotic predators are capable of attacking numerous species in both nutrient rich and poor soils (Zeph and Casida 1986). They also appear to cause the decline of some Cyanobacteria blooms (Rashidan and Bird 2001) and drive the population dynamics of purple sulfur bacteria (Esteve et al. 1992). Microbial predators may cause some species to form microcolonies, filaments, or other defensive structures (Hahn et al. 1999; Hahn et al. 2000; Shemesh and Jurkevitch 2004). These effects of microbial predators may be influenced by environmental conditions that define the interaction of predators with specific populations, and that also cause predatory evolution. The focus of this dissertation is the predatory ecology and evolution of the non-obligate bacterial predator *M. xanthus*.

Myxococcus species are ubiquitous in soil (Reichenbach 1999). They are capable of lysing and consuming various yeast, fungi, and bacteria but may also survive on dead organisms or synthetic nutrients (Dworkin 1962; Rosenberg and Varon 1984). However, they are best known for their strategy for survival in the absence of food. Under starvation conditions, ~10^5 *M. xanthus* cells aggregate and coordinate their movements to produce three-dimensional fruiting body structures (Kaiser 2003). This coordination is accomplished through propagation of a series of intercellular signals. A portion of the population within the fruiting body differentiates into spores, which are resistant to dessication and starvation. When

prey or nutrients become available again, the spores germinate into rod-shaped cells that swarm through the soil environment in search of prey (Kaiser 2003).

The ecology and evolution of predation in *M. xanthus* is largely unknown. Previous studies have focused on exploring the prey range of various strains (Beebe 1941; Bull et al. 2002) and observing the population dynamics of predation on Cyanobacteria (Burnham et al. 1981) or soil organisms (Liu and Casida 1983). There have been a few recent evolutionary studies of *M. xanthus*, but evolution always occurred in nutrient-rich settings that did not include prey (Velicer et al. 1998; Velicer et al. 2000; Velicer and Stredwick 2002; Fiegna and Velicer 2003). Thus, despite the fact that *M. xanthus* has been studied for many years, there remains much to learn about the ecological variables that impede or enhance predation, and how these variables and the genetics of the organism affect the course of evolution.

Physiology of predation by M. xanthus

Although there has not been much research on the ecology and evolution of *M. xanthus*, the physiology and genetics of this organism have been well-studied relative to other microbial predators. Most of the research that is applicable to predatory physiology has focused on searching-related traits. Searching involves two gliding motility systems that differ in mechanism, in their requirement for cell proximity, and in the range of surfaces over which they can provide movement.

Cells can move individually by the adventurous (A) motility system (Hodgkin and Kaiser 1979). It is thought that the A-motility system involves pushing cells by secreting slime onto the gliding surface out of pores in the cell exterior (Wolgemuth et al. 2002). This mechanism allows *M. xanthus* to swarm over hard agar surfaces (Shi

and Zusman 1993). The social (S) motility system enhances swarming by A-motility on hard agar, provides movement on soft agar surfaces, and requires close cell-cell proximity (Hodgkin and Kaiser 1979; Shi and Zusman 1993). S-motility uses long, thin, proteinacious extracellular appendages called pili that extend from the cell pole (Kaiser 1979). The cell is pulled forward by retraction of the pili after their tips have attached to a surface (Kaiser 2000). One surface moiety that serves as both anchor and retraction trigger for pili is the carbohydrate portion of fibril material (Li et al. 2003). Fibril material covers the exterior of *M. xanthus* cells and is involved in cell-cell cohesion (Arnold and Shimkets 1988).

Although *M. xanthus* has a variety of systems that enable it to orient its movement in response to its environment, it is unclear to what extent the predator actively directs its movement towards prey cells or clumps. Directed movement by A-motility towards prey clumps may occur by elasticotaxis. Elasticotaxis directs cells along stress lines in a surface and, apparently, directs *M. xanthus* swarms towards beads and prey colonies on plates (Dworkin 1996). Because elasticotaxis only seems to direct A-motility, and because swarming by A-motility is minimal on soft agar, it is unlikely to direct cells towards prey in environments that resemble soft agar (Fontes and Kaiser 1999). *M. xanthus* also responds chemotactically to lipids (phosphatidylethanolamines, or PE) that are present in the cell membranes of prey as well as other *M. xanthus* cells. The chemotactic response to this lipid may be involved in prey searching, but is just as likely to serve other functions (Kearns and Shimkets 2001). When PE is sensed by *M. xanthus*, the cell reverses direction less frequently, causing it to move primarily in one direction up the gradient of PE (Kearns and Shimkets 1998). The response to PE involves fibrils (Kearns et al. 2000) and two loci, *frz* and *dif*, which contain genes that are similar to the chemotaxis genes

of *E. coli* (Kearns and Shimkets 2001). A third group of chemotaxis genes, designated *che4*, also affects cellular reversal frequency, but it is unclear if this system is involved in the response to PE or if it responds to other environmental stimuli (Vlamakis et al. 2004). The *frz* system affects both A- and S-motility, but the *dif* and *che4* genes appear to primarily influence S-motility (Yang et al. 1998; Yang et al. 2000; Vlamakis et al. 2004).

These chemotaxis systems may also affect the movement of cells while feeding on prey by causing *M. xanthus* swarms to move away from negative stimuli or up steep gradients of amino acids. Dworkin and Eide (1983) were unable to demonstrate preferential movement of the swarm edge up moderate gradients of a variety of chemicals. In steeper gradients in soft agar, *M. xanthus* swarms expanded preferentially into compartments containing dense mixtures of amino acids and expanded away from compartments that contained various 'repellent' molecules (Shi et al. 1993). This behavior involved the *frz* chemotaxis genes (Shi et al. 1993; Shi and Zusman 1994). Other researchers were unable to demonstrate chemotaxis in steep and stable chemical gradients in slide cultures with a hard agar surface (Tieman et al. 1996). Thus, the *frz* chemotactic system of *M. xanthus* appears to respond to steep gradients of amino and repellent chemicals in soft agar, but not hard agar. This response affects the expansion of swarms up steep chemical gradients. Steep gradients of amino acids or repellent molecules are likely to occur when a swarm is feeding on a patch of prey.

Once prey have been found, *M. xanthus* 'handles' them by lysing them open and breaking down the components into molecules, such as amino acids, that it can take up and use as food. A variety of secreted molecules have been implicated in this activity. Several different bacteriolytic enzymes have been isolated from *M. xanthus*

(Hart and Zahler 1966; Sudo and Dworkin 1972). These enzymes were capable of cleaving the cell walls of gram-positive organisms, but not gram-negative organisms. *M. xanthus* also secretes several antibiotics. These may inhibit cell growth or kill actively growing cultures, making it easier to lyse prey cells (Rosenberg et al. 1973; Reichenbach and Höfle 1993). However, Noren and Raper (1962) showed that there was no relationship between antibiotic capacity and bacteriolytic activity, suggesting that antibiotics alone are not responsible for lysis. They proposed that antibiotics are used to suppress bacterial populations that cannot be killed, and thereby promote growth of prey (Noren and Raper 1962). Lipases, nucleases and proteolytic enzymes have also been identified (Hart and Zahler 1966; Rosenberg and Varon 1984). Proteolytic enzymes may cause lysis of dead organisms and break down released proteins into amino acids (Rosenberg and Varon 1984).

In addition to enzymes and antibiotics, prey handling may involve appendages that enhance cellular cohesion, such as pili and fibrils (Arnold and Shimkets 1988; Wu et al. 1997). Prey killing is significantly enhanced by close contact between *M. xanthus* and the prey (Rosenberg and Varon 1984). Lysis of Cyanobacteria by *M. xanthus* and *M. fulvus* within liquid cultures was caused by cells that formed spheres around the prey cells or attached to glass surfaces in a chemostat (Burnham et al. 1981; Burnham et al. 1984; Daft et al. 1985). Cohesion of these cells allowed *M. xanthus* to effectively lyse the Cyanobacteria even when the predator was only 1% of the population (Burnham et al. 1981; Burnham et al. 1984; Daft et al. 1985). The SEM images of these spherules and of populations attached to surfaces in chemostats show dense fibril matrices surrounding the Myxobacteria. Close cell contact may also affect lysis of live *E. coli*. McBride et. al. (1996) showed that individual *M. xanthus*

cells could lyse microcolonies of *E. coli*, but they did not cause lysis until they were in direct contact with the prey.

Overview of the dissertation

The remaining chapters of this dissertation present my work on characterizing the impact of three ecological variables - swarming surface, resource type, and resource density - on the predatory performance and evolution of *M. xanthus*. I manipulated these three variables and tested their impact on predation and evolution using predation arenas that consist of square petri dishes filled with a buffered agar medium that is covered in a grid of dense prey patches. In chapter 2, I describe these arenas and use them to assess how quickly *M. xanthus* can kill prey patches depending on surface type (hard *vs* soft agar), prey type (*Micrococcus luteus vs Escherichia coli*), and the density of prey patches. I also provide suggestions for potential modifications to the predation arenas that could be used to test additional hypotheses. The results of the predatory experiments in chapter 2 show that the swarming surface dramatically affected predatory performance.

In chapter 3, I test the hypothesis that the effect of prey surface results from a differential effect of food density on the functioning of A and S-motility. Using mutants, I explore the relative contributions of A-motility, S-motility, and fibrils to swarm expansion across several nutrient concentrations on hard and soft agar. I then discuss possible implications of the results in terms of the utility of each motility system in natural environments.

In the final two chapters, I address factors that affect the evolution of predatory performance in *M. xanthus*. In chapter 4, I test whether physiological or

genetic constraints limit the ability of *M. xanthus* to be a good predator and simultaneously excel in other performance features such as growth rate or swarming. To test for trade-offs between predatory performance and adaptation to environments that did not contain prey, I assessed the predatory abilities of sixteen populations that had evolved on a synthetic resource in either liquid or surface environments. Predatory performance was measured in predation arenas and in a well-mixed liquid environment.

Finally, in chapter 5, I used experimental evolution to test foraging theory by allowing sixteen independent populations of *M. xanthus* to evolve in each of two treatments that differed only in the density of prey patches. I predicted that if the rate of patch consumption was an important determinant of fitness, then low patch-density environments would favor better searchers and more thorough scavenging of prey patches. High patch-density was expected to favor faster handlers that would leave patches before they are fully exploited in order to move on to adjacent, unexploited patches.

CHAPTER 2

ECOLOGICAL VARIABLES AFFECTING PREDATORY SUCCESS IN *MYXOCOCCUS XANTHUS*

Abstract

The feeding efficiency of microbial predators depends on the availability of various prey species and abiotic variables. *Myxococcus xanthus* is a bacterial predator that searches for its bacterial prey by gliding motility and kills and lyses them with secreted compounds. I have manipulated three ecological variables to examine their effects on the predatory performance of *M. xanthus*. Predation arenas were designed to determine how surface solidity (hard *vs* soft agar), the density of prey patches (1 *vs* 2 cm grids), and type of prey (gram-positive *Micrococcus luteus vs* gram-negative *Escherichia coli*) affect predatory swarming and prey killing by *M. xanthus*. In these arenas, prey were dispersed in patches on a buffered agar surface. *M. xanthus* swarms attacked a greater proportion of available prey patches when patches were densely arranged on a hard-agar surface, compared to soft-agar surfaces or low patch-density arrangements. On hard agar, *M. xanthus* encountered more prey patches of *E. coli*

than of *M. luteus*. The opposite was true on soft agar. When *M. xanthus* was distributed across a patch in roughly equal proportion to the number of prey, it killed 99-100% of them within the first 3 h of incubation. During this initial period when most of the prey were killed, surface and prey type did not affect the rate of prey killing. However, surface type affected whether some of the remaining 1% of the population could escape prey killing by *M. xanthus* at later time points. After both 24 h and 14 days, prey were more frequently recovered from soft-agar surfaces than hard-agar surfaces. Neither prey species was significantly more likely to escape predation. These results indicate that as long as *M. xanthus* is near either *E. coli* or *M. luteus*, it will quickly kill most of them regardless of the swarming surface. However, the ability of *M. xanthus* to search out patches of these prey may be affected by surface hardness, the density of prey patches, and the type of prey species that is most abundant.

Introduction

Numerous predators of microorganisms exist, including viruses, protozoa, and bacteria (Martin 2002). Unfortunately, very little is known about the biology of most microbial predators or their roles in microbial communities (Martin 2002). It is known that predators, in general, influence the structure of a variety of biological communities (Mittelbach et al. 1995; Schmitz 1998; Jurgens and Matz 2002; Rønn et al. 2002). Protozoa, for example, affect taxonomic composition, substrate utilization patterns (Rønn et al. 2002), and the size distribution and shape of bacteria in communities (Jurgens and Matz 2002). The effect of predators on prey communities may be influenced by ecological factors that affect the rate of prey killing, such as

prey density (Messier 1994), or by differential predation on different prey types (Estes et al. 1998; Spiller and Schoener 1998; Bohannan and Lenski 2000; Jurgens and Matz 2002). A few studies have addressed how environmental variables such as temperature, prey density, and type may affect the performance of bacterial predators (Varon and Zeigler 1978; Varon et al. 1984; Jackson and Whiting 1992; Bull et al. 2002). The goal of this study was to examine relationships between three ecological variables and predatory performance in the bacterium *M. xanthus*.

Myxococcus xanthus is a soil inhabitant that preys on various other bacteria (Rosenberg and Varon 1984). When food becomes scarce, groups of ~10^5 cells aggregate and form multicellular fruiting bodies bearing spores that are resistant to starvation and dessication (Kaiser 2003). Predation involves searching for prey with two gliding motility systems, and lysing them with various secreted compounds (Dworkin 1996). Only a few of these secreted compounds have been characterized, and it is not clear which are responsible for killing live prey. Some antibiotics isolated from *M. xanthus* are bactericidal, whereas others suppress the growth of prey and may make them easier to lyse (Rosenberg et al. 1973; Reichenbach et al. 1988; Reichenbach and Höfle 1993). *M. xanthus* also secretes bacteriolytic enzymes that lyse whole cells (Hart and Zahler 1966; Sudo and Dworkin 1972) and proteolytic enzymes that degrade proteins released from prey organisms (Rosenberg and Varon 1984).

Myxococcus xanthus searches for prey using two motility systems which operate together to allow movement over a variety of surfaces (Hodgkin and Kaiser 1979; Hillesland and Velicer 2004/5). Cells can move on soft, wet surfaces with the social (S) motility system, which requires close cell-cell proximity (Shi and Zusman 1993). Movement by the S-motility system can be directed by the *frz*, *dif*, and *che4*

chemotaxis systems (Yang et al. 1998; Sun et al. 2000; Yang et al. 2000; Vlamakis et al. 2004). It is unclear, however, whether these systems direct cells towards prey, nutrients or other *M. xanthus* cells (Kearns and Shimkets 2001). The adventurous (A) motility system differs from the S-motility system in that cells can move individually, and it is responsible for most movement on hard agar (Shi and Zusman 1993). The A-motility system appears to push cells forward by the secretion of slime through cell-surface pores (Wolgemuth et al. 2002). A-motility may be directed by the *frz* system or by elasticotaxis, which causes cells to move along the stress-lines in a surface (Fontes and Kaiser 1999; Spormann 1999; Sun et al. 2000). Elasticotaxis can direct swarms toward dense objects such as glass beads or prey colonies (Dworkin 1996).

Surface hardness, patch density, and prey species may affect the ability of *M. xanthus* to search out and consume prey. The rate of predation by *M. xanthus* over a broad area may be limited by the predator's ability to search for prey organisms or by its ability to handle (kill and consume) them once they have been found (Holling 1959). Searching rate may be affected by the density of prey patches or by the differential functioning of the A- and S-motility systems on different surfaces. The rate at which *M. xanthus* handles prey clumps may depend on how easily the available prey species can be killed.

To examine these possibilities, I designed predation arenas in which prey were distributed as patches in a grid configuration on an agar surface with no other growth substrate added. This method of prey-patch distribution allowed me to vary both the density of patches and the prey species. In these predation arenas, *M. xanthus* was added to a patch in the center and allowed to swarm outward for two weeks. Overall predatory performance was assessed by counting the number of patches encountered by the expanding swarm and estimating the rate of individual prey killing within the

patch. The effects of prey type (gram-negative *vs.* gram-positive), patch density (1cm *vs.* 2 cm grid), and surface type (hard *vs.* soft agar) on predatory performance were compared.

Methods

Strains and culture conditions

Esherichia coli B and *Micrococcus. luteus* ATCC 4698 were the prey organisms. *M. xanthus* strain GJV1 is a clone of DK1622 (Kaiser 1979) and GJV2 is a spontaneous rifampicin-resistant mutant of GV1 (Velicer et al. 1998). All liquid cultures of *M. xanthus* strains were propagated in CTT (1% casitone dissolved in 10 mM Tris pH 8.0, 8 mM $MgSO_4$, 1 mM KPO_4 (Bretscher and Kaiser 1978)) at 32°C with constant shaking (300 rpm). To initiate all predation arenas, liquid cultures of *M. xanthus* were resuspended in TPM buffer (10 mM Tris pH 8.0, 8 mM $MgSO_4$, 1 mM KPO_4) to a density of 10^9 cells/ml and 10 µl were added to the center prey patch of each plate. After the addition of *M. xanthus* to a center prey patch, arenas were incubated at 32°C in plastic bags with slits to maintain plate moisture content while allowing oxygen flow. The incubator was kept humid by placing a pan of water near the fan in the incubator.

For assays of patch-encounter ability, prey suspensions were prepared from thick lawns of *E. coli* and *M. luteus* that grew overnight at 37°C on Terrific broth agar (1.5%) plates (Sambrook et al. 1989). Cells were scraped off of the plate, suspended in TPM, and centrifuged. After supernatant removal, cells were resuspended in TPM to a density of between 1.2 and 1.6 x 10^{11} cells/ml for *E. coli* and 1.2 and 1.4 x 10^{10}

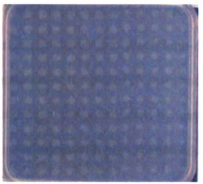
cells/ml for *M. luteus*. The biomass of *M. luteus* and *E. coli* was similar at these cell densities.

This method of procuring high quantities of prey to add to predation arenas was prone to contamination. Therefore, for subsequent assays of prey killing within patches, prey were grown in 500 ml Brain heart infusion broth (Difco) that was distributed in two 1-liter flasks and incubated at 32°C with shaking at 300 rpm. Liquid prey cultures were washed two times with TPM. Growing the cells in broth yielded far fewer incidents of contamination than the previous method. Brain heart infusion broth was chosen because it seemed to provide a higher yield of cells than other media, including Terrific broth. I do not expect the difference in prey culturing method between patch-encounter rate and prey-killing assays to affect the results for two reasons. First, in subsequent experiments using the Brain-heart infusion broth method, I have observed similar patch-encounter rate results to those presented here. Second, prey were always added to plates one day before the start of the assay, so their physiological state was likely to be similar regardless of which method had been used for culturing.

Assay of patch-encounter rate

Predation arenas are pictured in Figure 1. Prey patches were dispersed in a grid on buffered agar (0.5 or 1.5% agar dissolved in TPM buffer, 75 ml per plate) in 12 cm x 12 cm square petri dishes (PGC Scientific). Three parameters of the arena environment were varied. The two surface types were hard (1.5%) and soft (0.5%) agar. *E. coli* and *M. luteus* were used as representative gram-negative and gram-positive prey types, respectively. Finally, patches were arranged at either high (1 cm

a



b

Figure 1. Predation arenas. Prey were arranged on buffered agar as patches at either high density (a, 1 cm grid) or low density (b, 2 cm grid). *M. xanthus* was added to a central patch on the plate and allowed to swarm outward for two weeks. Patch-encounter rate was the percentage of total prey patches in an arena that were encountered by the predator after 14 days incubation. Images in this dissertation are presented in color.

grid) or low (2 cm grid) density.

Prey-patch grids were formed on the surface of predation arenas using a Bel-Blotter™ Replicator (Bel-Art Products). This polycarbonate blotter consists of 96 cone-shaped tips arranged to fit the spacing of a 96-well plate. Each tip can pick up cell suspension from a well by capillary action and deposit ~3 µl onto agar. Prey suspension (150 µl) was added to each well of a 96-well plate and transferred to the agar surface by the blotter. Each high-density arena had enough patches to extend to the boundaries of the plate (13 rows and 12 columns). Low-density prey configurations were constructed by leaving an empty well in the microtiter plate between each well with prey suspension. Thus, there were only 7 rows and 6 columns of prey patches, but they were 2 cm apart, so they also extended to the boundaries of the plate.

In this environment, *M. xanthus* swarms expand radially outward from the center, crossing prey-free regions and then traversing and consuming patches before crossing the next prey-free region in search of the next layer of patches. Patches in the center of the swarm after two weeks of incubation have been consumed, but the leading edge of the swarm may only be encountering the edge of prey patches. All of the patches covered or touched by the swarm were counted to estimate the number of patches encountered by the predator. Predatory assays were performed in a complete factorial design so that all eight combinations of prey type, patch density, and surface solidity were tested simultaneously. The experiments consisted of four complete temporal blocks with one replicate each of *M. xanthus* GJV1 and GJV2 for each treatment in each block. For purposes of our analysis, GJV1 and GJV2 were regarded as two replicates of the same genotype because they differ only by a genetic marker.

This design therefore provided eight independent replicates for each of the four treatments.

Estimate of prey killing efficiency within a patch

I evaluated two qualities of *M. xanthus*'s ability to kill individual prey within a patch. These included the rate of decline in the viable prey population in the first 24 h after inoculation, and the likelihood that some prey would escape predation, even after longer periods of incubation. This latter prey-killing measure was tested on predation arenas from both the patch-encounter rate assays and the prey-killing rate assays, which are described below. For both prey-killing measures, prey were harvested from the initially inoculated patch by cutting it out of the agar, suspending it in a microfuge tube with 1 ml of Davis minimal medium (DM) (Carlton and Brown 1981) and vortexing 12 times. This suspension was serially diluted in DM and spotted or spread on LB agar (Sambrook et al. 1989). After incubation, colonies were counted to determine how many prey remained within the patch. Predation arenas only contained buffer and agar. Therefore, prey death may have been caused by starvation or by predation. To control for the effects of starvation, the number of prey harvested was always compared to the number harvested from control plates that did not include the predator but were otherwise the same.

The rate of prey killing within a patch was estimated by counting the number of live prey cells remaining at several time points between 0 and 24 h after inoculation with *M. xanthus*. Prey-killing rate was assayed in predation arenas containing high-density grids of prey patches. Unlike the arenas used to measure patch-encounter ability, these were round and 6 cm in diameter. For ease of

comparison across prey types in the number of prey killed, both species were adjusted to the same cell number (1.2-1.6 x 10^{10} cells/ml) in suspensions that were used to form prey patches. Prey patches were formed from prey suspensions by the same method used for patch-encounter assays.

In the prey-killing rate assay, all four combinations of the two prey types (*E. coli* and *M. luteus*) and two surface types (hard and soft agar) were tested in two temporal blocks. In each block, there were two replicates of each environmental treatment with the predator added (experimental) and two with only prey (control). To determine the fraction of prey remaining, the number of prey recovered from an experimental plate was divided by the number recovered from a control plate of the same treatment and time interval.

Statistical analyses

All statistical analyses were performed with SAS software version 8.2 (SAS Institute 2001). To compare the rate of prey killing between ecological treatments, the regression procedure in SAS was used to obtain an estimate of the slope for each replicate in each treatment using log-transformed data from 0-3 h, when decline was approximately log-linear. These slopes were then compared in an ANOVA using the generalized linear model (GLM) procedure in SAS and the following statistical model: Slope = Block + Prey + Surface + Prey*Surface. 'Block' refers to the effect of the temporal block. 'Prey' refers to the effect of the two prey species and 'Surface' refers to the effect of the two surface types.

Results

Ecological effects on patch-encounter rate

The percentage of prey patches encountered by *M. xanthus* varied greatly across ecological treatments, ranging from 1.3 - 63% of patches depending on the combination of variables. Figure 2 shows that a higher proportion of patches was encountered in high-density arenas than in low-density arenas that had the same prey-type and surface. This difference is less dramatic in soft-agar arenas where some of the percentages overlapped (e.g. *E. coli* on soft agar at high *vs* low density). However, on soft agar the predator always encountered only the patch that it started on at low density, but sometimes encountered up to 9 patches (out of 156 total) at high density. I tested the overall effect of patch density by pairing high- and low-density data points that were obtained from arenas in the same temporal block and that also had the same prey and surface type. A Wilcoxan signed-rank test was performed on the difference between these points to determine if it was statistically different from zero. This test indicated a significant effect of patch-density on patch-encounter rate when all other ecological variables were held constant (Fig. 2a,b; $p < 0.001$). Surface type also had a general effect on patch-encounter ability that was independent of the other two ecological variables. A greater proportion of patches was encountered on hard agar than on soft agar for both prey types and at both patch-densities (Fig. 2a, b). This effect was again highly significant (Wilcoxan signed rank test, paired by block, patch-density, and prey-type, $p < 0.001$).

Surface type also modified the effect of patch density on patch-encounter rate. Pooling across prey types, the average difference in patch-encounter rate was ~27% for high *vs* low density arenas on hard agar (40% high density − 13% low density;

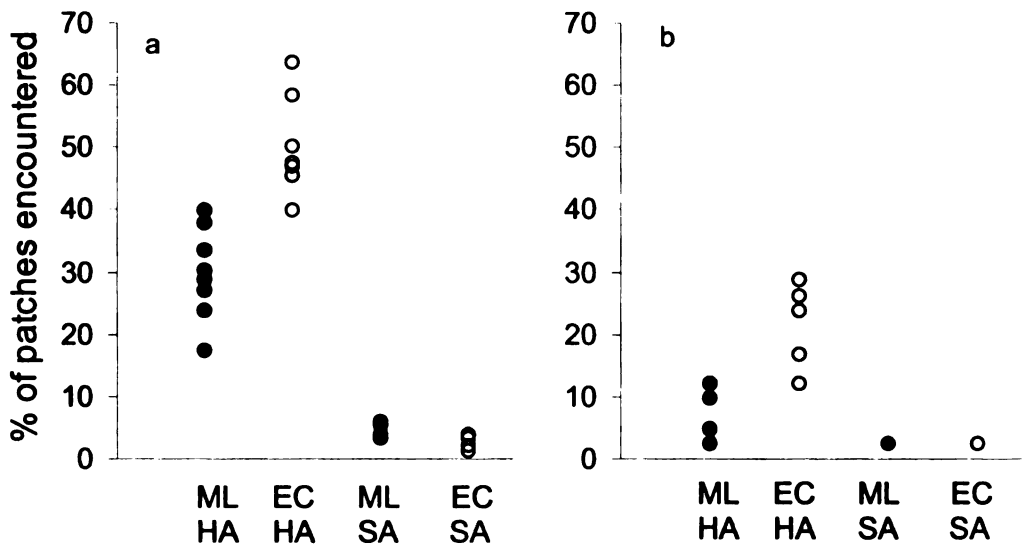


Figure 2. Effect of prey-patch density, prey species, and surface type on the ability of *M. xanthus* to encounter prey patches. The percentage of available patches encountered by swarms of *M. xanthus* was determined at (a) high patch density or (b) low patch density, and on hard agar (HA) or soft agar (SA). Prey were either *M. luteus* (ML, closed symbols) or *E. coli* (EC, open symbols). Each point on the graph represents one measurement. There were eight replicates in each treatment combination, but in some cases the same value was obtained for multiple or even all replicates.

Fig. 2, compare left columns of 'a' and 'b') but only ~2% on soft agar (4% high density – 2% low density; Fig. 2, compare right columns of 'a' and 'b').

Prey type also affected the percentage of patches encountered by the swarm, although these effects were less dramatic and varied across surfaces. On hard agar, *M. xanthus* consumed more prey patches when the prey was *E. coli* than when it was *M. luteus* at both high and low patch-density (Fig. 2a, b; Wilcoxan signed-rank test paired by block, $p = 0.0078$ for both high and low density). On soft agar at high patch-density, the ranking of prey types was reversed. More prey patches were consumed when the prey was *M. luteus* in this environment than when the prey was *E. coli* (Fig. 2a, $p = 0.0469$, paired Wilcoxan sign-rank test). On soft agar at low density, the only patch encountered in every replicate for both prey types was the patch that was initially inoculated (Fig. 2b).

Ecological effects on prey killing

In addition to testing how ecological variables affect the rate at which patches were encountered, I also wanted to know if they affected how many of the prey within the patch were killed. Therefore, at the completion of patch-encounter rate assays, the center patch initially inoculated with *M. xanthus* was extracted and the number of prey remaining quantified. This number was compared to the number of prey remaining on a plate that did not contain *M. xanthus*. After 14 days, 99.5 to 100% of prey relative to the number remaining in controls were eliminated from the patches where *M. xanthus* had been inoculated. Thus, none of the ecological variables had much effect on the ability of *M. xanthus* to kill prey in a patch over this long interval. However, surface hardness did affect the likelihood that any prey could be recovered

from the patch after 14 days of predation. Prey were recovered from the initial patch in 14 out of 24 trials on soft agar, but only 3 out of 24 trials on hard agar. The probability of such a difference in recovery frequency was very low (Fisher's exact test, two-tailed $p = 0.002$). Prey-patch density and prey species, on the other hand, only minimally affected the frequency of prey recovery. Prey were recovered from the patch 6 out of 24 times for *M. luteus* and 11 out of 24 times for *E. coli*. Obtaining these frequencies by chance is not unlikely (Fisher's exact test, two-tailed, $p = 0.2270$). Patch-density also had no significant effect on the likelihood that prey could be recovered from the patch. When patches were in the low-density configuration, prey were recovered 6 out of 24 times. In a high-density configuration they were recovered 11 out of 24 times. These frequencies are not significantly different (Fisher's exact test, two-tailed, $p = 0.2270$).

Even if almost all of the prey in the patch were eventually killed, the initial rate of prey killing may be influenced by the environment, and this could affect how quickly *M. xanthus* obtains food for population growth and additional foraging excursions. To test whether ecological variables affected the initial rate of prey killing, a separate experiment was performed in which prey patches were sampled at several early intervals within the first 24 h after inoculation. All of the plates in this assay had high-density grids, but surface hardness and prey type were varied as for patch-encounter assays. At time zero *M. xanthus* was added to the central patch so that it covered the patch in roughly equal numbers to the prey. Individual *M. xanthus* cells would therefore not have to move far to find prey if they were not deposited directly onto prey cells. In the first 3 hours, roughly 99% of available prey were killed by *M. xanthus* (Fig. 3). If the rate of prey killing varied across prey or surface type during this initial period of decline, then it would have the greatest impact on

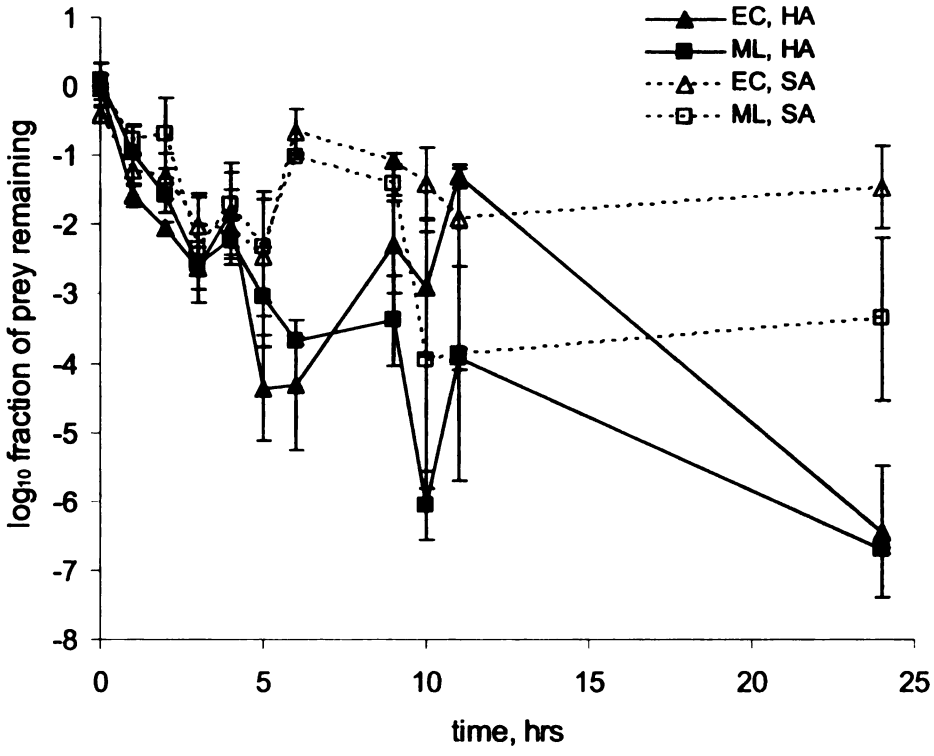


Figure 3. Effect of ecological variables on the rate of prey killing within a patch. *M. xanthus* was added to the center patch of a prey-patch grid. The number of prey remaining in that patch was quantified and divided by the number of prey remaining in a patch on a control plate that did not have predators to obtain the fraction of prey remaining over time. Each data point represents the mean of four replicates obtained from two temporal blocks. Error bars indicate the standard error. The rate of prey-patch depletion was quantified on two prey species, *Escherichia coli* (EC) and *Micrococcus luteus* (ML), and two surface types, hard agar (HA) and soft agar (SA).

how quickly *M. xanthus* was able to acquire food. However, the decline in prey population size appeared to be similar among treatments (Fig. 3). To formalize this visual impression, I tested whether the mean rate of prey-population decline in the first 3 h after inoculation was affected by prey or surface type by performaning an ANOVA (Table 1). Neither ecological variable nor their interaction significantly affected the rate of population decline during this period.

Table 1. ANOVA comparing initial rates (0-3 h) of prey-killing across surface types and prey species.

Source	df	SS	MS	F	*p*
Prey	1	0.043	0.043	0.30	0.595
Surface	1	0.267	0.267	1.85	0.201
Prey*surface	1	0.030	0.030	0.21	0.657
Block	1	0.291	0.291	2.02	0.183
Error	11	1.587	0.144		
Total	15	2.219			

In the last 21 h of the prey-killing rate assay (Fig. 3), the fraction of prey remaining tended to be greater on soft agar than on hard agar. To formalize this visual impression, I tested whether surface hardness and prey type affected the likelihood that prey remained at 24 h. At this time point, prey could be recovered from all 7 replicates on soft agar (one plate was lost due to contamination), but only 1 out of 8 times on hard agar. This result is very unlikely by chance alone (Fisher's exact test, two-tailed, $p = 0.0014$). In contrast, prey were recovered with roughly equal frequency whether the prey type was *E. coli* or *M. luteus* (4 out of 8 times for *M. luteus*, 4 out of 7 times for *E. coli*; Fisher's exact test, two-tailed, $p = 1.0$).

Discussion

Ecology of predation by Myxococcus xanthus

To characterize components of *M. xanthus* predation ecology, I utilized predation arenas that allowed me to vary three ecological parameters that may influence the rate of predation. I tested the effects of surface hardness, prey species, and prey-patch density on the rate at which swarms could encounter prey patches and reduce the size of prey populations within a patch. The rate of prey-patch encountering was greatest when patches were densely arranged on a hard-agar surface compared to low-density patch distributions or soft-agar surfaces. Prey species also affected patch-encounter rate, suggesting that the dynamics of prey handling could also affect the number of patches encountered. In fact, however, prey species did not influence the rate of prey killing within a patch.

If *M. xanthus* had swarmed at exactly the same rate on high and low patch-density arenas, it would have encountered the same proportion of patches. However, *M. xanthus* encountered a significantly lower proportion of the available prey patches at low density, indicating that it swarmed more slowly in the low-density environment. This result is not surprising because the amount of food per unit area is lower and the rate of swarming by *M. xanthus* depends on the density of food (Chapter 3). The effect of density on patch-encounter rate does emphasize the importance of swarming to predation. If a mutation were to increase the rate of swarming at low food densities, *M. xanthus* would be able to encounter prey patches at a higher rate at low densities. This hypothesis is tested in Chapter 5.

In addition, the effects of patch-density on the rate of patch encounter may be modified by other ecological variables. The dramatic effect of surface hardness,

which determines the relative input of the two motility mechanisms in *M. xanthus*, underscores the importance of searching (relative to handling) for predation in this patchy environment. Surface hardness affected the overall rate of patch encounter, but not the rate of prey killing within a patch. The physical structure of predatory environments can influence the rate at which prey are found by making it more difficult for predators to move or making it easier for prey to hide. For example, the surface hardness of snow affects how swiftly lynx can move in search of prey. This property leads to geographic variation in lynx population dynamics (Stenseth et al. 2004). Similarly, surface hardness dramatically affected how quickly *M. xanthus* could find and attack prey patches. Patches were encountered at a higher rate on hard compared to soft agar. This pattern was probably caused by differential performance of two motility systems across nutrient concentrations for the following reason. The surface between patches contained only residual nutrients from the agar. At low food densities, swarming by the S-motility system, which is responsible for movement on soft agar, is slower than swarming by the A-motility system (Chapter 3).

Within a patch, surface hardness did not affect the initial rate of individual prey killing, but it did affect the likelihood that a few prey escaped predation. Apparently prey are better able to 'hide' within the soft agar matrix, which may act as a prey refuge (Alexander 1981). How soft agar allows some prey to escape lysis is unclear. It could be a result of faster enzyme diffusion on soft agar, such that the local concentration is reduced, or perhaps some prey sink deeper into the agar matrix and are thereby protected from *M. xanthus* secretions. Regardless of the mechanism, these results show that soft surfaces affect prey consumption both by slowing swarming in search of patches at low nutrient concentrations and by providing a means for some prey to escape attack.

The effects of prey type on patch encounter were smaller in magnitude and less consistent than patch density and surface hardness effects. On hard agar, more prey patches were encountered when *E. coli* was the prey compared to *M. luteus*. This ranking was reversed on soft agar. These effects could have been caused by different mechanisms of lysis or movement across patches of the two prey types. Such differences in 'handling' of patches could lead to variation in patch-encounter rate. For example, *M. luteus* might, hypothetically, secrete a chemical that inhibits *M. xanthus* from swarming maximally across a patch on hard agar. If the concentration of the chemical differed on soft versus hard agar (e.g., diffusion effect), this could explain the reversed ranking of the two prey types on hard and soft agar. In support of this possibility, prey secreted compounds that inhibit *Myxococcus* swarming have been observed previously (Shi and Zusman 1994; Bull et al. 2002).

Additional applications of predation arenas

Many microbial predators live in soil and in other environments where surface structure may have important effects on predation (Martin 2002), yet most studies of the effects of variables like prey density on predatory rates have been performed in liquid environments (Varon and Zeigler 1978; Jackson and Whiting 1992). I have developed a system that allows me to vary environmental parameters and measure their effects on the proportion of patches encountered by *M. xanthus* and also the number of prey killed within a patch. In addition to the experiments presented in this chapter, I applied this system to address further the relationships between predatory swarming, nutrient concentration, and surface hardness (Chapter 3), for identifying a trade-off between adaptation to a prey-free environment and predatory capabilities

(Chapter 4), and as an environment for experimental evolution of a predator (Chapter 5). These predation arenas may also be used to expand our knowledge of microbial predation and its effects on prey by using them in experiments with other microbial predators that are capable of traversing the prey-free surface between patches.

In studying microbial predation, it may also be interesting to examine predatory rates under more complex scenarios than the conditions I tested. These predation arenas may be modified to address such issues. For example, predatory behavior may be affected by whether the prey population is growing, by whether prey are able to activate defenses, by the presence of the predator's own enemies, or by the presence of multiple prey species that vary in profitability (Stephens and Krebs 1986). To examine these scenarios, the predation arenas could be altered by adding nutrients to the agar surface, by expanding the range of prey species, and by adding protozoa or phage that can kill the microbial predator.

CHAPTER 3

RESOURCE LEVEL AFFECTS THE RELATIVE PERFORMANCE OF THE TWO MOTILITY SYSTEMS OF *MYXOCOCCUS XANTHUS*

Abstract

The adventurous (A) and social (S) motility systems of the microbial predator *Myxococcus xanthus* show differential swarming performance on distinct surface types. Under standard laboratory conditions, A-motility performs well on hard agar but poorly on soft agar, whereas the inverse pattern is shown by S-motility. These properties may allow *M. xanthus* to swarm effectively across a greater diversity of natural surfaces than would be possible with one motility system alone. Nonetheless, the range of ecological conditions under which dual motility enhances effective swarming across distinct surfaces and how ecological parameters affect the complementarity of A- and S-motility remain unclear. Here we have examined the role of nutrient concentration in determining swarming patterns driven by dual motility on distinct agar surfaces, as well as the relative contributions of A- and S-motility to these patterns. Swarm expansion rates of dually motile (A^+S^+), solely A-

motile (A^+S^-) and solely S-motile (A^-S^+) strains were compared on hard and soft agar across a wide range of casitone concentrations. At low casitone concentrations (0% – 0.1%), swarming on soft agar driven by S-motility is very poor, and is significantly slower than swarming on hard agar driven by A-motility. This relationship reverses at high casitone concentration (1% - 3.2%) such that swarming on soft agar is much faster than swarming on hard agar. This pattern greatly constrained the ability of *M. xanthus* to encounter patches of prey bacteria on a soft agar surface when nutrient levels between the patches were low. The swarming patterns of a strain that is unable to produce extracellular fibrils indicate that these appendages are responsible for the elevated swarming of S-motility at high resource levels. Together, these data suggest that large contributions by S-motility to predatory swarming in natural soils may be limited to soft, wet, high-nutrient conditions that may be uncommon. Several likely benefits of S-motility to the *M. xanthus* life cycle are discussed, including synergistic interactions with A-motility across a wide variety of conditions.

Introduction

Dual motility systems in bacteria may allow effective cell movement across a wider range of ecological conditions than would a single motility system. For example, flagellated bacteria can alter the type of flagella they produce in response to environmental cues (Möens and Vanderleyden 1996). The bacterial predator *Myxococcus xanthus* has two distinct motility systems that allow it to move in groups or as individual cells. Under some conditions, these motility systems allow *M. xanthus* to swarm effectively over a greater range of surfaces than would be possible with one system alone (Shi and Zusman 1993). Thus, it is likely that dual motility in

M. xanthus provides at least some degree of flexibility for moving throughout the complex soil environment in which it lives. The relative importance of such flexibility for the evolutionary maintenance of dual motility, however, is unclear.

Most species of myxobacteria, including *M. xanthus,* are microbial predators. They are likely to exert a significant impact on the composition of many soil microbial communities both because of their ubiquity and because they prey upon diverse types of bacteria, yeast, and fungi (Rosenberg and Varon 1984). Moreover, they exhibit a high degree of cooperative behavior relative to other prokaryotes. Their most distinctive feature is the ability to form multicellular fruiting structures that are produced by cooperative assemblages of individuals in response to nutrient deprivation (Dworkin 1996). Portions of aggregated populations differentiate into spores able to survive extended periods of starvation and other stresses (Dworkin 1996).

M. xanthus preys by swarming through the soil matrix using its dual motility system while secreting antibiotics, proteases, and bacteriolytic enzymes (Rosenberg and Varon 1984). These enzymes lyse prey and break down their components to provide nutrients for the swarm (Rosenberg and Varon 1984). Because prey are killed and hydrolyzed extracellularly, predation may be more efficient at higher *M. xanthus* cell densities due to increased local concentrations of predatory enzymes. In support of this hypothesis, growth rate on casein, which must be hydrolyzed prior to uptake by the cell, is density dependent (Rosenberg et al. 1977). Although individual *M. xanthus* cells are capable of lysing prey microcolonies (McBride and Zusman 1996), social foraging by *M. xanthus* may be favored under many circumstances.

The ability to search for prey either individually or socially is provided by two physiologically and genetically distinct motility systems (Hodgkin and Kaiser 1979).

The social (S) motility system is pilus-mediated and functions only under conditions of close cell-cell proximity (Kaiser 1979). Recent evidence indicates that pilus-mediated motility occurs by extension, attachment and retraction of Type IV pili in a manner triggered by contact with exopolysaccharides (Kaiser 2000; Li et al. 2003). Exopolysaccharides and extracellular protein together compose a fibril matrix that mediates cell-cell cohesion (Arnold and Shimkets 1988) and some chemotactic responses (Kearns et al. 2000) and is required for S-motility (Yang et al. 2000).

Individual cells can also move efficiently without cell-cell contact by means of adventurous (A) motility, which may result from slime secretions through cell-surface pores (Wolgemuth et al. 2002). Movement by A-motility can be directed by elasticotaxis, which orients motility along stress lines in a surface (Fontes and Kaiser 1999). Both motility systems are regulated by at least one chemotaxis operon, with the *frz* system regulating cell reversal frequency in both A- and S-motility (Spormann 1999; Sun et al. 2000). Alternatively, the *dif* and *che4* pathways are specific to S-motility, with *dif* regulating fibril biogenesis (Yang et al. 1998; Yang et al. 2000) and *che4* apparently regulating cell reversal frequency by directing the function of type IV pili (Vlamakis et al. 2004). The only demonstrated chemoattractants of *M. xanthus* are phosphatidylethanolamines (PE), including PE molecules isolated from *M. xanthus* cell membranes (Kearns and Shimkets 1998). It is unclear whether PE serves only as a within-species chemoattractant or may also serve as a means of prey detection (Kearns and Shimkets 2001).

Why does *M. xanthus* maintain two motility systems rather than only one? Shi and Zusman (1993) showed that under some laboratory conditions, dual motility expands the range of laboratory surface types on which effective swarming can occur because each motility system swarms most effectively over a different range of

surfaces. Mutants defective in S-motility (A^+S^-) move relatively well on hard (1.5%) agar, but are practically non-motile on soft (0.3%) agar. The inverse pattern was seen for mutants with defective A-motility (A^-S^+) (Shi and Zusman 1993). Dually motile (A^+S^+) genotypes move well on both surface types. These observations were made on agar medium containing high concentrations of homogeneously distributed pre-hydrolyzed nutrients, a very specific nutritional environment that differs greatly from most soils. It is unclear whether the differential swarming proficiency of A- and S-motility on distinct surface types observed by Shi and Zusman also occurs across environments that vary in respects other than surface type, such as nutrient concentration. If so, then maintaining both motility systems may enhance fitness by increasing the range of surfaces over which proficient swarming can occur in a wide variety of habitats.

To further understand the role of dual motility in the ecology of *M. xanthus*, I compared the swarming proficiencies of the dually motile wild-type and defined motility mutants on both hard and soft agar while varying overall nutrient concentration, resource type (bacterial prey vs. hydrolyzed casitone), and the spatial distribution of prey. In addition to revealing how wild-type swarming with dual motility is affected by resource level, type, and structure, these experiments allowed me to define more clearly the relative contributions of A- and S-motility to wild-type swarming across a range of environments. Moreover, I was able to test whether differential swarming proficiency by A- and S-motility across surface-types is independent of nutrient concentration. My results indicate that the two systems do show differential performance on distinct surface types that is general across multiple nutritional environments. However, the quantitative degree to which A- and S-motility differed in the surface-type specificity of their performance was dramatically

affected by nutrient concentration. In particular, S-motility swarming was much more sensitive to nutrient concentration than was A-motility swarming. These results have implications for understanding the relative contributions of A- and S-motility to the overall fitness of *M. xanthus* in the soil.

Methods

Strains and culture conditions

Escherichia coli REL 606 (a clone of *E. coli* B) was used as the prey organism. *M. xanthus* strain GJV1 is a clone of the standard lab strain DK1622 (Kaiser 1979). Strain GJV3 is a markerless *cglB* null mutant of GJV1 which has functional S-motility, but does not exhibit A-motility (A^-S^+). It was constructed with p113Δ*cglB* by the same method as Rodriguez and Spormann (1999). A markerless deletion in the *pilA* gene was constructed in GJV1 with pSWU365 using the same method as Wu and Kaiser (1996) to form GJV4. This strain has functional A-motility, but exhibits defective S-motility (A^+S^-). Both deletions are in-frame, with the *cglB* deletion (633 bp) comprising nucleotide positions 31-663 of NCBI *cglB* sequence AF032467(Rodriguez and Spormann 1999) and the *pilA* deletion (561 bp) comprising nucleotide positions 3442-4002 of NCBI *pilA* sequence L39904 (Wu et al. 1997; Velicer and Yu 2003). The presence of these deletions in GJV3 and GJV4, respectively, were confirmed by sequencing analysis. Strain DK3470 (here termed simply '*dsp*') is a derivative of DK1622 that does not produce fibrils due to a mutation in the *dsp* locus (Shimkets 1986), which maps to the *dif* chemotaxis genes (Lancero et al. 2002) required for fibril production (Yang et al. 2000). All liquid cultures of *M. xanthus* strains were propagated in CTT (1% casitone, 10 mM Tris pH

8, 8 mM $MgSO_4$, 1 mM KPO_4 (Bretscher and Kaiser 1978)) at 32°C and constant shaking (300 rpm). All plates were incubated in plastic bags to minimize dessication. Slits were cut in the bags to allow oxygen flow. The incubator was humidified by an open pan of water near the inflow fan.

Swarming comparisons across nutrient concentrations

The swarming rates of four *M. xanthus* strains (GJV1, GJV3, GJV4, DK 3470) were measured in the absence of prey in triplicate on hard and soft agar plates (50 ml medium per 15 cm round petri dish) containing 1.5% agar, TPM buffer, (10 mM Tris pH 8, 8 mM $MgSO_4$, 1 mM KPO_4 (Bretscher and Kaiser 1978)) and casitone at concentrations of 0, 0.001, 0.01, 0.1, 0.32, 1, 2, and 3.2% weight/volume. To measure swarming rates on agar, liquid cultures of the four strains were resuspended in TPM buffer to a density of 10^9 cells/ml and 10 µl of suspension was spotted in the center of the agar plates. Two perpendicular diameters at random orientation were measured after 3 and 14 days of incubation. The swarming rate was calculated as the change in average radius divided by the number of days. The experiment was performed in two complete, temporally independent blocks with triple replication within each block. Results are the grand mean of each treatment across both blocks.

Swarming comparisons in a predatory environment

Swarming performance was measured simultaneously in the following environments: i) an *E. coli* prey-patch grid on TPM agar, ii) an *E. coli* prey-patch grid on CTT agar, and iii) an *E. coli* lawn overlaid on TPM agar. *E. coli* was grown

in brain-heart infusion broth (Difco) at 32°C with shaking, washed two times with TPM and resuspended in TPM buffer to a density of 1.3-1.6 x 10^{11}cfu/ml. For the prey-patch environments, this suspension was distributed in a grid configuration in square Petri dishes (12 cm wide, PGC scientific) using a Bel-Blotter™ Replicator (Bel-art products) on 75 ml solid TPM agar. The polycarbonate blotter consisted of 96 cone-shaped tips arranged to fit the spacing of a 96 well plate. Prey suspension (150 µl) was added to each well of a 96-well plate and transferred by capillary action to the agar surface by the blotter. The resulting grid of 156 patches extended to the boundaries of the plate. To control for potential variation between plates in the amount of prey added per patch, predators and controls were randomly assigned to specific plates. *E. coli* lawns were prepared by covering a TPM agar plate with seven ml of an *E. coli* suspension (7 x 10^{10} cfu/ml) and drying the suspension on the plate in a laminar flow hood. All assays were performed on both 0.5 and 1.5 % agar surfaces. *M. xanthus* is resistant to gentamicin, which was added (5 µg/ml) to suppress *E. coli* growth on the CTT agar prey grid. Gentamicin was also added to all TPM plates. *M. xanthus* GJV1 was prepared as described above and inoculated onto prey in the center of each plate. On prey patch grids, swarming performance was assessed as the percentage of total prey patches that were encountered by an expanding *M. xanthus* swarm. All patches touched by the swarm were counted, even if the swarm had not yet overtaken the entire patch. On *E. coli* lawns, the average radius of the zone of lysis created by the expanding *M. xanthus* swarm was measured.

Results

Rates of M. xanthus swarming across diverse environments

Shi and Zusman (1993) showed that the presence of two motility systems in *M. xanthus* allows it to swarm on a greater range of agar surfaces than would be possible with only one system. I tested the degree to which this flexibility across agar concentrations is dependent on the high concentration of homogeneously distributed, pre-hydrolyzed amino acids used by Shi and Zusman (1993). As shown in Figure 1a, the swarming rate of the dually motile *M. xanthus* wild-type was measured across a range of casitone concentrations. At the lowest nutrient concentration, the swarm expansion rate on hard agar (0.29 mm/day) was significantly higher ($p = 0.001$; paired, one-tailed *t*-test) than the rate on soft agar (0.11 mm/day). Swarming was faster on hard agar than on soft agar at all five of the lowest casitone-level treatments (0 - 0.32%). At concentrations of 1% or higher, the ranking of hard and soft agar swarm expansion rates is reversed. At these higher nutrient levels, swarming is almost 2-fold faster on soft agar than on hard agar. Thus, dually motile *M. xanthus* can swarm on hard and soft agar across a wide range of nutrient concentrations, but its swarming rate is much more sensitive to resource level on soft agar than on hard agar.

Unpurified agar contains residual nutrients that can diffuse through water and serve as a resource for growing microorganisms. It is possible that at low nutrient concentrations swarms expand faster on hard agar because there are more residual nutrients available than on soft agar, thereby leading to larger population sizes and greater energy for swarming. Alternatively, the hard-agar surface *per se* may be more conducive to outward swarm expansion at low nutrient concentrations. If the superior

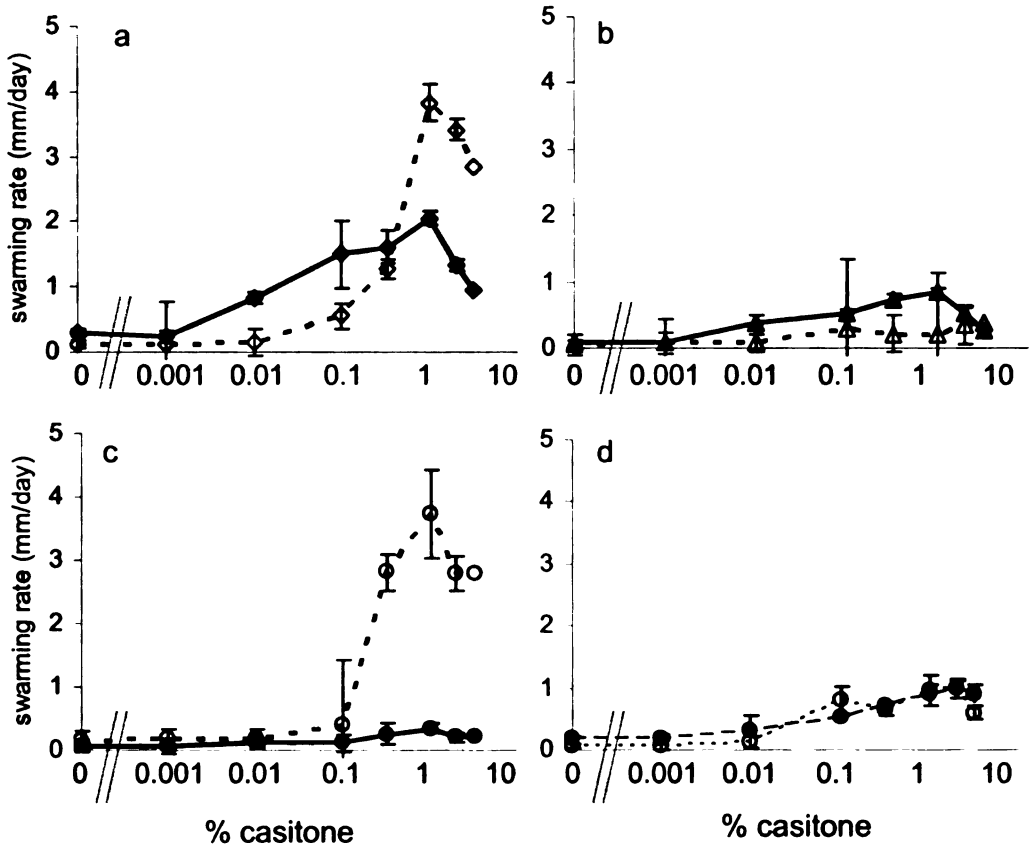


Figure 1. Absolute swarming rates of (a) dually motile (A^+S^+), (b) solely A-motile (A^+S^-), (c) solely S-motile (A^-S^+), and (d) fibril-less (*dsp*) strains. Swarming rate was estimated from the change in average radius of the expanding swarm after 14 days incubation on hard agar (closed symbols, solid line) and soft agar (open symbols, dashed line) with varying casitone concentrations. Error bars indicate bounds of the 95% confidence interval about the mean. Hatches across the x-axis indicate discontinuity of scale in all figures.

rates of hard agar swarming at intermediate casitone levels (0.01% – 0.32%) were caused primarily by excess residual nutrients in the hard agar treatment, then these rates should be relatively insensitive to casitone concentration over this range. This is not the case, as hard agar swarming rate increases significantly as a positive function of casitone concentration (from 0.001% to 0.1%), indicating that the faster swarming on hard agar is a function of surface type, and is not due to the potentially confounding variable of excess nutrients in high concentrations of agar. In addition, when agarose was substituted for agar in a similar swarming rate experiment, the difference between hard and soft agar was evident at intermediate nutrient concentrations (0.032% and 0.1%; Fig. 2).

Faster swarm expansion on soft agar at high casitone concentrations and hard agar at low casitone concentrations may be specific to the resource used and its homogeneous distribution. *M. xanthus* is capable of lysing and consuming prey bacteria, which may be a primary food source in many natural habitats. I tested whether the faster soft-agar swarm expansion (relative to hard agar) that is observed only at high casitone concentrations is specific to casitone or if it is rather a general response to food density that is independent of resource type. Predatory swarming was compared when prey were distributed as i) discrete patches on buffered (TPM) agar (0% casitone, Fig. 3a), ii) discrete patches on high-nutrient agar (1% casitone, Fig. 3b), and iii) a dense, continuous lawn on buffered agar (Fig. 3c). As expected, swarming was faster on hard agar than on soft agar when *M. xanthus* had to traverse regions of scarce resources (Fig. 3a), whereas swarming was faster on soft agar in both treatments containing a homogeneous distribution of abundant resources (Fig. 3b,c).

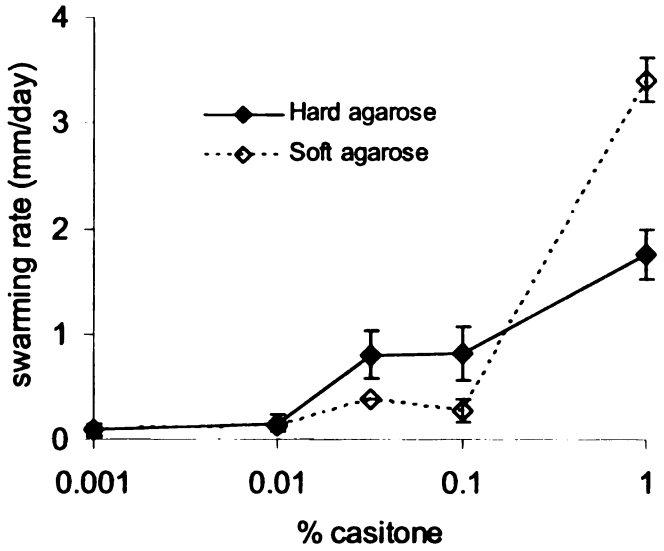


Figure 2. Swarming on agarose surfaces. The swarm expansion rate of the dually motile strain was measured on several concentrations of casitone on a hard agarose (1.5%) and a soft agarose (0.5%) surface.

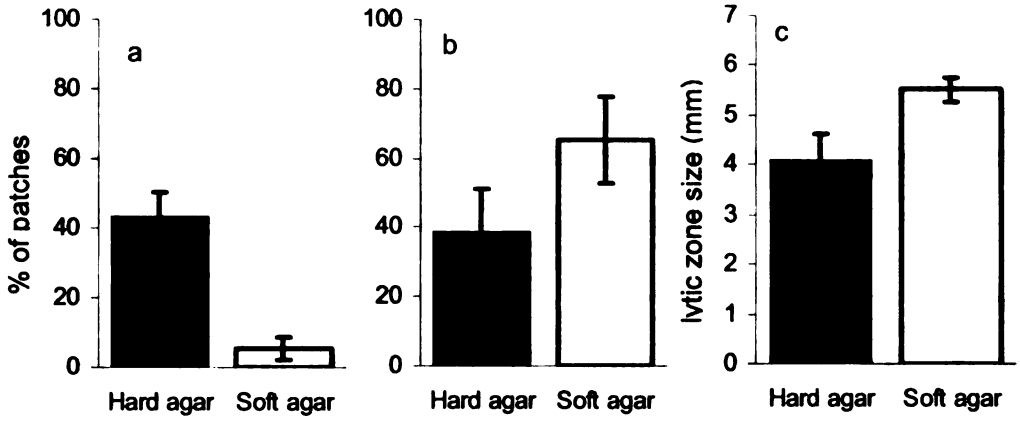


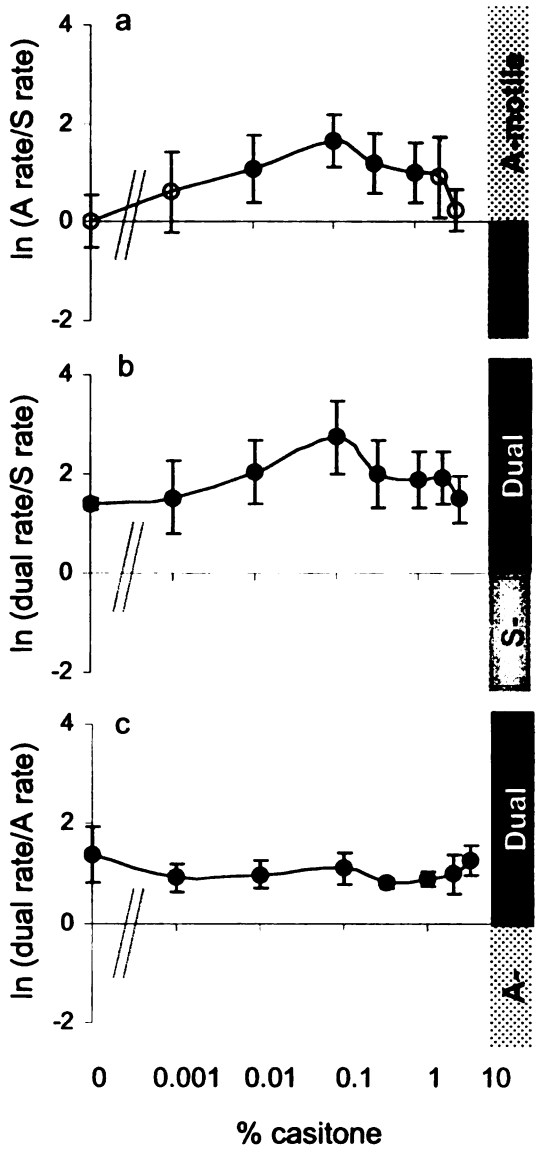
Figure 3. Effect of nutrient concentration on predatory swarming. The relationship between hard and soft agar predatory swarming was determined for the prey *E. coli* in three different nutritional environments. a*) E. coli* was dispersed as patches on buffered (TPM) agar supplemented with 5 μg/ml gentamicin. A higher percentage of patches was encountered on hard compared to soft agar after 14 days of swarming (one-tailed *t*-test; $p < 0.01$). b) *E. coli* patches were dispersed on nutrient-rich (CTT) agar supplemented with gentamicin. More patches were encountered on soft compared to hard agar (one-tailed *t*-test; $p < 0.01$). c) *E. coli* was dispersed in a lawn on buffered (TPM) agar supplemented with gentamicin. Lytic zone radius after 14 days was greater on soft agar (one-tailed *t*-test; $p < 0.05$). Error bars indicate bounds of the 95% confidence interval about the mean.

Nutrient-level, surface type, and motility system interactions

The increased range of surface types over which *M. xanthus* can effectively swarm due to the presence of two motility systems rather than one might result from the separate use of either system individually over a particular range of surface types, with the dually-motile individual switching discretely between the two motility systems as needed. Alternatively, the motility systems may synergistically interact during simultaneous activity to provide a swarming benefit above and beyond the ability of either A- or S-motility to drive motility in isolation from the other system. Shi and Zusman (1993) showed that A- and S-motility exhibit significant synergism at high nutrient concentration on hard agar surfaces, but not on very soft agar. To examine the relative contributions of the two systems and their interaction component to swarming over a wide range of surface-type and nutrient-level combinations, the swarming rates of solely A-motile (A^+S^-) and solely S-motile (A^-S^+) mutants were compared to their dually motile (A^+S^+) parental strain on hard and soft agar at multiple casitone concentrations. For ease of comparison, absolute swarming rates (Fig. 1a-c) were transformed into ratios (Figs. 4 and 5). This analysis also allows assessment of whether the differential performance rankings of A- and S-motility on hard and soft agar reported by Shi and Zusman (1993) are independent of nutrient concentration.

Hard agar: A-motility dominance and dual motility synergism. Shi and Zusman (1993) showed that in a dually motile strain swarming on rich medium, A-motility contributes relatively more than S-motility to swarming at high agar concentrations, whereas S-motility dominates at low agar concentrations. If this relationship between motility systems and surface types is independent of nutrient

Figure 4. Relative swarming rates on hard agar. Relative rates of a) A-motile vs. S-motile, b) dually motile vs. S-motile, and c) dually motile vs. A-motile strains were calculated as the natural log of the ratio of the absolute swarming rate for each strain (see Fig. 1). Shaded boxes indicate the half of the graph where data points should fall if the indicated strain swarms comparatively faster than the alternative strain. Closed symbols indicate ratios that were significantly different than zero in a one-sample, one-tailed *t*-test after sequential Bonferroni correction for multiple comparisons ($p < 0.05$ for all 8 comparisons in the graph). Error bars indicate bounds of the 95% confidence interval about the mean.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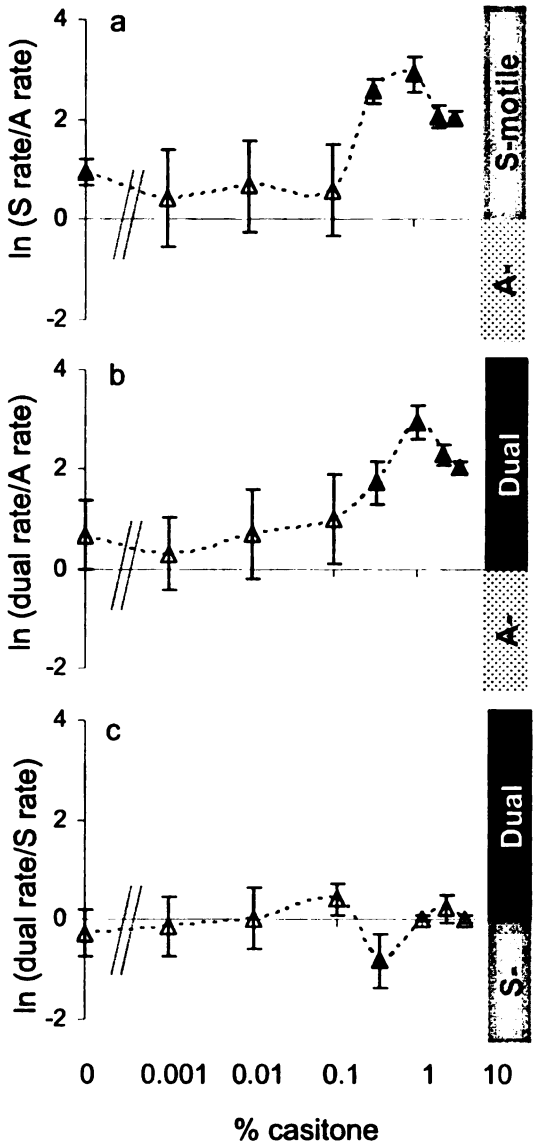

Figure 5. Relative swarming rates on soft agar. Relative rates of a) S-motile vs. A-motile, b) dually motile vs. A-motile, and c) dually motile vs. S-motile were calculated as the natural log of the ratio of the absolute swarming rate for each strain (see Fig. 1). Shaded boxes, and closed and open symbols and error bars represent the same meanings as in Fig. 3.

level, then a solely A-motile or dually motile (A^+S^- or A^+S^+) strain should swarm faster than a solely S-motile (A^-S^+) strain on hard agar at all casitone concentrations. The general superiority of A-motility on hard agar is in fact evident across a wide range of nutrient levels (Fig. 4a, b).

Although A-motility is generally superior to S-motility on hard agar, the two systems interact synergistically at all resource levels on a 1.5% agar surface (Fig. 4c). The dually-motile (A^+S^+) strain swarms significantly faster than both single-system mutants at all casitone concentrations (Fig. 4b,c). The benefit of combining the two systems relative to swarming by A-motility alone is relatively constant on hard agar across all nutrient levels (Fig. 4c).

Soft agar: S-motility dominance without synergism. Just as A-motility dominates over S-motility on hard agar at all nutrient levels (Fig. 4a,b), my results indicate that the rank superiority of S-motility over A-motility on soft agar is a general effect of the surface type that is independent of nutrient level (Fig. 5a). Both the solely S-motile and dually-motile strains swarmed faster than the solely A-motile strain at all nutrient levels on soft agar (Fig. 5a,b). At each of the four lowest casitone concentrations, the superiority of S-motility swarming was so small that it was not statistically distinguishable. If the data for these four nutrient concentrations are combined into one 'low casitone' group, then swarming by the solely S-motile and dually-motile (A^-S^+ and A^+S^+) strains is significantly greater than swarming by the solely A-motile (A^+S^-) strain within this low casitone category (0% - 0.1%; $p = 0.009$ and 0.005, respectively, one-tailed one-sample *t*-tests). The quantitative degree of superiority exhibited by the two strains with S-motility over the solely A-motile strain increased markedly at high nutrient concentrations (Fig. 5a,b).

In contrast to the synergism between the two motility systems observed on hard agar (Fig. 4c), there is no evidence for general synergism on soft agar. The dually-motile strain was superior to the solely S-motile strain on soft agar at only one nutrient level (0.1% casitone), and there only slightly so (Fig. 5c). The data from the 0.32% casitone treatment on soft agar suggest that combining A-motility with S-motility may actually hinder movement relative to swarming by S-motility alone on some soft surfaces.

Fibril mediation of S-motility nutrient sensitivity

Since fibrils are required for normal S-motility (Yang et al. 2000) and are also essential for some chemotactic responses (Kearns et al. 2000), I tested whether they were responsible for the sensitivity of S-motility to nutrient concentration. The *dsp* mutant used here (DK3470) does not produce fibrils but behaves similarly to the solely A-motile strain lacking functional pilin on hard agar (Fig. 1b,d). On soft agar, however, it swarms faster than the solely A-motile strain across a range of nutrient concentrations, indicating that the pili-mediated component of S-motility can drive a low rate of swarming even in the absence of fibrils (Wu et al. 1997; Velicer and Yu 2003). Unlike either fully S-motile strain (A^+S^+ or A^-S^+), the *dsp* mutant does not exhibit the same dramatic elevation of swarming rate at high casitone levels that is observed in the fibril producing strains (Fig. 1a,c,d). In particular, the ratio of soft vs. hard agar swarming rates increases greatly at high casitone levels (9.2-fold increase from 0.32% – 3.2% casitone) in the dually motile wild-type, whereas this ratio actually decreases more than 2-fold over the same interval in the *dsp* mutant (Fig. 1a,d). Because lack of fibrils eliminates the enhancement of soft agar swarming at

high nutrient concentrations, it appears that some component of the fibril matrix provides the causal mechanism of this enhancement.

Discussion

Predators such as *M. xanthus* may use a variety of behavioral strategies or physiological mechanisms to cope with temporally and spatially varying prey populations and environmental conditions. Shi and Zusman (1993) suggested that *M. xanthus* dual motility provides a means of adjusting to diverse environmental conditions by expanding the range of surfaces across which it can effectively move. Their observations were made primarily on homogeneous distributions of dense pre-hydrolyzed nutrients. My study of *M. xanthus* swarming across several distinct sets of laboratory conditions provides broader information for understanding the evolutionary benefits of maintaining dual motility. In particular, I have demonstrated that differential performance of the two *M. xanthus* motility systems on distinct surface types is in fact a general phenomenon across environments that vary dramatically in resource availability. In the case of S-motility, however, the quantitative benefit derived from soft-agar-specific performance varies dramatically with nutrient level.

The sensitivity of S-motility to resource level has implications for understanding the contribution of S-motility to the overall fitness of *M. xanthus*. This sensitivity caused low predatory performance across prey patches on soft agar that were separated by regions with very little growth substrate. Conversely, soft agar swarming was greatly enhanced (relative to hard agar swarming) during growth on continuous prey lawns and when prey patches were dispersed on rich casitone media. The conditions necessary for S-motility to drive substantial predatory swarming on

surfaces analogous to soft agar may be uncommon in soil environments. A variety of evidence indicates that most soils are nutrient limited (Williams 1985). For example, a significant percentage of soil microorganisms appear to be in a starved state (Bakken 1997) and dramatic increases in microbial growth and activity are commonly observed when energy sources are added to soils (Tate 1995). Moreover, many soils in which *M. xanthus* is found, particularly in arid climates, may only intermittently have a moisture content that approaches the water content in soft agar medium. Of course, inferences from swarming behavior on laboratory agar about swarming in soil are inherently limited due to the vast differences between these environments. Nonetheless, the laboratory data suggest that *M. xanthus* may only swarm at maximal S-motility rates under unusual soil conditions. In the laboratory, maximal social swarming rates require a combination of ecological conditions (nutrient abundance and moist surfaces) that appear to be uncommon in many soils.

Because S-motility is physiologically costly to maintain under conditions where it is not important for fitness (Velicer et al. 2002), its presence in wild-type strains of *M. xanthus* indicates that it provides a significant overall benefit in most natural environments. My results and those of previous studies suggest several benefits of possessing S-motility (in addition to A-motility) that likely contribute to the overall evolutionary advantage of maintaining dual motility in *M. xanthus* (Table 1). First, the A- and S-motility systems are synergistic on hard agar. Under almost all nutrient levels tested, *M. xanthus* swarms better on hard agar with both systems than with only one. Thus, even under the low resource conditions that are common in many soils and where S-motility does not greatly enhance swarming on soft surfaces (relative to swarming on hard surfaces), a dually-motile strain should still outcompete a strain lacking either system.

Table 1. Possible evolutionary benefits of *M. xanthus* S-motility.

Benefit	Mechanism
I. Synergism with A-motility on many surfaces (Shi and Zusman 1993)	Unknown
II. High within-group relatedness (Wu et al. 1997; Velicer and Yu 2003)	Fibril mediated cell-cell cohesion
III. Clustered spores (Wu et al. 1998) -enhanced dispersal? (Kaiser 2001) -enhanced spore survival/germination? (Wireman and Dworkin 1977)	Tight spore packing by S-motility
IV. Habitat-specific swarming enhancement that is independent of A-motility (Shi and Zusman 1993)	S-motility swarming on soft surfaces and fibril-mediated sensitivity to high nutrient levels

Second, the extracellular pili that drive S-motility enhance cohesion and a kin-clustered population structure. Genotypes lacking pilin are defective in cell-cell cohesion (Wu et al. 1997; Velicer and Yu 2003). In groups with low relatedness, competition and exploitation among individuals may degrade group-level benefits of cooperation (Turner and Chao 1998; Velicer et al. 2000; Fiegna and Velicer 2003). The high degree of cell-cell cohesion conferred by pili and fibrils during S-motility is undoubtedly important for determining the genetic structure of natural *M. xanthus* populations. They may ensure that the recipients of an individual's cooperative behavior are close relatives sharing a similar genotype.

Third, S-motility is important for tight packing of spores within fruiting bodies and this may provide several benefits to *M. xanthus*. Wu and Kaiser (1998) observed that, while many solely A-motile strains sporulated as or more efficiently than a dually-motile strain, the spores were not tightly packed within fruiting bodies, but rather many of them were interspersed between fruiting aggregates. It has been inferred from laboratory studies (Rosenberg et al. 1977) that the germination and

growth rates of spore populations are likely to benefit from being packed in fruiting bodies (Kaiser 1993) due to density-dependent feeding efficiency. In addition, well-formed fruiting bodies may also facilitate dispersal by vector organisms (Kaiser 2001) and enhance spore survival, possibly by providing spores proximate access to nutrients released by cells undergoing autolysis (Wireman and Dworkin 1977).

Finally, as demonstrated by Shi and Zusman (1993) and my results, the A- and S-motility systems do exhibit distinct surface-type-specific swarming abilities under abundant resource conditions such as those within and immediately surrounding animal dung. The degree to which such apparent surface specificity of the two motility systems contributes to evolutionary fitness should depend on how frequently the need to traverse variable surface types occurs when local resources happen to be abundant. Such surface-type specificity is unquestionably beneficial under a limited range of laboratory conditions; however, the overall importance of this fitness component under natural conditions remains unclear because the amount of reproduction that occurs in the commonly low-nutrient conditions of soil relative to that which occurs under rare nutrient-abundant conditions is unknown.

Nutrients, fibrils and S-motility

What causes the observed nutrient-level dependence of relative swarming rates on soft and hard agar in *M. xanthus*? More specifically, what causes faster swarming on hard compared to soft agar in the absence of nutrients and what causes enhanced soft agar swarming at high nutrient concentrations? Because cells reverse their direction at regular intervals (Blackhart and Zusman 1985), net rate of swarm movement in a particular direction is determined both by absolute cell speed and by

the relative amount of time spent moving in two opposite directions (as well as by cell orientation relative to the swarm and population growth rate). In previous studies, individual cells moved more slowly by A-motility on hard agar than by S-motility on soft agar at both high and low nutrient concentrations (Shi and Zusman 1993). Thus, it is unlikely that faster swarming on hard agar compared to soft agar at low nutrient concentrations was simply a result of increased cell speed. Shi, Köhler, and Zusman (1993) observed that cells moving on soft agar reversed frequently (once every 4 minutes) on MOPS buffer, but rarely reversed on high nutrient agar (CYE). Such a difference in behavior could contribute significantly to the elevated rate of swarming on soft agar at high nutrient concentration. In future work, it would be interesting to examine whether reversal frequency at low nutrient concentrations is greater on soft agar relative to hard agar. If reversal frequency on hard agar is lower than on soft agar, this could result in faster net cell movement away from the center of the swarm, and faster swarming at low nutrient levels even if individual cell velocity is lower on hard agar.

There is some evidence suggesting that enhanced swarming at high nutrient concentration may result from a chemotactic response to casitone mediated by the *frz* chemotaxis genes, which are homologous to the *che* genes that control chemotaxis in *E. coli* (Blair 1995). Shi, Köhler, and Zusman (1993) showed that swarms migrated asymmetrically from MOPS buffer towards casitone on soft agar in a steep and stable chemical gradient and that this response was correlated with methylation of FrzCD. Such chemotaxis was only observed at casitone concentrations of 0.2% or higher, which is within the range of casitone levels that resulted in more substantial soft agar swarming in our experiments. In the same study, only a weak chemotactic response was observed on hard agar. These results suggest that the *frz* system may function in

coordinating swarming away from nutrient-poor regions towards nutrient-rich regions. Consistent with my experiments, such an effect appears to be much stronger on soft agar than on hard agar.

My results expand on these previous observations by demonstrating that fibrils are likely to mediate the mechanism that causes increased swarming by S-motility on soft agar at high nutrient concentration. On soft agar, the presence or absence of fibrils had relatively little effect on swarming rate at low nutrient concentrations, but had a dramatic effect at higher concentrations (Fig. 1a,d). Thus, fibrils appear to mediate the stimulation of S-motility by abundant nutrients, but the mechanism by which they do so is not clear.

Because fibrils have been shown to be abundant at high cell densities under both nutrient-rich and starvation conditions (Behmlander and Dworkin 1991), a dramatic difference in fibril density as a function of nutrient concentration is unlikely to fully explain my results. However, the composition of the fibril matrix or the activity level of some fibril component may be altered as a function of nutrient concentration. Li, *et al.* (2003) showed that fibril polysaccharides containing amine sugars are the likely stimuli of pili retraction in S-motility. In their model, pili termini attach onto specific sugar moieties in the fibril matrix. These fibril attachment moieties then trigger pilus retraction and thereby generate a pulling force sufficient to move cells in the direction of the attached pili. One hypothesis that could be tested is that stimulation of pilus-retraction by these fibril attachment moieties is positively dependent on nutrient concentration.

Regardless of precisely how fibrils mediate the nutrient-level sensitivity of S-motility, the ecological significance of this sensitivity requires further investigation. The rate at which a swarm advances across a surface should determine the size of the

microbial community that *M. xanthus* influences as well as the overall rate of predation. My results provide information about how nutrient and agar concentration combine to influence the rate of advancement of vegetative cells across a surface, leading to hypotheses about the conditions that should result in significant predatory swarming by *M. xanthus*. These conditions include instances of high food density, especially when the predatory surface has properties similar to soft agar, and when the surface is similar to hard agar at either high or low food density. My data also lead to hypotheses about which *M. xanthus* genotypes may be favored in particular ecological conditions and the most frequent role of S-motility in the life-cycle of the organism.

CHAPTER 4

DECLINE IN PREDATORY PERFORMANCE DURING EVOLUTION IN THE ABSENCE OF PREY

Abstract

Myxococcus xanthus and some other microbial predators are capable of growing on soluble nutrients when prey are not available. I wanted to determine if adaptation to prey-free environments may frequently lead to a decline in predatory ability. To examine this possibility, I assessed the predatory ability of *M. xanthus* populations that had adapted to two different environments that did not contain prey. Eight populations that evolved on a surface were neither better nor worse than the ancestor at encountering patches of *Escherichia coli* or *Micrococcus luteus* dispersed on buffered agar, even though they were able to swarm across the buffered agar surface more quickly. In contrast, eight populations that evolved in a liquid environment were significantly worse than the ancestor at encountering prey-patches. These populations were also largely unable to lyse prey while shaking in a buffer solution. This consistent decline in predatory ability of liquid-evolved populations

indicated that there was a trade-off between adaptation to the prey-free liquid environment and predatory performance. The simplest explanation for decreased predatory performance is that liquid-evolved populations were either worse at searching for patches or were unable to lyse prey. However, liquid-evolved populations were able to lyse prey within patches as efficiently as the ancestor and did not appear to have diminished swarming ability on a buffered agar surface such as that between prey patches. Thus, adaptation to only one of two non-predatory environments resulted in degradation of predatory ability, but the mechanism of this trade-off is not clear.

Introduction

There are often diverse strategies for adapting to complex environments that cannot be simultaneously adopted by one organism. For example, in an environment containing a diverse array of potential prey, optimal exploitation of one prey type may require a morphology that interferes with the predator's ability to attack other prey species (Thompson 1994). This trade-off between attacking a diversity of prey or specializing on only one species leads to the evolution of generalist and specialist predators. Trade-offs arise because organisms are limited by their physiology and genetics and in the time and energy they have to invest in diverse activities such as foraging and reproduction.

The evolution of predators may be influenced by a variety of trade-offs. In addition to foraging for prey, some predators may themselves be prey to other species and trade-offs may exist between optimal prey consumption and optimal predator-avoidance behavior (Stephens and Krebs 1986). For example, garter snakes are able

to prey on newts because they are resistant to a toxin they produce. However, the mechanism of this resistance also impairs the ability of garter snakes to escape from their own predators (Brodie and Brodie 1999; Geffeney et al. 2002). There may also be trade-offs involved in adapting to multiple prey species. For example, adaptation of vesicular-stomatitis-virus (VSV) to a novel host (HeLa cells) resulted in a decline in the ability to infect its native host, baby hamster kidney cells (Elena 2002). Finally, there may be trade-offs that arise from energetic costs of predatory activities, such as searching for and handling prey, that generally decrease the energy spent on growth and reproduction (Stephens and Krebs 1986). This last trade-off may be especially relevant for bacterial predators that are capable of surviving on dead organisms or nutrients in addition to live prey (Casida 1988). The adaptations that allow these non-obligate predators to kill live prey or lyse dead organisms may be metabolically costly, or otherwise interfere with acquisition and growth on nutrients.

Myxococcus xanthus is a non-obligate prokaryotic predator that is ubiquitous in soil (Dworkin 1996). It kills prey by secreting antibiotics, bacteriolytic and proteolytic enzymes that together lyse the prey cell and break down its components into amino acids which it can use as food (Rosenberg and Varon 1984). Some evidence suggests that close contact with prey cells significantly enhances predatory efficiency, especially in aquatic systems (Burnham et al. 1981; Burnham et al. 1984; Daft et al. 1985). To find prey, *M. xanthus* uses two gliding motility systems that differ in their response to agar and nutrient concentration. The adventurous (A) motility system allows cells to move individually on hard-agar surfaces along stress lines that may lead the organism to clumps of prey cells (Shi and Zusman 1993; Dworkin 1996; Fontes and Kaiser 1999). A-motility is thought to propel cells by secreting 'slime' material through pores of the rear cell pole (Wolgemuth et al. 2002).

Movement on hard agar can be enhanced by social (S) motility, which pulls cells that are in close contact with each other via attachment and retraction of long, thin, appendages called pili (Shi and Zusman 1993; Dworkin 1996; Kaiser 2000). Swarming by S-motility is much faster on soft agar compared to hard agar and is sensitive to nutrient concentration (Chapter 3; (Shi and Zusman 1993)).

Almost all ecological and evolutionary studies with *M. xanthus* have taken place on synthetic media that contain high concentrations of amino acids (Velicer et al. 1998; Velicer and Stredwick 2002; Velicer and Yu 2003). In one evolutionary study, *M. xanthus* evolved for 1000 generations in this prey-free media in shaking flasks (Velicer et al. 1998). All twelve populations independently evolved faster growth rates relative to their common ancestor but declined in their ability to form multicellular fruiting bodies and spores and in rates of movement by A and S-motilty (Velicer et al. 1998). In a second evolution experiment, twelve independent populations were propagated on hard agar containing rich nutrients to select for improved motility (Velicer and Stredwick 2002).

I wanted to determine if there was a loss in predatory ability associated with adaptation to these two evolution environments, which have dramatically different structures but share the same resource. The advantage of comparing two evolution experiments is that I can explore the range of conditions under which a trade-off between predatory performance and growth in the absence of prey might occur. For example, a loss in performance could occur if there was a general trade-off between growth rate on the amino acid medium that was used in both experiments and performance on a live prey resource. If this trade-off did exist, then I would expect all of the populations from both evolution experiments to decline in predatory ability, since both contain the resource. Alternatively, the source of the trade-off may be

adaptation to both the resource and the structure of the evolution environment. If this were the case then predatory ability may degrade in only one of the two evolution environments.

To explore these possibilities, I assessed the predatory performance of eight randomly chosen populations from each evolution environment. Each population was placed on a prey patch in the center of a square, buffered agar plate that was covered in a grid of patches. After 14 days, I counted the number of prey patches encountered by the expanding swarm of the evolved population and compared it to the patch-encounter ability of its ancestor. Each population was tested on two prey types that differed in their cell-wall structure so that it would be evident if losses of performance were limited to either gram-positive or gram-negative prey species.

Overall performance in this predatory environment depends on how quickly the population can move across the low-nutrient surface between patches and the prey-coated surface within patches, as well as the rate of individual prey killing. Each of these activities may involve several traits. Some of these traits may be disadvantageous in either the liquid or surface environments or both. One trait that could affect predatory performance and may be disadvantageous in both the surface and liquid environments is production of compounds required for prey killing. These compounds may be physiologically costly to produce when they are not needed. Based on our current understanding of secreted predatory compounds, there is no reason to expect that such molecules contribute positively to fitness in an environment abundant in soluble nutrients. Another trait that is clearly important for effective predation in the patchy environment is motility, which is necessary for swarming toward food sources across any solid surface. This trait degraded in the liquid environment, but not in the surface environment (Velicer and Stredwick 2002).

Because of the crucial role of motility in predatory performance across the patchy environment, I expected liquid-evolved populations to lose predatory ability but surface evolved populations to maintain it unless predatory traits other than motility declined.

Methods

Liquid-evolution experiment

Two clones of *M. xanthus* that differed only in their resistance to rifampicin were used to initiate twelve independent populations (six per clone) that evolved for 1000 generations as described by Velicer et al. (1998). These clones were GJV1, which is a clone of the standard lab strain, DK1622 (Kaiser 1979) and GJV2, which is a spontaneous rifampicin resistant mutant of GJV1 (Velicer et al. 1998). The evolution environment consisted of a 50 ml flask filled with 10 ml of CTT broth (1% casitone dissolved in 10 mM Tris pH 8.0, 8 mM $MgSO_4$, 1 mM KPO_4 (Bretscher and Kaiser 1978)) at 32°C with constant shaking at 120 rpm. CTT broth is rich in amino acids. Every day, 100 µl was transferred to fresh media. The following eight populations were randomly chosen for further study: S1, S2, S3, and S5 (labeled L1-4, respectively in this study), R1, R3, R4, R5 (labeled L5-8 in this study).

Surface-evolution experiment

M. xanthus clones GJV1 and GJV2 were also used to initiate a total of twelve independent populations (six per clone). These populations evolved for 16 two-week transfer cycles consisting of approximately 14 days on CTT agar (1.5% agar dissolved

in CTT broth) and two days in CTT liquid. To initiate each plate phase, 10 µl of the preceding liquid population was spotted onto the center of a plate. Populations swarmed radially outward during incubation at 32°C. After 14 days, a portion of the swarm edge (5 mm deep band around 25% of perimeter) was cut out of the agar and added to a flask with CTT broth, which was incubated for approximately 40 h while shaking at 120 rpm. This culture was then used to initiate the next transfer interval. Eight populations were randomly chosen for further study.

Patch-encounter ability assay

Patch-encounter ability was assessed in "predation arenas". Predation arenas consisted of 12 cm square petri dishes filled with 75 ml TPM agar (1.5% agar dissolved in TPM buffer: 10 mM Tris pH 8.0, 8 mM $MgSO_4$, 1 mM KPO_4 (Bretscher and Kaiser 1978)). Patches of either *E. coli* B or *M. luteus* ATCC 4698 were distributed in a grid configuration on top of the agar. The distance between patches was approximately 1 cm.

Prior to adding prey to predation arenas, cultures of each prey organism were grown in brain heart infusion broth (Difco), washed two times in TPM buffer, and then resuspended to a density of 1.2 -1.6 x 10^{11} cells/ml for *E. coli* and 1.2 - 1.4 x 10^{10} cells/ml for *M. luteus*. At these cell densities, the prey solutions contained a similar biomass. This solution was added to a 96-well microtiter plate (150 µl per well) and blotted onto the predation arena using a Bel-Blotter™ Replicator (Bel-Art Products). This polycarbonate blotter consisted of 96 cone-shaped tips arranged to fit the spacing of a 96-well plate. Each tip picked up cell suspension by capillary action from a well and deposited approximately 3 µl of it onto agar. To control for potential variation

between arenas in the amount of prey added per patch, predators were randomly assigned to specific plates. Predation arenas were always prepared one day in advance of inoculation with *M. xanthus*.

Ten µl of a suspension of *M. xanthus* in TPM buffer (1 x 10^9 cells per ml) were added to a center patch in the predation arena to initiate the patch-encounter rate assay. The predation arena was then incubated (32°C) in plastic bags with slits cut in it to allow oxygen flow while maintaining plate moisture. The incubator was kept humid by including a pan of water near the fan in the incubator. During the incubation period, the *M. xanthus* swarm moved radially outward across the prey-patch grid. Patch-encounter ability was the percentage of patches (out of 156 total) on the plate that were touched by the *M. xanthus* swarm after 14 days incubation, even if the swarm had not overtaken the entire patch.

Patch-encounter ability was assayed in four temporal blocks which included one replicate of each evolved *M. xanthus* population (L1-8 and S1-8) on each prey type and four replicates of each ancestral clone (GJV1 and GJV2) per prey type.

Swarming rate assay

Swarming rate in the absence of prey was measured by adding 10 µl of a suspension of *M. xanthus* (1 x 10^9 cells/ml in TPM) to the center of a buffered agar plate (50 ml TPM agar in a round, 15 cm, petri dish) and measuring the change in radius of the expanding swarm after 3 and 14 days incubation. Two perpendicular diameters at random orientation were measured at each time point. The average of these two diameters was divided by two to obtain an average radius. This number was divided by the number of days to obtain the swarming rate. Plates were

incubated as for patch-encounter ability. Swarming rate was measured in three temporal blocks that included one plate for each evolved population and four replicate plates for each ancestral clone.

Prey killing in a liquid environment

To measure prey killing in liquid, *M. xanthus* (100 µl of TPM buffer with 1 x 10^9 cfu/ml) was added to a 50-ml Erlenmeyer flask containing 6 ml of a suspension of prey (*M. luteus*, 6.6 x 10^7cfu/ml; or *E. coli*, 6.6 x 10^6cfu/ml) in TPM buffer. Prey were cultured and washed by the same procedure as in the patch-encounter assay, and added to flasks one day prior to inoculation with *M. xanthus*. Flasks were incubated at 32°C with constant shaking at 300 rpm for 24 hours. The concentration of prey remaining was determined by diluting a sample of the suspension in Davis Minimal medium without glucose (DM (Carlton and Brown 1981)) and plating on Luria-Bertani broth plates (Sambrook et al. 1989). The number of prey colonies was counted to estimate the number of prey in the sample. This assay was conducted in two temporal blocks. In each block, a different set of four populations from each of the liquid- and surface-evolution experiments were assayed along with two replicates of each ancestor and four controls. The controls contained only prey.

Prey killing in patch environment

Prey killing in a patch environment was determined by assaying the number of prey that remained in a patch after 24 hours incubation with *M. xanthus* and comparing that with a control that did not contain predator. Prey patches were formed

by the same method as for patch-encounter ability with the following exception. They were dispersed on a 6 cm round petri dish containing 11.9 ml TPM agar. A 10-µl spot of *M. xanthus* suspension (1 x 10^9 cells/ml in TPM buffer) was added to the patch. The spot covered the patch completely. After incubation at 32°C for 24 h, the prey patch was cut out of the agar and added to a 1.5-ml microfuge tube containing 1 ml DM. The microfuge tube was vortexed 12 times. The suspension was then diluted and plated on LB. The number of prey colonies was counted to estimate the number of viable prey in the sample. The experimental design for the prey-killing assays in the patch environment was the same as for the prey-killing assays in a liquid environment.

Prey killing in a lawn environment

To investigate prey-killing ability further, I quantified the degree of clarity in a dense *E. coli* or *M. luteus* prey lawn caused by inoculation of either an evolved *M. xanthus* population or the ancestor. A preliminary experiment showed that the clarity in the prey lawn correlated with the density of surviving *E. coli*. Five µl of suspension of each evolved population (1 x 10^9 cells/ml in TPM buffer) were spotted onto dense lawns of *E. coli* and *M. luteus* that covered TPM agar plates. Four spots of each ancestral clone were also included on the same plate. Evolved populations from both evolution experiments were arranged in columns on the plate with spots of their ancestor in between. After 19 h of incubation, the plates were photographed. The photographs were converted into black and white images, and the mean grey value of each spot was measured using the software Image J (Rasband 2004). These numbers were converted to "uncalibrated optical densities" by the software. To control for

variation in the density of *E. coli* or *M. luteus* across the plate, the optical density of the surface between the spot of each evolved population and its ancestor was subtracted from the optical density of the evolved population. For measurements of the ancestor, the mean of the optical density on either side of the ancestor spot was subtracted as a control. This experiment was repeated four times, with one plate of each prey species per repetition.

Statistical analyses

To quantify evolutionary change in patch-encounter ability, prey killing on a lawn, and swarming rates, each measurement of an evolved population was paired with and then divided by a different randomly chosen measurement of its ancestor from the same block in order to calculate a relative value. Then each data point was log-transformed. The ancestral value used for comparison for prey killing in a lawn was always the spot closest in proximity to the evolved population. A t-test was used to determine if the mean log-ratio of a particular population was significantly different from zero, indicating evolutionary change.

Results

Liquid-evolved populations: Patch-encounter ability

Twelve replicate populations of *M. xanthus* evolved independently in nutrient-rich, prey-free, liquid broth for 1000 generations. The evolved populations had improved growth rates, but were deficient at swarming in high-nutrient conditions and forming fruiting bodies and spores. I wanted to know if adaptation to this prey-free

environment resulted in loss of predatory ability. To test this hypothesis, eight evolved populations and the ancestor were added to a prey patch at the center of an agar surface that had been covered with a grid of patches. After 14 days, I counted the proportion of available patches that were encountered by the swarm as it expanded radially from the center. No nutrients were added to the agar, so prey were the main food source for *M. xanthus* in this environment. Patch-encounter ability was tested on both a gram-positive (*M. luteus*) and a gram-negative (*E. coli*) species to assess the generality of changes in predatory ability.

The patch-encounter ability of each evolved population relative to the ancestor is plotted in Figure 1a. All eight populations were worse than the ancestor at encountering prey patches of both prey species. The overall decrease in patch-encounter ability of populations that evolved in liquid was about 3-fold relative to the ancestor and was significantly different from zero (One sample, two-tailed t-test; $p < 0.001$). Prey type did not affect the magnitude of decrease in patch-encounter ability (Paired, two-tailed t-test; $p > 0.05$). The consistency of responses, as indicated by all 8 populations showing the same direction shift on both prey species, demonstrates that evolution in the prey-free liquid regime led to correlated losses in prey-patch encounter rates.

One explanation for evolutionary decreases in patch-encounter ability is that the evolved populations are worse than the ancestor at moving across the surface between prey patches. Previous experiments indicated that all of the liquid-evolved populations had motility defects to some extent (Velicer et al. 1998). Therefore, I also tested the swarming rate of the evolved populations and the ancestor on a buffered-agar surface that did not contain prey patches (Fig. 1b). There was no

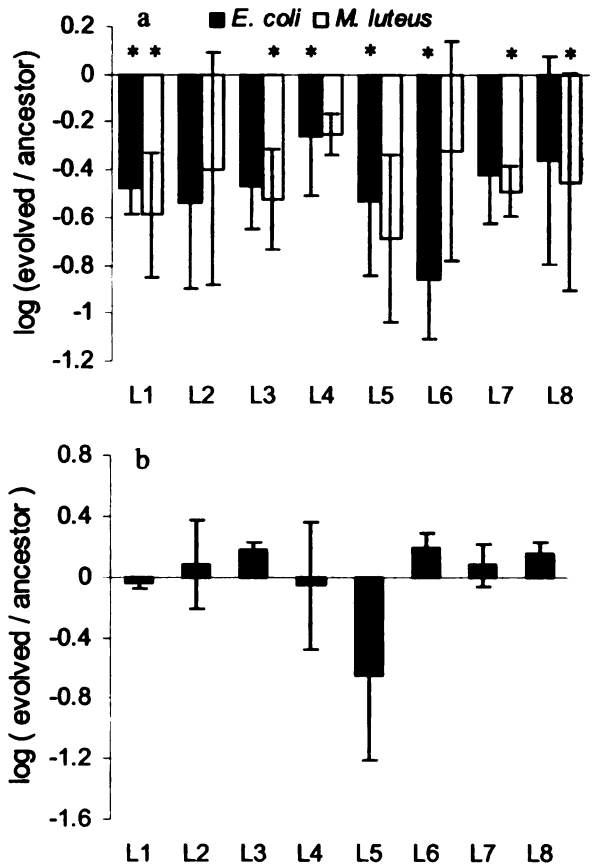

Figure 1. Evolutionary changes of liquid-evolved populations in swarming rate and patch-encounter rate. The mean log of the ratio of the evolved population relative to the ancestor for a), patch-encounter rate with *E. coli* or *M. luteus* as prey, and b) swarming rate on buffered agar. A positive number would indicate that the evolved population was superior to the ancestor, a negative number would indicate that it was worse than the ancestor, and zero would indicate that the evolved population and ancestor were the same. Error bars indicate 95% confidence intervals. Asterisks (*) indicate that the mean log ratio was significantly different from zero in a two-tailed, one-sample, t-test after sequential Bonferonni correction for all eight comparisons on the same prey type ($p < 0.05$ for eight comparisons combined).

consistent trend towards improvement or decline in swarming on buffered agar in the absence of prey. Three populations out of eight were slightly worse than the ancestor at swarming in this environment. However, the mean change across populations was not significantly different from zero (one-sample, two-tailed t-test; $p > 0.9$). This finding indicates that decreased patch-encounter ability in the evolved populations could not be explained simply by decreased population-level swarming on buffered agar in the absence of prey. The presence of prey patches must somehow affect the rate of swarming across the patchy environment.

Liquid-evolved populations: Prey killing ability

In addition to encountering fewer prey patches, liquid-evolved populations may kill fewer prey per patch than the ancestor. Within each patch, the rate of prey killing may be affected both by how quickly *M. xanthus* can find the individual prey within the patch and by how efficiently it lyses those prey. I used two strategies to separate prey lysis ability from motility rate. In one strategy, dense solutions of *M. xanthus* were added to prey on a surface such that they were distributed across the prey population in roughly equal proportion to the prey. In this situation the predator would not have to search very far for prey and they could maintain contact with prey individuals as long as was necessary to kill them. The second strategy consisted of measuring prey-killing rate in shaking liquid, where motility would not be possible. In contrast to the first strategy, individual *M. xanthus* cells would have difficulty maintaining contact with individual prey in the vigorously shaking flasks. Prey-killing ability was determined by counting the number of prey remaining that were still capable of forming colonies after several hours incubation with *M. xanthus*. In

these predatory environments there were no additional resources for the prey or predator to grow on, so some prey may have died from starvation rather than predation over the course of incubation. To account for this possibility, the number of prey remaining was compared to the number remaining in a control that did not contain predator.

The abilities of the liquid-evolved populations and their ancestors to kill prey in vigorously shaken buffer are presented in Figure 2a. In all eight trials of the ancestor on each prey type, the fraction of prey remaining was very low (between 0.00371 and 2.84%). This result indicates that almost 100% of the prey were killed by the ancestor within 24 hours. In contrast, prey-killing by many of the liquid-evolved populations was undetectable. Fifty-eight to 100% of the viable prey population remained after incubation with liquid-evolved populations. This difference between the ancestor and liquid-evolved populations was statistically significant in a paired signed-rank test ($p = 0.0078$ for each prey type), indicating that liquid-evolved populations were highly deficient in their ability to kill prey in a liquid environment that discouraged sustained contact between predator and prey.

Liquid-evolved populations eliminated prey at rates similar to the ancestor on surfaces, however. In this environment, the necessity for prey searching was minimized by distribution of the predator throughout the prey patch or lawn, but sustained contact with prey was possible. Prey-killing ability was measured on surfaces in prey patches by counting the number of prey remaining and on prey lawns by measuring the optical density of lysed areas. In the prey-patch environment, the ancestor and most of the liquid-evolved populations killed nearly 100% of the prey (Figure 2b). Although a few of the liquid-evolved populations left a large proportion

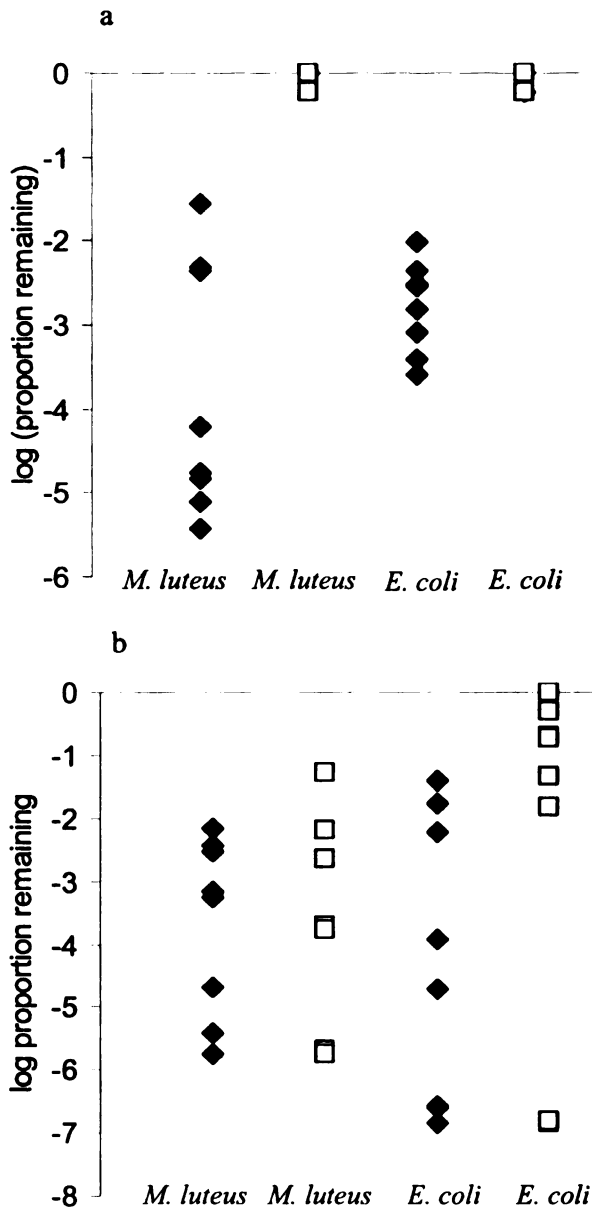

Figure 2. Prey killing ability of liquid-evolved populations. The logarithm of the proportion of prey remaining relative to control after 24 hours of predation by the ancestor (black diamonds) or liquid-evolved populations (open squares) in a) a shaking flask, or b) a patch in a predation arena.

of the prey in patches of *E. coli*, there was not a significant overall decrease in prey killing on surfaces among the liquid-evolved populations whether *E. coli* (Wilcoxan signed-rank test; $p = 0.2188$) or *M. luteus* (Wilcoxan signed-rank test; $p = 0.4375$) was the prey. A similar result was obtained when prey-killing ability was measured on a dense lawn of each prey type. None of the liquid-evolved populations exhibited a statistically significant difference from the ancestor in the ability to clear a prey lawn (Figure 3).

Surface-evolved populations: Patch-encounter ability

Twelve replicate populations evolved independently for 32 weeks on a nutrient-rich, prey-free, agar surface under selection for improved swarming (Velicer and Stredwick 2002). I wanted to determine if predatory ability was lost during adaptation to this prey-free environment. The patch-encounter ability of eight of these populations and the ancestor was assayed with both *E. coli* and *M. luteus* as prey. The results are shown in Figure 4a. Similar to the liquid-evolved populations, the magnitude of evolutionary change in patch-encounter ability was not affected by the prey-type used in the assay (Paired, two-tailed t-test; $p > 0.05$). Unlike the liquid-evolution experiment, however, evolution on a surface did not result in a decrease in the ability to encounter prey patches. Only two populations (S3, S4) showed a decline in patch-encounter ability on both prey types and these were not significant. Five out of eight of the surface-evolved populations showed small improvements in their ability to encounter patches of both prey species. This frequency of improvement could suggest an evolutionary trend towards slight improvement in patch-encounter ability. However, the mean change in patch-encounter ability relative to the ancestor

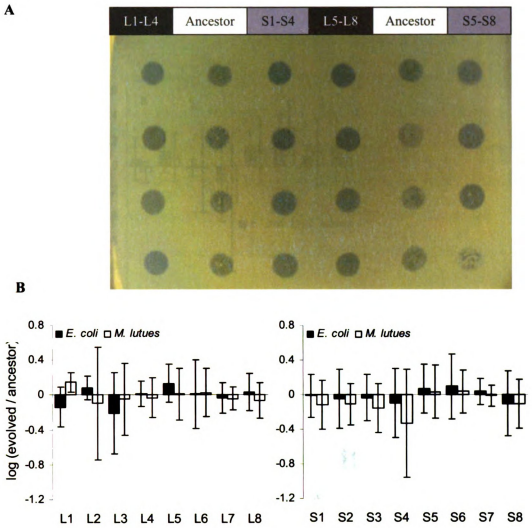

Figure 3. Evolutionary changes in prey lysis efficiency. Populations were spotted onto a dense lawn of *E. coli* or *M. luteus* and the plate was photographed after 19 hrs incubation at 32°C. One plate from four replicate experiments is shown in panel A. Liquid-evolved populations (L1-L8) were spotted in columns marked with black boxes and surface-evolved populations (S1-S8) are indicated with grey boxes. Columns of replicate spots of the ancestor are marked with a blank box. Panel B shows the log of the ratio of the mean spot intensity of evolved populations relative to the ancestor. Error bars and asterisks have the same meaning as in figure 1. Images in this dissertation are presented in color.

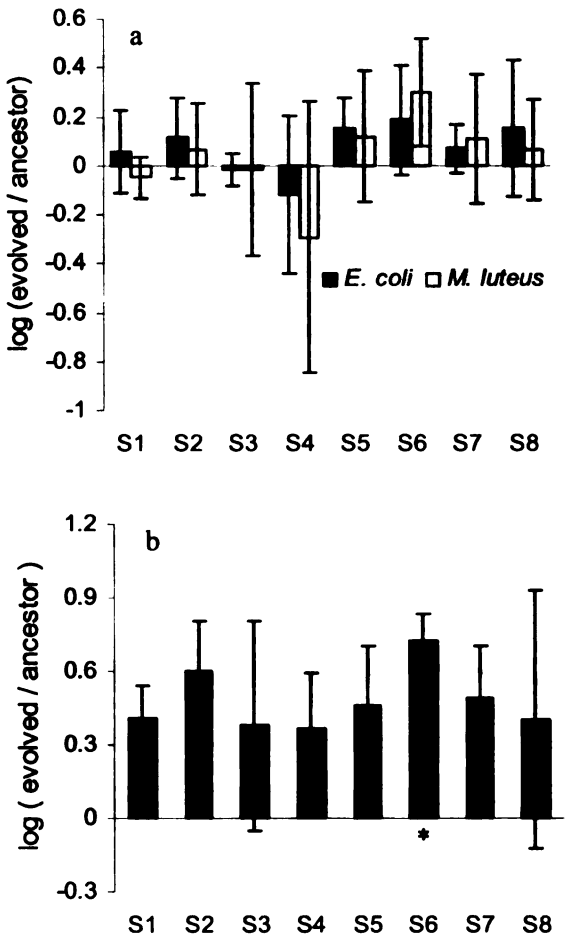

Figure 4. Evolutionary changes of surface-evolved populations in swarming rate and patch-encounter rate. The mean log of the ratio of the evolved population relative to the ancestor for a) patch-encounter rate with *E. coli* or *M. luteus* as prey, and b) swarming rate on buffered agar. Error bars and asterisks have the same meaning as in Figure 1.

was not significantly different from zero (One-sample, two-tailed t-test; $p > 0.05$), indicating that there was no significant trend towards improvement or decline.

Although patch-encounter ability did not consistently improve or decline during evolution in the surface-environment, there was evidence that populations evolved to swarm faster than the ancestor across a buffered-agar surface, which is the same as the surface between patches. All eight surface-evolved populations swarmed at a faster rate on prey-free buffered agar than the ancestor (Fig. 4b). This 3-fold overall evolutionary improvement in swarming was highly significant (One-sample t-test, $p < 0.001$).

Surface-evolved populations: Prey-killing ability

The surface-evolved populations improved in their ability to swarm across the surface between patches, but not in their overall patch-encounter ability. I tested whether they were deficient in their ability to kill individual prey once they have encountered them. Unlike the liquid-evolved populations, most surface-evolved populations were able to kill prey proficiently in the shaking liquid environment (Fig. 5a). They caused declines in *M. luteus* prey populations that were indistinguishable from those caused by the ancestor (Wilcoxan signed-rank test, paired, $p = 0.4688$). When *E. coli* was the prey, the average proportion of prey-remaining after incubation with surface-evolved populations was somewhat greater than when *E. coli* was incubated with the ancestor (Wilcoxan signed-rank test, paired, $p = 0.0156$). However, all but two of the populations killed almost 100% of their prey populations, indicating that most surface-evolved populations were able to proficiently kill both prey species in liquid. The surface-evolved populations were also able to clear prey

lawns of *E. coli* or *M. luteus* (Fig. 3b) and kill as many prey within patches as the ancestor (Fig. 5b; Wilcoxan signed-rank test, paired, $p = 0.8438$ and $p = 0.4375$, on *E. coli* and *M. luteus*, respectively).

Discussion

The predatory abilities of the bacterium *M. xanthus* may be limited by trade-offs that occur during adaptation to non-predatory conditions. If such a trade-off is general, predatory ability should consistently decline during evolution in a variety of different prey-free environments. I tested this hypothesis by evaluating the predatory abilities of two sets of populations that had evolved for many generations under two very different prey-free environments (shaken liquid and a solid agar surface) that were both rich in nutrients. The eight populations which evolved in prey-free liquid were worse than the ancestor at encountering prey patches of both *E. coli* and *M. luteus* (Fig. 1a). They were also largely unable to kill either prey in a liquid environment (Fig. 2a). However, these populations were comparable to the ancestor at lysing prey on an agar surface (Figs. 2b and 3). By contrast, eight populations that evolved under selection for improved fitness during swarming and growth on agar did not consistently improve or decline in predatory ability in any environment (Figs. 4a, 3 and 5). Together these results show that in the absence of prey, predatory ability consistently declined under selection for improved fitness in an unstructured environment where motility is not important for fitness, but not necessarily in a structured (agar) environment in which motility is important.

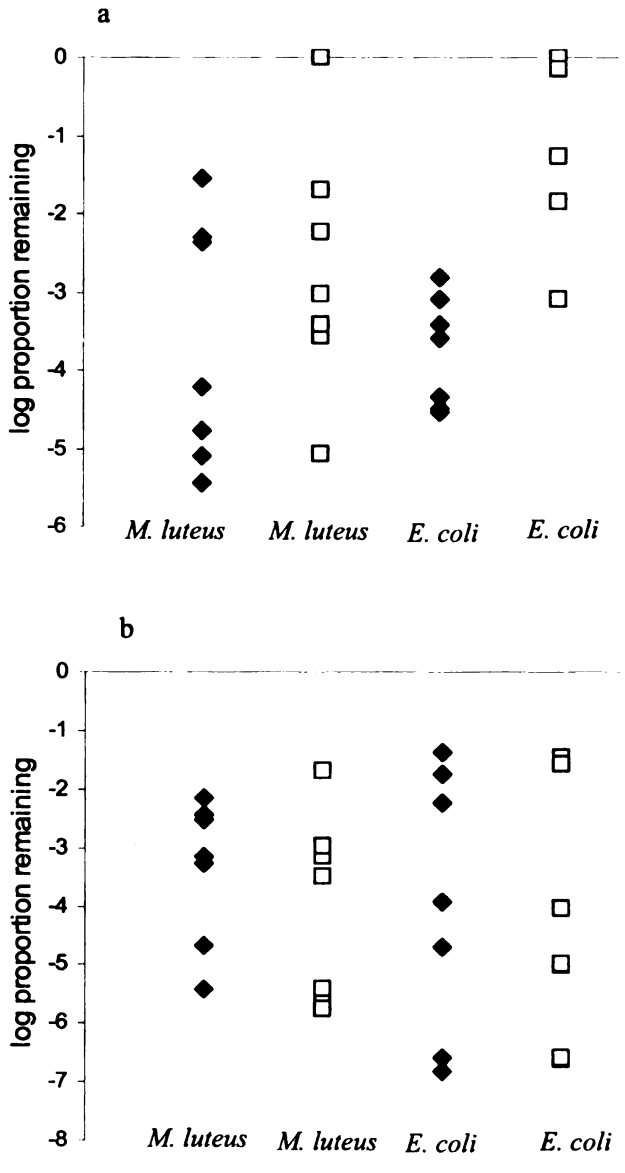

Figure 5. Prey killing ability of surface-evolved populations. The logarithm of the proportion of prey remaining relative to control after 24 hours of predation by the ancestor (black diamonds) or surface-evolved populations (open squares) in a) a shaking flask, or b) a patch in a predation arena.

Evidence for trade-offs affecting predatory performance

Consistent declines in predatory performance among the liquid-evolved populations indicate a trade-off between adapting to the unstructured liquid environment and maintaining predatory traits. Some trade-offs may arise from interrelationships among genes and phenotypes. An example is mutations that cause phage resistance but also decrease competitive ability in the absence of phage (Lenski 1988; Bohannan et al. 1999). Such antagonistic pleiotropy may lead to ecological specialization (Cooper and Lenski 2000) and may promote coexistence of multiple ecological types (Rozen and Lenski 2000; Bohannan et al. 2002). Parallel declines in predatory ability of *M. xanthus* within 1000 generations suggest an antagonistic pleiotropic relationship caused by genes that are adaptive in the liquid environment but also adversely affect predatory ability. The source of this antagonistic pleiotropy may have been loci that affect fibril and pili production. Fibrils and pili are extracellular appendages involved in motility and cell-cell cohesion (Arnold and Shimkets 1988; Wu et al. 1997; Kaiser 2000; Li et al. 2003; Velicer and Yu 2003). Decrease in fibril and pili production may have increased the energy and biomass that can be allocated to growth (Velicer et al. 2002) but simultaneously caused a decline in predatory ability (see below).

This trade-off, like others caused by antagonistic pleiotropy, is not necessarily an evolutionary dead-end. In some conditions the severity of trade-offs may be diminished by mutations in another locus. For example, the fitness cost of resistance to phage can be reduced by mutations in a second gene that improves competitive ability (Lenski 1988; Bohannan et al. 1999). In addition, Turner and Elena (2000) showed that there was a trade-off in adaptation of vesicular-stomatitis-virus (VSV) to

two novel hosts, but this trade-off did not limit the ability of VSV to adapt to both hosts simultaneously. Similarly, the fitness cost of *M. xanthus*'s predatory alleles in conditions that select for improved growth rate could allow for the coexistence of multiple 'types' with varying predatory capabilities and growth rates. However, some other form of cost savings might evolve in such an environment if it included both prey and simple nutrients as food sources. Predatory ability may not have been lost, for example, if the liquid environment alternated between nutrients and prey as the main resource.

Evolution in prey-free liquid led to diminished overall predatory ability, but evolution in a second prey-free environment, solid agar, did not. Although the eight surface-evolved population did not consistently decline in predatory ability, their swarming rates on buffered agar (the surface between prey patches) were significantly higher than their ancestor. This result prompts the question of why the plate-evolved populations were not consistently better at predation given their improved motility on buffered agar. Two potential explanations for this phenomenon are described at the end of this discussion.

Because motility is necessary for swarming on surfaces in search of nutrients or prey patches, it is perhaps not surprising that evolution on surfaces did not produce extensive or rapid losses in patch-encounter ability. However, *a priori*, it seemed possible that *M. xanthus* could have lost individual prey-killing ability leading to a predatory decline even if motility did not change. In fact, all sixteen populations that evolved in environments that were absent of prey or other complex resources maintained the ability to kill and lyse prey on surfaces. Other researchers have also noted that prey lysis ability was not lost after prolonged propagation in prey-free environments (Singh 1946). Why was prey-killing ability on surfaces maintained?

Prey killing and lysis may be caused by a diversity of secreted molecules (Rosenberg and Varon 1984). Thus, it is likely that several independent mutations would be required to completely eliminate the capability of causing prey lysis. Alternatively, production of the molecules that cause prey lysis may provide unidentified fitness benefits for *M. xanthus*.

Liquid-evolved trade-off may be caused by variable roles of pili and fibrils

What is the physiological and genetic basis for the trade-off between adaptation to the liquid environment and predatory ability? A previous study demonstrated that several of these populations lost the ability to produce pili, and that this loss provided a fitness benefit in the liquid environment (Velicer et al. 2002). Pili are extracellular appendages that are required for S-motility and enhance cohesion (Wu et al. 1997; Kaiser 2000; Velicer and Yu 2003). Restoration of pili production through genetic complementation decreased fitness in the liquid environment and substantially restored swarming ability (Velicer et al. 2002). Fibrils are another extracellular appendage that enhance cohesion (Arnold and Shimkets 1988) and swarming at high nutrient concentrations (Chapter 3). It is probable that the mutations affecting pili production and possibly other mutations affecting fibril biogenesis, could have caused decreased patch-encounter ability and decreased prey lysis in liquid. Indirect support for this hypothesis is described below.

Fibrils and pili are likely to enhance prey killing in liquid. When wild-type *M. xanthus* preys on *E. coli* or *M. luteus* in liquid, rings form on the sides of the flask. Formation of these rings requires cohesion, which is caused by pili and fibrils (Arnold and Shimkets 1988; Wu et al. 1997; Velicer and Yu 2003). Unlike the ancestor, the

liquid-evolved populations do not form these rings. Unfortunately, my attempts to determine if ring formation was necessary for prey killing by physical disruption were inconclusive. Therefore, I cannot confirm that the ability to form a ring on the side of the flask is a prerequisite for killing prey in liquid, but previous predatory studies have suggested that cohesion is important for effective lysis of some organisms. Efficient lysis of cyanobacteria by *Myxococcus* strains involved encapsulating the prey within spheres or trapping them in cell clumps that cohered to vessel walls and glass beads (Burnham et al. 1981; Burnham et al. 1984; Daft et al. 1985).

Cell clumping could enhance predation by increasing the density of *M. xanthus* cells, or by facilitating the action of lytic agents that may be bound to the cell (Rosenberg and Varon 1984). In support of this latter possibility, I was never able to cause lysis of live *E. coli* or *M. luteus* with cell-free supernatants, even if the supernatant was concentrated with a filter with a 10,000 MW pore size. This pore size is smaller than the estimated size of previously isolated enzymes that were active against lyophilized *M. luteus* (Sudo and Dworkin 1972). The high density of *M. xanthus* cells within rings or clumps might increase the efficiency of lysis by increasing the local concentration of enzymes. Previous studies have shown that growth of *M. xanthus* on resources that require processing with secreted enzymes is density dependent (Rosenberg et al. 1977).

In addition to possibly enhancing predation efficiency in liquid through cohesion, pili and fibrils may also be necessary for swarming in a prey-dense environment. The liquid populations were able to lyse prey within a patch and swarm across the surface between patches, but they still had decreased patch-encounter ability. One explanation for this result is that they declined in their ability to swarm across the prey-coated surface within a patch. There are two lines of evidence

suggesting that the liquid-evolved populations may be deficient in this trait. All eight of the liquid-evolved populations were worse than the ancestor at swarming across a high-nutrient surface that did not contain prey (Velicer et al. 1998). The surface within a patch should be rich in nutrients once a few prey have been lysed, so it is possible that they also do not swarm across a patch very quickly. In addition, when the liquid-evolved populations were placed on a dense lawn of *Micrococcus luteus* (a different strain from that used here), the lytic zone produced was much smaller in size than that produced by the ancestor (Velicer and Stredwick 2002). This observation suggests they do not swarm as quickly in this environment. Pili and fibrils could facilitate swarming across nutrient-rich prey patches in a prey-specific manner or by general enhancement of A-motility swarming on nutrient-rich hard agar (Chapter 3, (Shi and Zusman 1993)).

In addition to fibril and pili mediated swarming across prey patches, other changes in predatory physiology may have contributed to the decrease in patch-encounter ability in liquid-evolved populations. These aspects of predatory physiology might have also affected the surface-evolved populations and could explain how improved swarming on buffered agar did not lead to corresponding changes in patch-encounter ability. First, even though the liquid- and surface-evolved populations are capable of killing and lysing prey effectively on surfaces, they may not be as proficient as the ancestor at converting the prey into food. This deficiency would decrease effective food density, and hence the growth and swarming rate of both liquid- and surface-evolved populations. Second, the rate of swarming across buffered agar may be different in a patchy environment than on the prey-free surface I used to assay swarming. The physiological state of *M. xanthus* as it leaves a patch may affect its subsequent swarming rate on buffered agar. Thus, in addition to

possibly swarming more slowly across a patch, the presence of prey patches may also negatively affect the liquid-evolved populations' swarming rate between patches. For the surface-evolved populations, the presence of patches may limit the impact of evolutionary improvements in buffered agar swarming on their ability to move between patches.

In conclusion, evolution of *M. xanthus* in two different prey-free environments had diverse effects on predatory performance. Evolution on a surface did not noticeably affect predatory performance, although the ability of these populations to swarm in a low nutrient environment did improve. On the other hand, populations that evolved in the absence of prey in a liquid environment consistently lost some degree of predatory ability. This trade-off may have been caused in part by a decrease in fibril or pili production along with possible evolutionary changes in other aspects of predatory physiology. Most importantly, this trade-off could lead to the coexistence of different *M. xanthus* 'types' that vary in growth rate and predatory performance in some conditions.

CHAPTER 5

PREY-PATCH DENSITY AFFECTS EVOLUTION OF PREDATORY SEARCHING IN *MYXOCOCCUS XANTHUS*

Abstract

The efficiency of a predator is determined both by its own capabilities and environmental features such as prey distribution. Evolution can change the relationship among these factors by altering the capabilities of predators. Predation efficiency should be more dependent on search time when prey are far apart (low density) than when they are close together (high density). Extending this ecological relationship to evolution, a predator that evolves in a low prey-density environment should show greater improvement in searching efficiency than the same predator evolving in an environment with high prey density. To test this hypothesis, I allowed replicate populations of the microbial predator *Myxococcus xanthus* to evolve in predation arenas containing patches of *Escherichia coli* on grids at high (1 cm) or low density (2 cm). *M. xanthus* populations swarmed outward from a prey patch in the

center of each arena, and at two week intervals a cross-section of each swarm was transferred to a fresh arena. After 24 transfer cycles, the predatory ability of evolved populations was compared simultaneously to that of their ancestors by measuring the percentage of prey patches encountered by expanding swarms under identical conditions. Populations from both evolutionary treatments improved in their ability to encounter prey patches. However, these improvements were greater and more consistent in populations that evolved at low prey density. Further experiments compared swarm expansion rates in the low-nutrient environment that existed between prey patches. On average, populations from the low-density evolution treatment swarmed about 7-fold faster than the ancestor in this environment, whereas those from the high-density treatment swarmed only about 2-fold faster, indicating greater evolutionary improvements in searching efficiency when prey patches were farther apart. In addition, all sixteen evolved populations were much worse than the ancestor at forming fruiting bodies, suggesting a trade-off between predatory adaptation and developmental proficiency.

Introduction

The efficiency of a predator is determined both by its own capabilities and features of its environment, such as the distribution of prey or abiotic variables (Holling 1959; Pitt and Ritchie 2002; Stenseth et al. 2004). Evolution can alter this relationship by changing the capabilities of predators. To the extent that natural selection is involved, it may be possible to make general predictions about the likelihood and direction of evolution of particular predatory traits depending on what features of the environment limit prey consumption. This information could be useful

for two reasons. First, if we can link particular variables such as prey distribution to the evolution of specific predatory traits, this will increase our knowledge of the causes of natural selection (Endler 1986). Second, if it is possible to define the relationships between the ecology and evolution of predation, this could further our understanding of community structure and what causes it to change over time. Predator-prey interactions can be important determinants of community structure (Mittelbach et al. 1995; Schmitz 1998; Jurgens and Matz 2002; Rønn et al. 2002). Both theory and empirical evidence show that evolution of predators and prey can affect the structure of communities (Thompson 1998; Bohannan and Lenski 2000; Johnson and Agrawal 2003; Yoshida et al. 2003). Theoretically, predator evolution can affect the stability of predator-prey interactions (Abrams 2000). Predators may also evolve to become more or less specialized to various prey, affecting the strength of connections in foodwebs (Thompson 1998).

One way to determine if there are general relationships between predatory evolution and variables such as prey density is to compare results from observations of selection in natural populations. Evolution of predators has been documented in a variety of systems (Endler 1986; Reznick and Ghalambor 2001). For example, evolution of predatory traits in finches, crossbills, and garter snakes has been linked to spatial variation in prey defenses or temporal variation in the composition of prey populations (Grant and Grant 1995; Benkman 1999; Geffeney et al. 2002). By comparing many such studies, it might be possible to identify some common evolutionary responses to particular variables. However, relying entirely on observations of natural selection in the wild may limit the breadth of examples available because it is difficult to observe and identify the causes of natural selection in wild populations (Endler 1986). In addition, if we focus entirely on natural

populations, we are limited to exploring the specific combinations of ecological conditions that nature provides us with (Conner 2003). Thus, it is important to supplement our investigations of evolution in natural populations with experimental manipulations in the lab and field.

Another approach to identifying general relationships between prey distributions and predatory evolution is to compare natural predators to general "optimization" models. This is the approach used by foraging theory to identify behavioral adaptations to varying prey distributions (MacArthur and Pianka 1966; Stephens and Krebs 1986; Perry and Pianka 1997). Foraging theory models are based on dividing the act of prey consumption into two phases, searching and handling. Searching consists of the time it takes to find prey. Handling consists of the time it takes to capture, kill, and consume prey once they have been found. Both of these phases are limited by the capabilities of the predator, and the distribution and type of prey available (Holling 1959). Foraging models generally assume that a predator will use behaviors that maximize their energy intake given the challenges presented by the distribution of prey and its effects on searching or handling rates (MacArthur and Pianka 1966; Stephens and Krebs 1986; Perry and Pianka 1997). Generally, if a predator's characteristics fit the model, this implies that the behavior is an adaptation to varying prey distributions. The assumption that predatory traits are optimized to maximize energy intake may not always accurately reflect reality (Perry and Pianka 1997). Other factors, such as avoiding predation risk, may also have an affect on fitness and may require different traits. Therefore, when a predator does not fit the model, it is difficult to determine whether the assumption about energy intake is wrong, if the behavior is not adaptive, or if the proposed relationship between behavior and prey distribution is simply incorrect. This ambiguity is a general

difficulty with applying optimality models to infer adaptation (Gould and Lewontin 1979; Endler 1986; Stephens and Krebs 1986).

Nevertheless, these models provide explicit hypotheses about how prey distributions could influence the course of predatory evolution. Microbial systems provide the opportunity to investigate such hypotheses experimentally. With a microbial system, prey density can be controlled in the laboratory, evolution can be repeated by starting several independent populations from a single clone, and evolution can be observed over many generations (Elena and Lenski 2003). Microbial systems of phage and bacteria have been used successfully to study predator-prey ecology and evolution in a variety of conditions (Bohannan and Lenski 2000), and to test aspects of foraging theory (Abedon 1989; Abedon et al. 2003). There are also several bacterial species that prey upon other bacteria and even yeast and fungi. These organisms may use a variety of mechanisms to kill prey (Martin 2002) and could have profound effects on microbial communities, yet their ecology and evolution remain largely unexplored. I have chosen to study the effect of prey-patch density on the evolution of predatory traits in the bacterium *M. xanthus*.

M. xanthus is a soil microbe best known for the fact that cells cooperate to produce multicellular fruiting body structures and spores in response to environmental stress (Dworkin 1996). It preys by swarming through the soil matrix with gliding motility and lysing prey with secreted enzymes (Dworkin 1996). Proteases, bacteriolytic enzymes, and antibiotics secreted by *M. xanthus* may all be involved in 'handling' prey (Rosenberg and Varon 1984), which involves lysing bacteria, yeast, and fungi and breaking their components down into amino acids that can be taken up and used as food.

M. xanthus searches for prey using a combination of two physiologically distinct forms of gliding motility and multiple sensory systems that direct movement in response to the environment. The adventurous (A) gliding motility system is thought to push the cell by secreting slime out of pores onto a surface (Wolgemuth et al. 2002). Cells moving by the A-motility system may be directed towards prey clumps or dense particles through elasticotaxis, which directs cells along stress lines in a surface (Fontes and Kaiser 1999). Cells can move individually by A-motility, but movement by the social (S) gliding motility system requires close cell proximity (Hodgkin and Kaiser 1979). Movement using S-motility is accomplished through the action of two cell-surface appendages, pili and fibrils. Pili attach to carbohydrate moieties of the fibrils of neighboring cells (Li et al. 2003) and retract to pull the cell forward (Kaiser 2000). The *dif* and *che4* chemotactic systems affect the direction of movement by S-motility (Yang et al. 1998; Yang et al. 2000; Vlamakis et al. 2004). It is unclear whether these chemotactic systems function to direct cells towards prey or towards other *M. xanthus* cells (Kearns and Shimkets 1998). The *frz* system also affects movement by both motility systems (Spormann 1999; Sun et al. 2000), probably by directing cells in response to nutrient availability (Shi et al. 1993).

How might patch density affect evolution of *M. xanthus*? The marginal value theorem of optimal foraging theory predicts that a predator should exploit each prey-patch more fully when the distance between patches is greater (Stephens and Krebs 1986; Giraldeau and Caraco 2000). This theory has been applied to a variety of organisms (Stephens and Krebs 1986; Abedon et al. 2003; Thiel and Hoffmeister 2004). For example, phage mutants with a short latent period have higher fitness than wild-type at high host densities but lower fitness where hosts are sparse (Abedon 1989; Abedon et al. 2003). In *M. xanthus*, mutations in chemotaxis systems may

affect the likelihood that it will exploit a prey patch completely. McBride and Zusman (1996) observed that a mutant in the *frz* system more frequently left prey microcolonies before they were consumed than did wild-type *M. xanthus*. At high-patch densities, therefore, *frz* mutants might have an advantage over individuals with wild-type *frz* systems because they will be more likely to leave a largely depleted patch and move on to a fresh patch as the rate of prey consumption in the former patch declines.

Patch-density could also affect the evolution of a predator's searching and handling rates. Searching rate is affected both by the capabilities of the predator and the distance the predator must travel to find a patch. At high-patch densities, there is relatively little distance between prey, so searching rate is negligible and overall consumption rates depend entirely on the handling capabilities of the predator (Holling 1959). A mutation that increases handling rate will be beneficial in such an environment. At low patch-densities, on the other hand, the rate of patch consumption is limited by the searching ability of a predator (Holling 1959). A mutation that increases the searching rate of a predator without pleiotropically harming other fitness components will be beneficial in a low density environment. Therefore, we would expect more evolutionary improvements in searching at low density and greater evolutionary improvements in handling at high density. Predatory searching in *M. xanthus* could evolve by altering one or both of the gliding motility systems or any of the sensory systems that function in prey detection, including chemotaxis and elasticotaxis systems.

To determine how the density of prey patches affects the evolution of predatory traits, *M. xanthus* populations were allowed to evolve on agar surfaces covered with patches of the prey *E. coli* that were distributed as grids at either high or

low density. There was no additional resource in this environment beyond the prey, except any residual nutrients present in commercial agar. I assessed the effect of prey-patch density on the evolution of predatory traits by comparing several predatory traits in the ancestor and evolved populations. First, the rate at which patches were encountered was assessed to determine if evolved populations could reach more of the prey population than the ancestor. This encounter rate depends on how quickly the population swarms across both the buffered agar surface between patches (searching) and the prey-lawn within the patch (handling). These two predatory traits were then also quantified by measuring the swarming rate of a population on the relevant surface. The degree of clearing caused by lysis of a prey lawn was used to estimate the efficiency of prey-patch depletion. Finally, I also examined evolutionary changes in the ability to form fruiting bodies, a trait that is not required for predation but which may be affected by some genes that also underlie aspects of predation.

Methods

Evolution experiment

Sixteen independent populations of *M. xanthus* were derived from two clones, GJV1 and GJV2. GJV1 is a clone of the standard lab strain, DK1622 (Kaiser 1979). GJV2 is a spontaneous rifampicin-resistant mutant of GJV1 (Velicer et al. 1998). These populations were allowed to evolve for one year in "predation arenas", which consisted of square Petri dishes (12 cm wide, PGC Scientific) filled with 75 ml of TPM agar (1.5% agar plus TPM buffer: 10 mM Tris pH 8.0, 8 mM $MgSO_4$, 1 mM KPO_4 (Bretscher and Kaiser 1978)). A grid of patches of the prey organism *E. coli* B was laid down on top of the surface. For half of the evolved populations (4 of each

clone type), *E. coli* patches were 1 cm apart in grids (high patch-density); and for the other half, patches were 2 cm apart in grids (low patch-density). In these predation arenas, *M. xanthus* was always added to a central prey patch and allowed to swarm outward for two weeks at 32°C in an incubator that was humidified by an open pan of water near the inflow fan. All plates in the evolution experiment and subsequent assays were kept in plastic bags with slits cut in them to minimize dessication while allowing oxygen flow.

Transfers to fresh predation arenas were performed every fourteen days by scraping two perpendicular diameters of the swarm, always across prey patches, with a sterile, wooden dowel that was 1 mm in diameter. The end of the dowel was then rubbed in a central patch on the fresh predation arena. Populations were transferred 23 times for a total of 24 two-week selection cycles. For 15 of the 23 transfers, the diameters that were chosen for transfer were in the center of the swarm, including the patch that was initially inoculated. For the other 8 transfers, the diameters chosen for transfer consisted of columns of patches that were one patch away from the center. Populations were frozen at -80°C at every third transfer by scraping the entire population (minus the amount transferred) off the plate, and then depositing it in a slant containing CTT agar (1.5% agar and 1% casitone dissolved in TPM buffer (Bretscher and Kaiser 1978)) plus 5μg/ml gentamicin to kill any remaining *E. coli*. After 3 days incubation at 32°C, the population was then scraped from the slant and suspended in a solution containing 3 parts CTT plus gentamicin and 1 part 80% glycerol. This suspension was distributed into vials and frozen. To initiate each assay of predatory traits, 50 μl of freezer stock of the ancestors and each evolved population were inoculated into CTT broth and allowed to grow at 32°C with constant shaking.

Each culture was then centrifuged and resuspended in TPM buffer to a density of 1 x 10^9 cfu/ml.

Prey patches were added to the agar surface of predation arenas with a Bel-Blotter replicator (Bel-Art Products), which is a polycarbonate blotter that consists of 96 cone-shaped tips arranged to fit the spacing of a 96-well plate. Each well of a 96-well plate was filled with 150 µl of prey suspension. The blotter used capillary action to pick up some of the prey suspension and deposit a few µl of it onto the agar surface. The prey suspension was prepared by washing a culture of *E. coli* grown in brain-heart infusion broth (Difco) two times with TPM buffer and then resuspending it at a density of 1.3-1.6 x 10^{11} cfu/ml. To control for potential variation between arenas in the amount of prey added per patch, arenas were randomized prior to inoculation with the evolving *M. xanthus* populations.

M. xanthus produces a dense matrix of fibrils and pili at high population densities that causes cells to stick together (Kaiser 1979; Behmlander and Dworkin 1991). This clumping makes it impossible to estimate the population size of cultures grown on an agar surface by dilution plating and counting the number of colony forming units on selective media. Each resulting colony may represent one or many cells from the original population. In this experiment it is also difficult to estimate population sizes through microscopic counts because both *M. xanthus* and *E. coli* are gram-negative rods, and they are therefore difficult to distinguish under the microscope. For these reasons it is technically challenging to calculate the number of generations that occurred in each two-week selection cycle. A crude estimate of the number of generations can be obtained, however, by comparing the fold-change in the area covered by the population over the 14-d incubation period. I obtained this estimate by measuring two perpendicular, randomly chosen diameters of swarms of

the ancestor in several predation arenas of each density treatment. From these numbers I was able to calculate an average area of the swarm. By multiplying the average diameter of a swarm by twice the width of the toothpick (given two scrapes per transfer) I was able to obtain an average transfer area. I then divided the average area of the swarm by the average transfer area to estimate the fold change in area during each transfer cycle. When this value was multiplied by the total number of transfers, the number of generations in the low-density populations was 102, and the number of generations in the high-density populations was 123. It is likely that only a portion of the population scraped by the wooden dowel actually rubbed off the dowel into the prey patch of the next plate. Assuming that as little as one-tenth of the population scraped actually made it to the next transfer, the numbers of generations experienced by the low- and high-density populations were 182 and 203, respectively.

Assay of patch-encounter rate

To assess patch-encounter rate, 10 μl of predator suspension were added to the center patch of both high- and low patch-density predation arenas. After two weeks incubation, the number of patches touched by the swarm was counted, even if the swarm had not overtaken the entire patch. This number was divided by the number of patches on the plate (156 for high patch-density, 42 for low patch-density) to determine the percentage of patches encountered. This experiment included five temporal blocks. Each block included four replicates of each ancestral clone and one replicate of each evolved population in both high- and low-density predation arenas.

Assay of searching and handling

Searching and handling were measured as the rates of swarm expansion on TPM agar and on a lawn of *E. coli* distributed on TPM agar, respectively. These surfaces were chosen because they are the same as those between prey patches (TPM agar) and within a prey patch (prey lawn). To obtain a swarm expansion rate, 10 µl of predator suspension were added to the center of a plate, and two perpendicular diameters of the swarm were measured after 3 and 14 days of incubation. This experiment consisted of two temporal blocks with two replicate plates of each evolved population on each surface per block. There were also eight replicate plates of each ancestor on each surface per block.

Assay of prey-lysis efficiency

Prey-lysis efficiency was assessed as the degree of clarity in a dense *E. coli* prey lawn caused by inoculation of either an evolved *M. xanthus* population or the ancestor. A preliminary experiment showed that the clarity in the prey lawn was strongly and inversely correlated with the density of surviving *E. coli*. Five µl of suspension of each evolved population were spotted onto a dense *E. coli* lawn overlaid on buffered agar. Four spots of each ancestral clone were also included on the same plate. Evolved populations were arranged in columns on the plate with spots of their ancestor in a column between the evolved populations from each treatment. After 19 hours of incubation, the plates were photographed. The photographs were converted to black and white images, and the mean grey value of each spot was measured using the software image J (Rasband 2004). These numbers were

converted to "uncalibrated OD's" (optical densities) by the software. To control for variation in the density of *E. coli* across the plate, the OD of the surface between the spot of each evolved population and its ancestor was subtracted from the OD of the evolved population. For measurements of the ancestor, the mean of the OD on either side of the ancestor spot was subtracted as a control. This experiment was repeated five times, with two plates per repetition that were averaged prior to analysis.

Assay of fruiting-body morphology

To characterize fruiting-body morphology, each evolved population and the two ancestral clones were added to the central patch of high- and low-density predation arenas. After 14 days incubation, the central patch and occasionally other prey patches within the swarm were photographed under a dissecting scope at approximately 9x magnification.

Statistical analyses

All statistical analyses were performed with the SAS software package v. 8.2 (SAS Institute 2001). For patch-encounter rates, searching, handling, and lysis-efficiency, each measurement of an evolved population was divided by a different randomly chosen measurement of its ancestor from the same block in order to calculate a relative value. Each data point was then log-transformed. A t-test was used to evaluate if the mean log-ratio of a particular population was significantly different from zero, indicating evolutionary change. An effect of the evolution environment on the degree of evolutionary change in each of these traits was tested in

mixed model ANOVAs in Proc GLM with the following basic model: log-ratio of trait of interest = EvolEnv + Block + Ancestral marker type + Population(EvolEnv x Marker). The term 'EvolEnv' indicates the patch-density treatment effect and 'Marker' indicates the effect of being descended from GJV1 versus GJV2. 'Population' refers to any effect caused by the populations themselves, irrespective of density treatment. Parentheses indicate that the population is nested within the interaction between EvolEnv and Marker. The effects 'Block' and 'Population(EvolEnv x Marker)' were modeled as random effects while 'EvolEnv' and 'Marker' were fixed effects.

Results

Evolutionary changes in patch-encounter rate

Sixteen independent populations of *M. xanthus* were allowed to evolve in a patchy prey environment for one year. Eight of these populations evolved in a high-density environment (Figure 1a), and eight evolved in a low-density environment (Figure 1b). The common ancestor of these populations was able to encounter many more patches in the high-density environment compared to the low-density environment in the two-week interval between transfers (Figures 1c and d). I sought to determine if the evolved populations had improved in their ability to encounter prey patches, and if either environment had increased the likelihood of this evolutionary outcome. This measure could serve as an indication of fitness, because the number of patches encountered should determine the amount of food the bacteria consume, and hence their rate of reproduction. To determine if changes had occurred, the evolved populations and their ancestors were placed in both high- and low patch-

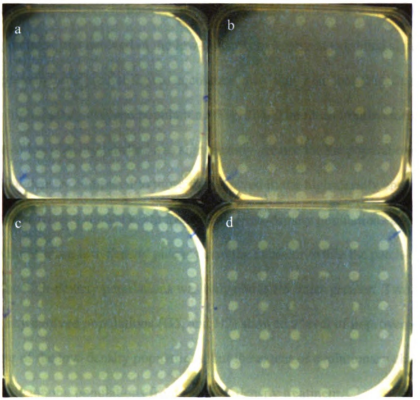

Figure 1. Ancestral conditions in predatory evolution environments. GJV1 was added to a central patch in each evolution environment. Photos were taken after 1 day of swarming at high (a) and low (b) patch density and again after 14 days of swarming at high (c) and low (d) patch density. Each plate consisted of buffered (TPM) agar which was overlaid with thick patches of *E. coli* (see text for details). Images in this dissertation are presented in color.

density environments for two weeks, at which point the percentage of patches reached by the swarm was calculated.

Figure 2 shows the percentage of patches encountered by each of the 16 evolved populations in both environments relative to their ancestor. All eight populations that evolved in the low-density environment exhibited significant improvement in their ability to encounter low-density patches (Fig. 2a), as did two of the high-density evolved populations (Fig. 2b). The mean improvement in encounter rate of low-density patches was significantly greater for the populations that evolved at low-density compared to those that evolved in the high-density environment (Table 1, EvolEnv, $p = 0.0001$). On average, the low-density populations encountered about 4.5 times more low-density patches than the ancestor, while the patch-encounter rate of the high-density populations was only about 1.8 times greater. Two of the high-density evolved populations (H5, and H7) showed a level of improvement similar to some of the low-density populations, but the extent of evolutionary change varied greatly among populations from the high-density treatment.

Table 1. Mixed model ANOVA of effect of evolution environment on degree of evolutionary improvement in ability to encounter prey patches in low-density environment.

Source	df	SS	MS	F	*p*
EvolEnv [2]	1	3.604	3.604	28.71	0.0001
Marker[2]	1	0.733	0.733	5.84	0.0311
Population(EvolEnv x marker)[1]	13	1.632	0.126	2.69	0.0048
Block[1]	4	0.561	0.140	3.00	0.0251
Error	60	2.800	0.047		

[1] Modeled as random factors in proc GLM

[2] Population(EvolEnv x Marker) Mean Square term served as error

For most populations, evolution led to only a slight improvement in the ability to encounter patches in the high-density configuration. (Figs. 2c, d). Seven of the eight low-density populations and seven of the eight high-density populations showed a directional trend toward improvement in patch-encounter rate, but these evolutionary trends were small in magnitude. In only four cases was the mean change significantly different from zero. Nonetheless, it is highly unlikely that 14 out of 16 populations would evolve in the same direction by chance alone (binomial test, one-tailed, p = 0.0021). This overall pattern suggests that there is an evolutionary trend towards improvement in encountering high-density patches. However, there was no evidence that the density of patches during evolution had affected the magnitude of improvement in encountering high-density patches. The mean improvement in the low-density populations was slightly higher than in the high-density populations (1.4- and 1.2-fold improvement for low and high-density populations, respectively), although this difference was not significant (Table 2, EvolEnv, p = 0.2888).

Table 2. Mixed model ANOVA of effect of evolution environment on degree of evolutionary improvement in ability to encounter prey patches in high-density environment.

Source	df	SS	MS	F	p
EvolEnv [2]	1	0.066	0.066	1.22	0.2888
Marker[2]	1	0.342	0.342	6.38	0.0253
Population(EvolEnv x marker)[1]	13	0.697	0.054	6.89	<0.0001
Block[1]	4	0.145	0.036	4.68	0.0024
Error	60	0.467	0.008		

[1] Modeled as random factors in proc GLM

[2] Population(EvolEnv x Marker) Mean Square term served as error

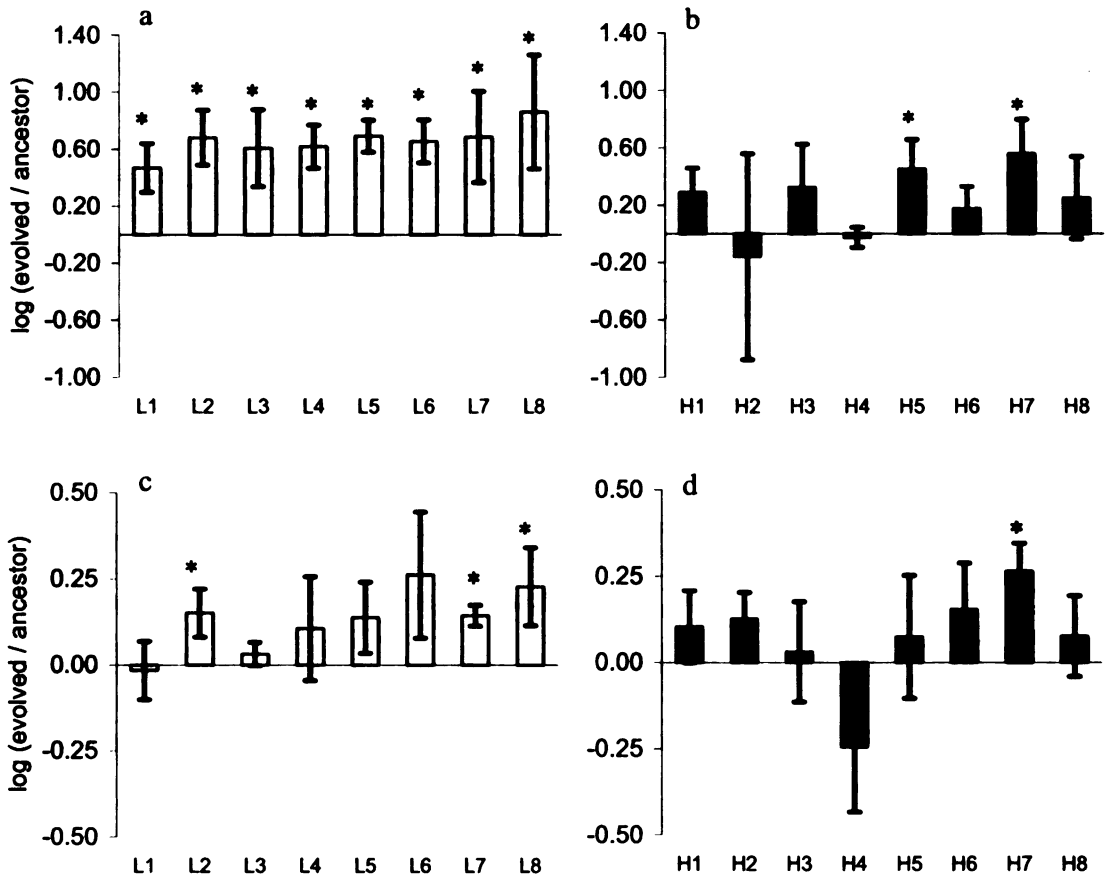

Figure 2. Patch-encounter rates of the evolved populations in comparison with their ancestor. Each bar represents the mean log-transformed ratio of the patch-encounter rate of the indicated evolved population relative to its ancestor. If the evolved population is superior to the ancestor, the log-ratio will be a positive number. If they are equal, it will be zero. Error bars indicate 95% confidence intervals, asterisks (*) indicate that the mean log-ratio is significantly different from zero after a sequential Bonferroni correction ($p < 0.05$ for the combination of all eight populations). a) and b) show low and high-density populations, respectively, assayed in low-density arenas. Low- and high-density populations assayed in high-density arenas are shown in panels c) and d). Notice the difference in scales between a, b and c, d.

The rate at which patches are encountered should depend on both the amount of time it takes to move across the buffered agar surface in search of patches (searching) and the time it takes to move across the prey-covered surface of each patch (handling). Thus, I set out to determine if there were evolutionary changes in these properties. Evolutionary changes in searching and handling were assessed by measuring how far a swarm of an evolved population expanded in comparison to its ancestor on the relevant surface in a defined period. To estimate searching ability, swarms were allowed to expand on buffered agar without prey. Handling ability was determined by allowing populations to swarm on a lawn of *E. coli* that was dispersed on buffered agar.

Figure 3 shows the evolutionary changes in searching and handling. All but one of the 16 evolved populations exhibited searching ability that was significantly greater than that of the ancestor (Figs. 3a, b). The 7.2-fold average improvement in searching ability for the populations that evolved in the low-density environment was significantly greater than the 2.4-fold average improvement of the high-density populations (Table 3, EvolEnv, $p < 0.0001$). This difference indicates that populations that evolved in a low patch-density environment tended to improve their searching ability to a greater extent than populations that evolved at high density.

There was also an overall trend towards improvement in handling rate over the course of evolution (Fig 3c, d). All but one population showed a trend towards improvement in handling, although the improvement was statistically significant in only one case. However, the fact that 15 of 16 populations showed a trend towards improvement in handling is itself highly significant (binomial test, one-tailed $p =$

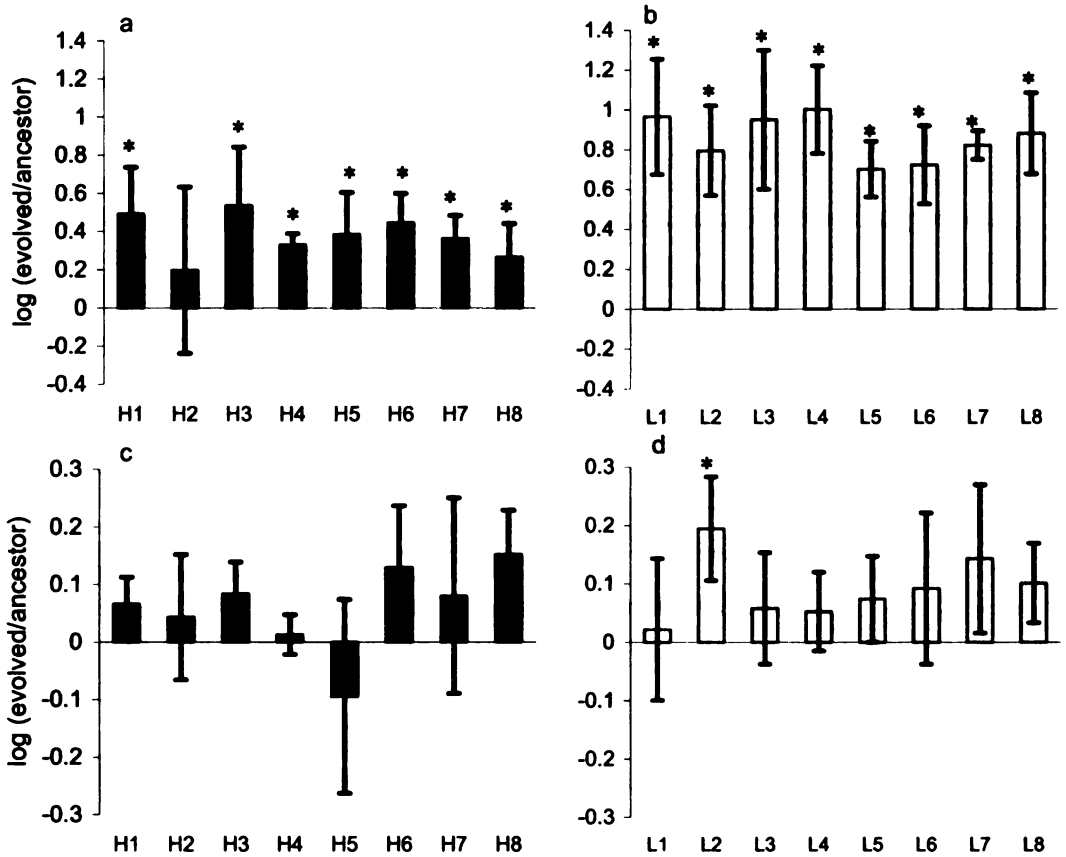

Figure 3. Evolutionary changes in searching and handling. Searching of a) high-density populations, and b) low-density populations, and handling of c) high-density populations and d) low-density populations and their ancestors were estimated by measuring the swarming rate on the relevant surface. Each bar indicates the mean log-transformed ratio of the rate measured for an evolved population relative to its ancestor. Error bars and asterisks have the same meaning as in Figure 2. Note difference in scales between a, b and c, d.

0.0003). Thus, there was an overall trend towards evolutionary improvement in patch-handling, but the extent of improvement in this trait was quite low. There was no compelling evidence that the evolution environment affected the degree of improvement in handling (Table 4, EvolEnv, p = 0.3499).

Table 3. Mixed model ANOVA of effect of evolution environment on degree of evolutionary improvement in searching.

Source	df	SS	MS	F	*p*
EvolEnv [2]	1	1.833	1.833	79.58	<0.0001
Marker[2]	1	0.057	0.057	2.49	0.1383
Population(EvolEnv x marker)[1]	13	0.299	0.023	3.83	0.0076
Block[1]	1	0.154	0.154	25.57	0.0001
Error	15	0.090	0.006		

[1] Modeled as random factors in proc GLM
[2] Population(EvolEnv x Marker) Mean Square term served as error

Table 4. Mixed model ANOVA of effect of evolution environment on degree of evolutionary improvement in handling.

Source	df	SS	MS	F	*p*
EvolEnv [2]	1	0.009	0.009	0.94	0.3499
Marker[2]	1	0.003	0.003	0.28	0.6061
Population(EvolEnv x marker)[1]	13	0.121	0.009	2.76	0.0317
Block[1]	1	0.002	0.002	0.48	0.4998
Error	15	0.051	0.003		

[1] Modeled as random factors in proc GLM
[2] Population(EvolEnv x Marker) Mean Square term served as error

Evolutionary changes in prey-lysis efficiency

In addition to moving through patches of prey more quickly, there may have been evolutionary changes in the ability of the evolved populations to lyse the prey

within patches. To test this, the evolved populations and the ancestor were spotted onto dense lawns of *E. coli* and the clarity of the lytic zone was measured from a photograph of the resulting plates. Figure 4a shows such a plate, while Figure 4b summarizes the log-ratio of evolved versus ancestral lytic zone clarity. No obvious pattern of evolutionary change in this trait was detected. Several populations appeared to be worse than the ancestor at lysing prey and a few populations were better. Three high-density populations (H4, H5, and H8) were sometimes much worse than the ancestor at lysing prey, but these differences were inconsistent across blocks and therefore not statistically significant. Also, the mean evolutionary change of the high-density populations was not significantly different from that of the low-density populations (Table 5, EvolEnv, $p = 0.3364$).

Table 5. Mixed model ANOVA of effect of evolution environment on degree of evolutionary change in prey-lysis efficiency.

Source	df	SS	MS	F	*p*
EvolEnv [2]	1	0.236	0.236	1.00	0.3364
Marker[2]	1	0.017	0.017	0.07	0.7929
Population(EvolEnv x marker)[1]	13	3.119	0.240	2.29	0.0163
Block[1]	4	1.379	0.345	3.29	0.0170
Error	57	5.974	0.105		

[1] Modeled as random factors in proc GLM
[2] Population(EvolEnv x Marker) Mean Square term served as error

Evolutionary changes in fruiting-body morphology and distribution

I examined fruiting body morphology of the evolved populations and the ancestor on both high- and low-density plates by looking at the center patch of each plate under a dissecting scope after 14 days of incubation. Photographs of the evolved populations and the ancestor on low-density plates are shown in Figure 5.

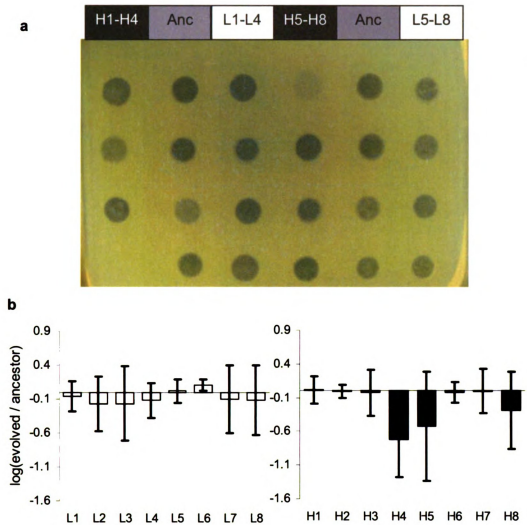

Figure 4. Evolutionary changes in prey lysis efficiency. Populations were spotted onto a dense lawn of *E. coli* and the plate was photographed after 19 h incubation at 32°C. One plate from five replicate experiments is shown in panel A. Populations that evolved at high-density (H1-H8) were spotted in columns that are marked with black boxes and low-density populations (L1-L8) are indicated with blank boxes. Columns of replicate spots of the ancestor are marked with a grey box. Panel B shows the log of the ratio of the mean spot intensity of evolved populations relative to the ancestor. Error bars and asterisks have the same meaning as in figures 2 and 3. Images in this dissertation are presented in color.

Figure 5. Fruiting bodies of evolved populations and ancestor on prey-patch plate. Photos were taken at 9x magnification of the center patch of an *E. coli* predation arena after two weeks incubation with the following predator populations: a) A1, High-density plate; b) A1, Low-density plate; c) A2, High-density plate; d) A2, Low-density plate, e) H1; f) H2; g) H5; h) H6; i) H3; j) H2; k)H7; l) H8; m) L1; n) L2; o) L5; p) L6; q) L3; r) L4; s) L7; t) L8. Unless otherwise indicated, all photos were taken on low patch-density plates. Images in this dissertation are presented in color.

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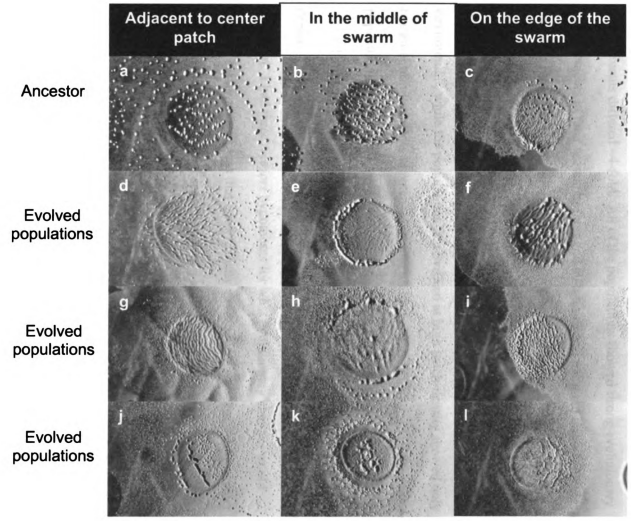
Results were qualitatively similar in the high-density environment. The ancestor produced many spherical fruiting bodies throughout the patch with a somewhat regular arrangement in the space between patches (Figs. 5a-d). The morphology and distribution of fruiting bodies of all the evolved populations differed substantially from the ancestor (Figs. 5e-t). Some of the evolved populations (e.g. Fig. 5j) did not even form noticeable cell aggregates. Others formed aggregates that were round, but not as large or as well shaped as those of the ancestor (e.g. Fig. 5m) and their spatial distribution was not as well organized. There were a few populations that formed cell clumps that were much more irregular in shape (e.g. Fig. 5o). Finally, a few populations failed to aggregate except to make one large mound of cells (e.g. Fig. 5l). Seven populations from the low-density treatment (Figs 5m-r, t) maintained the ability to form many aggregates, but only two of the high-density populations (Figs. 5f and g) exhibited much aggregation. The probability that, by chance, seven of eight low-density populations form cell aggregates when only two of eight high-density populations make aggregates is low (Fishers exact test, two-tailed, p=0.0406).

Many of the evolved populations also exhibited interesting patterns of aggregation on other patches that were encountered during the two-week incubation period. Examples of these patterns are shown in Figure 6. Many of the patterns reveal the direction of swarm migration, as seen in panels c, d, f, g, j, and l (Fig. 6). Although there are aggregates or mounds of cells in some cases (e, f, h, j, k), again there were no well-formed fruiting bodies like those of the ancestor. Together these data suggest that the evolved populations have retained the ability to aggregate to varying degrees, but they are no longer capable of making the organized fruiting bodies produced by the ancestor.

Figure 6. Phenotypes of patches not in the center of predation arenas. Photos are arranged in columns according to the location of the patch within the swarm. a) ancestor 1; b) ancestor 1; c) ancestor 2; d) evolved population L2; e) evolved population H5; f) evolved population L7; g) evolved population H8; h) evolved population L3; i) evolved population H7; j) evolved population H2; k) evolved population L4; l) evolved population L1. Images in this dissertation are presented in color.


Adjacent to center patch
In the middle of swarm
On the edge of the swarm
Ancestor
Evolved populations
Evolved populations
Evolved populations
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Discussion

Sixteen populations of *M. xanthus* evolved in either of two patchy-prey environments for a period of one year. Patch density between these two environments differed ~4-fold. All of the populations showed strong evolutionary improvements in their searching rates and all but one had marginal improvements in handling rate. The degree of improvement in searching was much greater for the populations that evolved at low patch-density. These same populations also improved significantly in their patch-encounter ability, especially in the low-density environment, while the populations that evolved at high patch-density showed only marginal improvements in patch-encounter ability overall. Finally, all of the evolved populations lost the ability to make well-formed fruiting bodies.

I allowed *M. xanthus* to evolve in two environments that differed only in patch density in order to determine how this variable might influence the course of predator evolution. I had three *a priori* hypotheses for what evolutionary changes would occur. One hypothesis was that a low-density environment would select for greater improvements in the rate of searching than a high-density environment. Reciprocally, evolution in a high density environment would select for greater improvements in the handling rate than a low density environment. A third hypothesis was that evolution in a high-density environment would be more likely than evolution in a low-density environment to select for predators that would move on to search for a new patch before having exhausted all of the resources in the first. These hypotheses are not mutually exclusive. My data were consistent with the first hypothesis, but provide little evidence for the second or third.

The pattern of evolutionary improvements in searching across density treatments suggest that it was under direct selection, and that in the low patch-density treatment it was more important than in the high-density treatment. It is highly unlikely that searching would improve in almost all sixteen populations by chance alone. Parallel improvements in searching imply that it either enhanced fitness or was genetically and functionally correlated with traits favored by natural selection. Without somehow isolating the searching trait and measuring its independent effect on fitness in the evolution environment, it is difficult to be sure which traits were under direct selection, and which were correlated. In fact, pleiotropic gene effects may make such an experiment impossible, even in principle. However, it is quite likely that searching was under direct selection, especially in the low-density environment. Among low-density evolved populations, a substantial improvement in searching, or intrinsic movement across the buffered agar surface, was correlated with a significant increase in patch-encounter rate, which should be a key aspect of the opportunity for growth and thus an important determinant of fitness. Populations that evolved in the high-density environment were also better at searching than the ancestor, but their improvement was not as great in magnitude and resulted in only a marginal improvement in patch-encounter ability. Improvements in searching therefore affected fitness, and the density of patches influenced the degree of improvement in searching. This result is consistent with the *a priori* hypothesis that searching improvements would provide a greater fitness benefit in a low-density environment compared to a high-density environment.

Evolutionary improvements in handling also occurred in parallel across almost all sixteen populations, but the pattern across density treatments is inconsistent with the second *a priori* hypothesis that there would be greater selection for handling at

high density. Improvements in handling were marginal and roughly similar across density treatments. These results indicate that efficient handling was not more beneficial in the high-density treatment than in the low-density treatment. Moreover, improvements in handling were not as correlated with improvements in patch-encounter ability as searching improvements were, so it is difficult to determine if improvements resulted from selection on handling ability or if they were selected because they were correlated with searching or loss of the ability to form fruiting bodies.

The third *a priori* hypothesis was that *M. xanthus* would evolve to exploit patches less fully in a high patch-density environment, but not in a low density environment. This hypothesis is consistent with the marginal value theorem of foraging theory (Stephens and Krebs 1986). If it were accurate, then high density populations would move through a patch more quickly and tend to lyse prey within a patch less fully. The rate of movement across a patch surface increased in almost all sixteen populations, indicating that they all probably moved through a patch more quickly than the ancestor. There were a few high-density evolved populations that also exhibited a decline in prey lysis ability, although these changes were not significant, nor were they a consistent result of evolution in the high-density environment. Thus, patch density hardly affected the evolution of patch-exploitation behavior in this experiment.

By studying the evolution of a microbial predator in a laboratory environment, I was able to identify an effect of low patch-density on the evolution of searching, a predatory adaptation. This result is important because it supports the assumption that the rate of prey consumption strongly affects fitness, and predatory traits (including behavior) will evolve so as to increase this rate. It is assumed that a predator will

maximize the rate of prey consumption with respect to prey density either behaviorally (MacArthur and Pianka 1966; Stephens and Krebs 1986) or by evolution of predatory traits (which may include behavior) (Abrams 1997; Abrams 2000). Either situation can affect predator-prey dynamics (Abrams 1992; Schmitz et al. 1997; Abrams 2000; Yoshida et al. 2003) and the structure of communities (Thompson 1998; Bohannan and Lenski 2000). Even in controlled environments, however, it is often difficult to predict what aspect of the environment will have the greatest impact on fitness and which phenotypes will change to meet an environmental challenge. Moreover, organismal physiology and morphology may sometimes constrain evolutionary outcomes (Gould and Lewontin 1979; Lenski and Levin 1985). In spiders, for example, locomotor performance is inhibited substantially by the presence of reproductive organs (Ramos et al. 2004). In the experiment reported here, the basic design of the motility motor might have imposed physical constraints, or mutations that improved searching might have negatively affected another trait important for fitness. The density-dependent evolution of predatory searching that I observed in *M. xanthus* provided support for the foraging hypothesis that searching will evolve differently across prey densities according to its effects on prey consumption rates.

Predatory searching was not the only trait that evolved in a patchy-prey environment. An unexpected but significant evolutionary change was the consistent loss of fruiting body formation ability in all sixteen evolved populations. These losses most likely occurred as a result of selection imposed by the transfer regime, but also could have resulted from potential genetic correlations between fruiting-body formation ability and predatory searching. At each transfer, a cross-section of the population, consisting of genetically diverse individuals in spore or vegetative form, were mixed together on the end of a toothpick and transferred at high density

to a fresh patch. Spores may have been at a competitive disadvantage compared to vegetative cells when inoculated into fresh prey because they would have to germinate before they could use the resource. In addition, transferred individuals were always able to start the next round of predation with a large group of individuals whether they were in a fruiting body or not. It is thought that fruiting-body formation may benefit *M. xanthus* by ensuring that a high density of spores are available to start predation when food becomes available after starvation (Kaiser 1993). Thus, the transfer regime most likely selected against fruiting body and spore formation, and may also have simply eliminated the advantage of fruiting-body formation.

Another explanation for the loss of fruiting-body formation ability is that the mutations causing improvements in searching also interfered with the process of fruiting-body formation *via* antagonistic pleiotropy. On a nutrient-limited surface such as buffered agar, fruiting bodies are produced in the center of the swarm, but these fruiting bodies are surrounded by a ring of vegetative cells. These vegetative cells are most likely to encounter fresh prey patches. A mutation that ensures a cell will be part of this vegetative ring may be favored in the patchy-prey environment. There are several categories of genes that might affect the location of cells within a nutrient-limited swarm. One category is the genes responsible for the intercellular signaling that leads into fruiting-body formation. In nutrient-limited conditions, *M. xanthus* initiates a stringent response that directs cells to arrest growth, produce the C-signal, and activate the export of proteases that hydrolyze proteins into amino acids, which serve as the A-signal (Shimkets 1999). Both the A- and C-signals are intercellular signals required to initiate fruiting-body formation and sporulation (Shimkets 1999; Kaiser 2003). A mutant with an altered stringent response system,

such that it does not respond to the A-signal or does not produce the C-signal, may not respond normally to density signals and may move away from cell dense regions towards the vegetative swarm edge.

Other genes that may affect both searching and fruiting-body formation are motility and chemotaxis genes. Mutational targets for improved motility could have included either A- or S-motility motors or loci in the systems that organize cell movement such as the *che4*, *frz*, and *dif* chemotaxis systems. There are some examples in the literature of increased swarm expansion rates that occurred in part because of mutations in motility or chemotaxis systems. In nutrient-rich media, mutations in the response regulator of the *che4* operon led to increased swarming (Vlamakis et al. 2004). Evolutionary increases in the production of fibrils led to dramatic motility improvements in some environments (Velicer and Yu 2003). Mutations in motility loci are known to also affect fruiting-body formation (Hodgkin and Kaiser 1979; MacNeil et al. 1994). In fact, several of the observed fruiting-body phenotypes were indeed similar to those previously reported for motility mutants (MacNeil et al. 1994). Thus, it is possible that evolutionary improvements in searching could have been caused by motility or chemotaxis genes and these same mutations could have pleiotropically affected fruiting-body formation.

Conclusion

The effect of natural selection is determined both by ecological variables, such as resource distribution, and by the unique features of the organism that might constrain or promote improvement in some traits (Gould and Lewontin 1979; Bohannan and Lenski 2000; Brakefield 2003). It is important to establish general

relationships between evolutionary outcomes and specific ecological or genetic properties in order to improve our understanding of adaptation and its effect on communities. Because evolution of predators and prey can affect the dynamics of their interactions (Abrams 2000; Yoshida et al. 2003) and community structure (Thompson 1998; Bohannan and Lenski 2000), it is important to understand the causes of natural selection in these systems. I was able to determine the effect of a specific ecological variable, prey-patch density, on the course of evolution of the prokaryotic predator *Myxococcus xanthus* by allowing replicate populations to evolve at two different patch densities. One expected effect of patch density on the course of evolution was repeatedly demonstrated. That is, natural selection consistently favored a greater extent of improvement in predatory searching in the low patch-density treatment than in the high patch-density treatment. However, an unexpected decline in fruiting-body formation also happened repeatedly. This result may have been caused by unexpected sources of selection in the transfer regime, by trade-offs in searching ability and fruiting-body formation, or a combination of both. In conclusion, these results support the hypothesis of general relationships between patch density and searching rate that influence the course of predator evolution. They also highlight the difficulty of making comprehensive predictions of evolutionary outcomes for any particular organism.

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