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CHAPTER 1.8

Cell-Cell Interactions

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Discovery and Distribution

Roland Thaxter published a time bomb in December 1892. He reported that *Chondromyces crocatus*, before then considered an imperfect fungus because of the morphological complexity of its fruiting body, was actually a bacterium. Thaxter had discovered the unicellular vegetative stage of *C. crocatus*; the cells he found were relatively short and they divided by binary fission, unlike the mycelium of a fungus. *C. crocatus* was, he concluded, a "communal bacterium." Thaxter described the locomotion, swarming, aggregation and process of fruiting body formation of *C. crocatus* and its relatives, which are collectively called myxobacteria, with an accuracy that has survived 100 years of scrutiny. He described the behavior of myxobacteria in fructification in terms of a "course of development" because it was "a definitely recurring aggregation of individuals capable of concerted action toward a definite end" (Thaxter, 1892). These qualities of an integrated multicellular unit clearly implied positive interaction between the cells. Existence of favorable cell-cell interactions is one hallmark of multicellular life and the theme of this essay.

Thaxter's discovery called attention to the transition from single, apparently independent cells to an integrated multicellular unit. There is general agreement that this step has been taken many times in the course of the organic evolution of plants and animals. For example, the sponges are thought to have arisen from solitary cells separately from all other animals; moreover the seed plants, the fungi, and the algae are all believed to have gained their multicellular condition independently (Whittaker, 1969). Given the apparently strong natural selection for multicellular life, it would be surprising if prokaryotes also had not adopted the multicellular condition many times, and perhaps even earlier in time.

One current estimate suggests that more than 99% of the bacteria on earth live as cell masses (Costerton et al., 1995), a condition conducive to cell interactions. As the broader significance of multicellular microbial life has been recognized, the cell interactions that facilitate multicellularity have been revealed.

Evolution of Cell Interactions

To imagine how two partners to a cell interaction could coordinately evolve high, yet complementary, specificity seems difficult. To start with a weak and poorly specific signal would seem to have little, if any, selective advantage. But what other way is there to start? The relative structural simplicity of bacteria belies their metabolic versatility and their sophistication in responding to the environment by their sensory systems. Given current knowledge of bacteria and given some advantage for an interaction, at least two evolutionary scenarios are plausible. 1) The progressive evolution of a cell-cell interaction between dissimilar cells could start with cells of one type feeding on the metabolic products of cells of another type. Mutation and selection on both partners would then optimize syntrophic growth. While this cooperative growth was taking place, the release by one cell type of a metabolic regulator for the other type would constitute a primitive signal, subject to improvement by selection. 2) Instead of metabolic cross-feeding, a cell-cell signaling interaction might evolve from an existing sensory system. The wide variety of two-component sensory, regulatory systems would be ground for such evolution (Hoch and Silhavy, 1995). Bacterial sensory systems are robust, having sensitivity and reliability required for an effective interaction (Barkai and Leibler, 1997). Release or display of a signaling factor by cells could trigger one of the sensory systems. Immediately after this event, selection would need to act only on signal production. Then the specificity of matching between trigger and sensor could be improved, or a higher level of sensitivity could evolve. 3) Bacteria have stimulons. If the output of a sensory system as in 2) or a metabolic regulator as in 1) becomes the input to a stimulon, responses would govern many genes. From cell-cell interactions, the evo-

lutionary path to timed gene expression and a circuit that "develops" in Thaxter's terms is simple. Starting with signal 1 and its response, including expression of a set of genes, let one of those genes encode production of signal 2. Iteration of that process would give something like the signaling pattern of fruiting body development in Myxobacteria Fruiting Body Development signaling. 4) Because haploid bacteria are less insulated from their environment than diploid organisms, natural selection can constantly play a role in shaping and improving the interactions between cells, speeding such evolution. Loss of cell-cell interaction in less than one thousand generations after relaxing social selection indicates experimentally the force of social selection (Velicer et al., 1998).

Types of Cell Interactions

Four kinds of cell interactions can be distinguished: 1) Transfer of a chemical signal from one cell to another. The variety of such transfers is presented in several examples below. 2) Signaling by direct physical contact between two cell bodies, which may involve their surfaces or cell appendages, such as fibrils, pili, or flagella. Direct physical contact is often involved in cell swarming. 3) Syntrophic metabolism. Because syntrophic interactions are the subject of another chapter by Schink (Syntrophism Among Prokaryotes in Volume 2), this essay will focus on the other three types. 4) Gene transfer from one cell to another. Eubacterial gene transfer interactions are widespread. Transfer within the Archaea has recently been observed, and their genetics is being developed (Stedman et al., 1999; Whitman et al., 1999). Prokaryotes have three mechanisms for unidirectional gene transfer from a donor to a recipient. These mechanisms are *transformation* in which naked DNA from the donor is taken up by the recipient, generalized *transduction* in which a phage has packaged a head-full of donor DNA and injects that DNA into the recipient, and *conjugation* in which a specialized apparatus in the donor transfers a long DNA segment directly into a conjugating recipient. The three mechanisms have different qualities: transforming DNA fragments may be 10 to 50 kb in length and are vulnerable to extracellular nucleases. Competence of the recipient to accept DNA may be restricted to certain nutritional states: *Bacillus subtilis* growing in minimal salts with glucose and amino acids develops competence postexponentially (Dubnau, 1991), but growing without amino acids, exponential cells are competent when two peptide pheromones are added (Lazazzera et al., 1999). In *Haemophilus influenzae*, competence is restricted to exponential cells after nutritional downshift in a medium that supports protein synthesis but not cell division (Herriott et al., 1970; Kahn et al., 1983), and *Streptococcus* cells are competent in the presence of a secreted competence peptide (Havarstein and Morrison, 1999). The *Bacillus* and *Streptococcus* peptides may be quorum sensors.

Transduced DNA fragments may range up to 100 kb, a limit imposed by the capsid volume of the transducing phage. Although the DNA is shielded from nuclease attack by the capsid proteins, the host range of the phage limits transduction. That typically narrow range is set by the distribution of the adsorption receptor for the phage among bacteria. Phage P1 is an exception because its receptor is the common lipopolysaccharide (LPS) core lipid (Lindberg, 1973), which suggests that practically all Gram-negative bacteria could be P1 recipients (Murooka and Harada, 1979). Transfer is by syringe-like injection that is triggered by phage adsorption (of P22 or P1, for example), or the virus may enter the cell intact and release its DNA once inside (M13 is an example).

In conjugation, most or all of the donor chromosome may be transferred. Conjugal DNA transfer is mediated by physical contact and a multi-molecular machine, consisting of many proteins. Conjugation mediated by F-plasmids begins when the F-pilus binds to the ompA protein on the surface of an *Escherichia coli* recipient (Manoil and Rosenbusch, 1982). A mating signal is sent back to the donor cell (Kingsman and Willetts, 1978), the donor and recipient are then drawn closely together, and an electron dense junction forms at the cell-cell interface (Durrenberger et al., 1991). DNA is then transferred with synthesis in both cells. After transfer and chromosome segregation, the cells actively separate (Achtman et al., 1978; Durrenberger et al., 1991). More than 15 proteins may be in a conjugation machine, including the pilus proteins. This degree of structural complexity suggests a strong selective advantage for conjugation.

Gene transfer by any of these mechanisms may be lateral (in which transfer is from one cell species to another) as well as vertical (in which the donor and recipient belong to the same species). Whereas transduction is restricted to certain members of particular species, transformation and conjugation may have very wide ranges that facilitate lateral gene transfer. Broad host-range plasmids such as RSF 1010 (Frey and Bagdasarian, 1989) can transfer between a wide variety of bacterial species, and RP4 can even transfer to *Saccharomyces cerevisiae* (Heineman and Sprague, 1989). The transkingdom transfer by RP4 requires its five *tra* genes (*tra FGIJK*)

and ten *trb* genes (*trb BCDEFGHIJ* and *L*; Bates et al., 1998), which include the pilin gene, *trb C*. Moreover, in nature, a sector of the pTi plasmid laterally transfers from *Agrobacterium* to a variety of plant cell species, thereby producing crown gall tumors.

Widespread infectious transfer of drug resistance between bacterial species is circumstantial evidence for lateral transfer (Mazodier and Davies, 1991). But recently, as a consequence of whole genome studies and of the construction of phylogenetic trees based on particular proteins, discrepancies between trees have suggested that lateral transfer may be more general. A strong case for the transfer of the gene for glucose-phosphate isomerase from a plant to *E. coli* has been made (Smith et al., 1992). Pathogenicity islands in, for example, *Salmonella*, are large clusters of virulence genes not present in related but benign organisms, and they appear to have been transferred from another organism(s) (Baumler, 1997; Groisman and Ochman, 1997). Ongoing lateral transfer of the retrons that encode production of multicopy single-stranded DNA is evident in *E. coli* and other enteric bacteria. Ten percent of *E. coli* clinical isolates are found to have them, and most retron isolates in these strains are different (Herzer et al., 1990). The retron Ec107 may be spreading from one strain of *E. coli* to others (Lampson, 1993). By contrast, retrons are ubiquitous in the Myxobacteria, and all members of the myxococcus subgroup have a version of Mx162, while members of the other subgroup lack it (Rice and Lampson, 1995). The retron element Mx162 apparently transferred laterally into the common myxococcus ancestor about 100 million years ago and has subsequently been inherited vertically (Rice and Lampson, 1995).

Lateral transfer requires a mechanism for DNA integration that does not depend on perfectly homologous recombination. Transposons such as Mu, Tn*5*, or Mariner insert within short target DNA sequences, and their transposases are thus present in bacteria. The Mariner target, for example, may simply be the AT dinucleotide (Hartl et al., 1997). Site-specific and other nonhomologous recombination mechanisms are also common in bacteria where they are associated with repair of damage to DNA and help preserve genome integrity.

Chemical Signals and Direct Physical Contact

Some themes recur frequently. Certain small molecules like amino acids and their derivatives are frequently used as signal molecules. Examples are homoserine lactones and peptides shorter than ten amino acids. Multicellular sensing is more accurate than unicellular and may be able to compensate for cell-to-cell variations in metabolism, transcription and translation. One example is the A-factor of *M. xanthus*, which is a mixture of 6 amino acids and is a cell-density signal (Kuspa et al., 1992a). The amount of A-factor released is proportional to the number of cells per unit volume, and a certain minimum quantity of A-factor is required to continue development. Thus the A-signal ensures a cell density sufficient to complete a proper fruiting body (Kuspa et al., 1992b). A-factor, which is released about 2 hours after the beginning of starvation, is also a way for cells to vote their individual assessment of nutritional conditions. Since new proteins must be made during aggregation and sporulation, some protein synthetic capacity must be retained, and the cells must begin to aggregate before they have exhausted all their sources of amino acids and energy. To initiate development or to grow slowly is an important choice on which long-term survival depends. An optimal choice is one that anticipates the future. A decision jointly made by a population of cells rather than by one cell is likely to be more reliable. Multicellular, rather than unicellular, feeding on polymeric substrates is more efficient when extracellular hydrolases are employed for digestion.

One example is the social feeding in myxobacteria. The selective advantage for the evolution of multicellularity in myxobacteria is likely to have been cooperative feeding. Myxobacteria feed on particulate organic matter in the soil by means of extracellular bacteriolytic, proteolytic, cellulolytic, and other digestive enzymes (Reichenbach, 1984). Based on their secretion of lytic enzymes, Dworkin (1973) likened myxobacteria to "packs of microbial wolves." Rosenberg et al. (1977) measured the growth rate when the only source of carbon and nitrogen for *M. xanthus* cells in liquid culture was the polymeric substrate casein and found that proteolysis was required for growth. A two-fold increase in growth rate was observed as the cell density was raised above 104 cells/ml. When intact casein was replaced with hydrolyzed casein, the cells grew independent of cell density at the more rapid rate. Evidently extracellular digestion of protein is enhanced by cooperation between cells, akin to syntrophism.

A swarm may be the unit of efficient cooperative feeding. Reichenbach has shown that a single germinating sporangiole of a *C. apiculatus* fruiting body forms an active swarm that behaves much like a swarm of bees (Bonner, 1952; Kuhlwein and Reichenbach, 1968; Quinlan and Raper, 1965). Forming a multicellular fruiting

body ensures that, when conditions favorable for growth are restored, the myxospores can germinate, and the new phase of growth can start as a pre-formed community of efficiently feeding cells. The success of the myxobacterial design is evident in their distribution; they are common inhabitants of soils drawn from all over the world regardless of climate (Reichenbach, 1984).

Multicellularity and cell density effects are prominent in starvation or nutrition-limiting states. Starvation requires management of scarce resources, and for that reason, requires sharper, more accurate perceptions. Examples include sporulation, biofilms, and fruiting bodies.

Possible Strategies for Detecting Signals

Common themes suggest the following possible strategies for detecting signals: 1) Look for cell-density effects (Lazazzera et al., 1999; Rosenberg et al., 1977). Test the response of low-density cells to high-density conditioned medium. 2) Look for signal-production defective mutants that can be rescued by co-cultivation with wild type cells. Such mutants can be divided into classes by mixing mutants with each other, possibly identifying different signal specificities (Hagen et al., 1978). 3) Isolate signal-response-defective mutants (Gorski and Kaiser, 1998). For this purpose, reporter gene constructs are likely to be useful. 4) Signal molecule bioassays can be built upon the provision of signal molecules in crude form (Hastings and Greenberg, 1999). 5) Starvation survival strategies often involve cell interactions. 6) Interactions are common among cells in biofilm communities (Davies et al., 1998). 7) Two-component sensory systems may perceive intercellular signals.

Examples of Interactions

These include: 1) Autochemotaxis; 2) Vibrio, Squid, Lux, autoinducer; 3) Microbial Biofilms in this Volume; 4) Agrobacterium and crown gall; 5) Rhizobium and nodules; 6) Myxobacteria and fruiting bodies: A-signaling and C-signaling; 7) Pheromone-inducible plasmid transfer in *E. faecalis*; 8) Swarming, type IV pili, twitching and gliding; 9) Heterocysts in filamentous cyanobacteria (Anabaena); 10) Sporulation in *B. subtilis*; 11) Syntrophism Among Prokaryotes in Volume 2.

Aggregation by Autochemotaxis

A striking illustration of the power of a single cell-cell interaction to produce complex cellular patterns is shown in Figure 1. This pattern of aggregates of *E. coli* cells developed in 3 days following a single inoculation made in the center of a plate containing succinate and a small amount of methylaspartate.

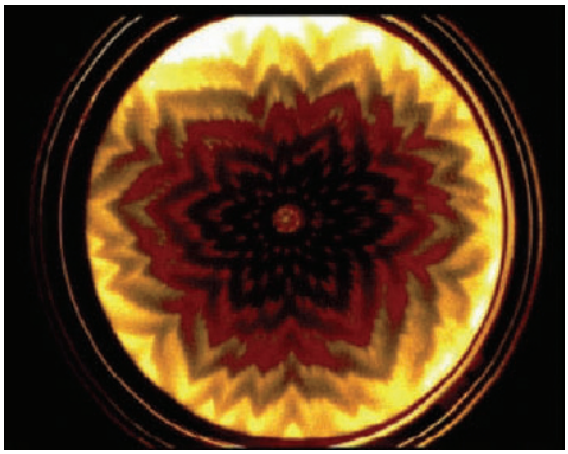

Fig. 1. Organized *E. coli* cells in a pattern generated by autochemotaxis. This pattern of cell aggregates developed in 3 days from a single inoculation made in the center of a plate. Color added for contrast. From Budrene and Berg, 1995.

When chemotactic *E. coli* cells are inoculated near the center of a Petri plate containing semi-solid agar, they swarm outward in concentric bands (Adler, 1966; Adler, 1969). The cells swim in semisolid (0.3%) agar almost as if it were water. Concentric bands form because of chemotactic responses to spatial gradients generated by transport and metabolism. As the cells take up metabolizable attractants from a complex medium, they grow, divide, and generate spatial gradients of attractants, which they subsequently chase. This leads them to swarm outward in concentric bands.

In medium rich in amino acids, a leading band of *E. coli* cells consumes most of the aspartate aerobically. The Tar methyl-accepting chemotaxis protein detects aspartate (Macnab, 1987), which binds to the extracellular domain. This binding then triggers the phosphorelay cascade of chemotaxis (che) proteins. The cascade signals the proteins at the base of the flagellum, which cause the cell either to continue swimming in the same direction or to tumble and swim in an arbitrary new direction. That the aspartate ring of a swarm depends on chemotaxis has been shown with a series of mutant strains made deficient in one of four chemotaxis receptors or in one of the cytoplasmic chemotaxis proteins (Wolfe and Berg, 1989). The spreading of chemotactic bands has been shown to require the entire regulatory network for chemotaxis, including desensitization and resensitization of the receptor by methylation and demethylation of its highly conserved carboxy tail located in the cytoplasm.

Formation of concentric bands and spots requires a particular set of metabolic conditions, unlikely to be found in nature, but instructive nonetheless. Growing in minimal medium on one of the more highly oxidized dicarboxylic acids of the tricarboxylic acid (TCA) cycle (e.g., 5 mM succinate), *E. coli* forms into a pattern of small, compact, focal aggregates, like those in the center of Fig. 1. If the concentration of succinate is doubled, the pattern becomes one of concentric rings. The pattern begins to form in the central part of the colony, then spreads sequentially outward.

The pattern depends on chemotaxis toward aspartate: the Tar receptor and all the downstream components in the chemotaxis phosphorelay are necessary (Wolfe and Berg, 1989). Ring formation can be suppressed by adding saturating amounts of chemicals that are sensed by the aspartate receptor (e.g., the nonmetabolizable aspartate analog, a-methyl-aspartate), implying in this case that a (self-generated) gradient of aspartate is involved. Formation of a focal pattern requires a high initial cell density in a ring; these cells then redistribute into foci (Budrene and Berg, 1995). Addition of succinate causes the cells to excrete aspartate. (A secretion of 4×10^{-17} moles of aspartate per cell has been measured.) Succinate would be oxidized by the TCA cycle to fumarate that in turn would be aminated to aspartate, some of which is released from the cell. A swarm ring forms when an aspartate gradient is established at the periphery of the spreading colony. The process of focal aggregate formation from a ring has been observed to continue in both directions around a ring until a complete set of aggregates has formed. Cells in the aggregates can be seen moving around, like a swarm of bees that are held together by their mutual attraction. While appearing to be colonies, they are swarms. By changing substrate concentrations and strains, a series of related patterns have been obtained. The formation of swarm rings and focal aggregates has been subjected to a thorough physical-mathematical analysis (Brenner et al., 1998). Thus, remarkable cellular patterns can be generated by chemotaxis to a single diffusible substance released by the cells themselves.

Autoinducer and Quorum Sensing

One of the earliest cell-cell interactions to be investigated is found in the symbiotic luminous vibrios. In nature they inhabit the light organs of certain marine bony (monocentrid) fishes and the bobtail squids (Nealson and Hastings, 1979). The bacterial light is crucial to the survival of these squid in their coastal water habitat. The squid's usual black ink trick is not very effective at night when the squid forages. Instead they emit light from their cultivated bacteria. This light helps camouflage the squid from their predators, which live below them on the floor of the relatively shallow coastal waters. This emitted light erases the shadow cast by the squid from the moonlight and starlight shining down on them from the night sky. A sophisticated "stealth" technology this, in which the squid by means of an iris and lens adjusts the intensity of light to match the light from above.

Luminous *Vibrio fischeri* release energy when they jointly oxidize $FMNH_2$ and tetradecanal with molecular O_2 (Meighen, 1994). The oxidation energy is emitted as fluorescent light, and luciferase catalyzes the oxidation coupled to light emission.

Light production by *Vibrio* is unusual in that the intensity of light produced per cell increases with cell density (Hastings and Greenberg, 1999). In dilute cultures, each cell is very dim. As their cell density is increased, however, the amount of light produced per cell rises as much as 100-fold. During this density-dependent induction, luciferase protein as well as the enzymes that synthesize the aldehyde substrate for luciferase increases. This augmentation is associated with release into the medium of a small molecule, β-ketocaproyl homoserine lactone, the *V. fischeri* autoinducer, or VAI (Eberhard et al., 1981).

An amide bond links homoserine lactone to an acyl chain, which arises from fatty acid biosynthesis.

Why should cells become bright when they have grown to high density and stay dim at low cell density? Light production requires a large amount of chemical energy to synthesize the fatty aldehyde and to keep FMN reduced. The idea is that the luminescent vibrios live two different lives: one when they are growing in the open sea, another when they are populating the light organ of an animal.

Since luminescence is energetically expensive, energy that could be used for growth in the free-living state would be squandered by emission of light. Life in the ocean is hard; carbon, fixed nitrogen, and energy sources are all difficult to find. Thus, the typical cell density of *V. fischeri* in seawater is low, perhaps 10 cells per ml.

A different economy prevails within the light organ of a squid or fish. There, *V. fischeri* reaches very high cell densities of 10^{10}–10^{11} cells per ml;

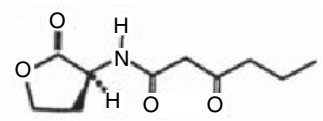

Fig. 2. The structure of *V. fischeri* autoinducer β-ketocaproyl homoserine lactone, or VAI. From Eberhard et al., 1981.

there it is luminescent. The animal pays the energetic cost by feeding its vibrios carbon and fixed nitrogen, allowing them to grow to very high density. The animal pays willingly because in return for food, *V. fischeri* makes the light which allows the animal to escape its predators.

Because of the low density of *V. fischeri* cells in sea water where they are nutrient limited, the extracellular autoinducer concentration never builds up to a significant level, and thus *V. fischeri* is not luminescent in its free-living state. Autoinduction may be viewed as an intercellular signaling system that allows *V. fischeri* to discriminate between a free living, low-density state and a host-associated, high-density state. Accordingly, the luminescence genes are activated only when a host is there to pay the high energy price of light production (Ruby and McFall-Ngai, 1992). The minimum number of bacteria necessary for high level luminescence in the light organ is thought of as a "quorum." This mechanism of regulatory discrimination between low density in the sea and high density in the light organ exemplifies the concept of quorum-sensing (Fuqua et al., 1994).

The autoinducer (VAI) and the regulatory response to its presence in the medium discriminate between the low- and high-density states. Two divergent lux operons, as shown here, have this purpose in *V. fischeri*.

The structural genes responsible for the synthesis and activity of luciferase (*luxA* and *B*), of tetradecanal (*luxC*, *D*, and *E*; Engebrecht and Silverman, 1986), and of the autoinducer synthase (*luxI*) make up one operon in *V. fischeri*. The substrates for autoinducer synthase are S-adenosylmethionine, which donates its methionyl portion, and the appropriate fatty acid conjugated to acyl carrier protein, which is an intermediate in fatty acid biosynthesis. The transcription unit is regulated by *luxR* (Figure 3), which encodes a DNA-binding protein that mediates the effect of autoinducer (Kaplan and Greenberg, 1987; Shadel et al., 1990). Gene *luxR* is divergently transcribed and separated by 155 bp from the *luxI* operon. Between these two transcription units lies a 20 bp inverted repeat, the "lux box," a sequence consensus element which has been found ahead of lux promoters in several organisms. The box is required for activation of both leftward and rightward transcripts and is thought to be the site of LuxR protein binding.

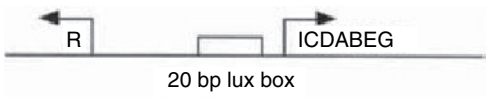

Fig. 3. Map of the two divergently transcribed lux operons. From Stevens and Greenberg, 1999.

The autoinducer is produced at a low constitutive rate during the early stages of *V. fischeri* growth. Once the cell density rises above a critical level, and the concentration of VAI in the medium rises to such a level that its intracellular concentration is high enough to bind LuxR, expression of the lux/CDABEG rises and there is luminescence. Because *luxI* is part of this transcription unit, a positive feedback loop is established which induces higher levels of VAI and locks in the luminescent state (Engebrecht et al., 1983).

Lux R protein is cytoplasmic and has no hydrophobic α-helical regions typical of membrane-spanning proteins. *V. fischeri* cells are freely permeable to acylated homoserine lactone, which is hydrophobic (Stevens et al., 1994). The acylated homoserine lactone (autoinducer) apparently enters and exits cells by passive diffusion through the membrane. Hydrophobic signal molecules have a double advantage: they require neither a specialized secretor nor a specialized transporter for uptake. Then why doesn't a cell signal itself with its own autoinducer? The cell does but the signal is too weak—the cell has no way to retain its "own" autoinducer. A hydrophobic signal molecule that is membrane diffusible in both the signal donor and the signal receiver is a starting point from which a plausible step-by-step Darwinian path could lead to highly specific signal molecules and to cognate and highly specific transmembrane receptors. In addition to the autoinducers of luminescent marine *V. fischeri* and *V. harveyi*, other acylhomoserine lactones are produced by a diverse and growing number (25 could be listed in 1997) of different terrestrial Gram-negative bacteria.

The LuxI homologs in all these organisms direct the synthesis of acylhomoserine lactones (HSL) with saturated or partly unsaturated acyl chains of 4 to 14 carbons, having substitutions at C-3 of either a hydroxyl group or a carbonyl group. Species specificity is provided by the acyl chain—its length, the oxidation state of C-3, and the degree of unsaturation of the hydrocarbon chain. All such acylated HSL compounds are expected to be membrane permeable, as has been demonstrated for β-ketocaproyl homoserine lactone (Kaplan and Greenberg, 1985). Various HSLs regulate diverse physiological processes including bioluminescence, swarming, antibiotic biosynthesis, plasmid conjugal transfer, and the production of exoenzyme virulence determinants in human, animal, and plant pathogens.

Biofilms

In natural aquatic environments, most bacteria are to be found within biofilms, which is the sub-

ject of the chapter by de Beer (Microbial Biofilms in this Volume). Biofilms are polysaccharide-enclosed bacterial masses adhering to a surface, the polysaccharide having been secreted by the bacteria themselves. As the bacteria grow and divide following their initial colonizing adhesion to a surface, the cells may cluster in pillar- and mushroom-like structures (Costerton et al., 1995). A three-dimensional structure, penetrated by anastomosing channels, is believed to provide a circulatory system that brings oxygen and other nutrients in while it flushes metabolic wastes out. Under favorable conditions, a biofilm can achieve a relatively high local density of cells, and the point here is that those cells can interact with each other. Cells within a biofilm are found in different regulatory states from their planktonic siblings, in part due to cell-cell interactions. Cells in the biofilm may be more antibiotic resistant and more exuberant in polysaccharide production and in enzymes for exopolysaccharide biosynthesis, such as alginate (Costerton et al., 1995).

Biofilms form on human epithelia bathed in nutrient-rich fluids. Many human bacterial infections occur in biofilms, such as common dental plaque leading to tooth decay and periodontal disease. *Pseudomonas aeruginosa* can form a biofilm on the skin of burn victims. Such a film tends to shield the bacteria from otherwise protective antibodies. At least one cell-to-cell signal molecule is necessary for the normal development of *P. aeruginosa* biofilms (Davies et al., 1998). The small molecule, *N*-(3-oxododecanoyl)-L-homoserine lactone, is essential for exopolysaccharide production by the Pseudomonad. Initial attachment of cells to a fresh surface requires motility and type IV pili (O'Toole and Kolter, 1998) but not the homoserine lactone. The lactone signal is needed in the subsequent step of biofilm differentiation. Two different acyl-homoserine lactones are produced by *P. aeruginosa*, which are specified by the *lasI* and *rhlI* genes. Only the first is required for biofilm differentiation as shown by specific mutant knockout and by rescue of the mutant with pure *N*-(3-oxododecanoyl)-L-homoserine lactone (see Autoinducer). There is no evidence for chemotaxis; the circumstances of rescue tend to rule out a gradient of the acyl-homoserine lactone. Wild type cells but not *lasI* mutants build a biofilm in which the cells are resistant to the detergent sodium dodecyl-sulfate (SDS). Resistance to SDS can be rescued in the *lasI* mutant by adding pure *N*-(3-oxododecanoyl)-L-homoserine lactone. Thus, cells within a biofilm can be functionally integrated with each other as well as physically attached to a surface. At least one cell-cell signal is required for the multicellular regulatory state of *P. aeruginosa* in a biofilm. The biofilm also integrates metabolic interactions between the cells through the polysaccharide matrix, growth substrates, and waste elimination.

Agrobacterium and Crown Gall

Agrobacterium tumefaciens, a plant pathogen, produces crown gall tumors. Agrobacteria are able to grow within the tumor as a consequence of both genetic and physiologic interactions with plant cells and of genetic interactions among the bacteria.

Virulent agrobacteria harbor a large plasmid called Ti (for tumor inducing). Responding to signal molecules released by wounded plants, a set of virulence (vir) genes on pTi are expressed. The proteins encoded by these genes process and transfer a segment of the Ti plasmid (the T-DNA) from the bacterium to the susceptible plant during infections (Kado, 1998). Following transfer, the T-DNA integrates into the nuclear genome of the plant cell. Expression of some genes on the integrated DNA results in an oncogenic transformation of the plant cell by means of phytohormone induction. The tumor cells also produce novel low-molecular-weight compounds called opines, whose synthesis from normal intermediary metabolites is directed by genes expressed from the T-DNA. Although the genes for opine biosynthesis are present on a bacterial replicon, they are expressed properly only in plant cells, a remarkable evolutionary adaptation (Hong et al., 1997). The genes for opine biosynthesis lack identifiable bacterial promoters but contain 5′ and 3′ sequence motifs characteristic of plant transcriptional signals. In turn, the causative bacteria can utilize the opines produced by the tumors as a source of carbon and energy, and nitrogen in some cases. Thus, *A. tumefaciens* redirects plant cell metabolism to produce specific metabolites which the bacterium can use as growth substrates.

The opine released by the host plant induces production of 3-oxo-octanoyl-HSL (Zhang et al., 1993). In turn, the HSL enhances the transfer of Ti plasmid from *A. tumefaciens* cells that carry the Ti plasmid to any non-carrier *Agrobacteria* within the tumor. The plasmid carries genes for the breakdown of a particular opine; Ti plasmid T-DNA, which encodes plant proteins involved in the biosynthesis of opines in crown gall tumors, is matched by Ti plasmid genes, which confer on the bacterium the ability to take up and utilize the particular set of opines produced by the tumor. For example, tumors that produce octopine, an arginine derivative, contain bacteria that specifically degrade octopine, while tumors that produce agropine contain bacteria that specifically degrade that agropine. Conjugation

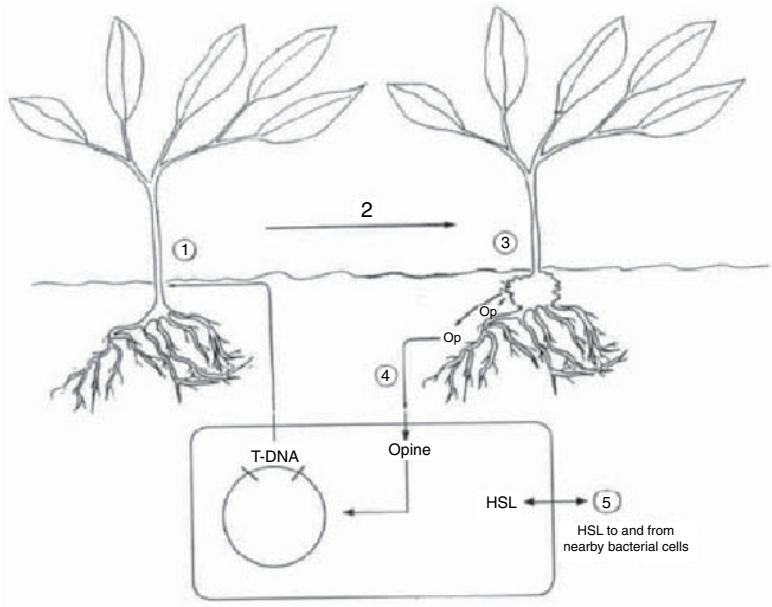

Fig. 4. Diagram showing infection of a plant by *Agrobacterium tumefaciens* and production of a tumor. The pTi plasmid is shown within the bacterium from which T-DNA is transferred to the plant and opine induces homoserine lactone (HSL) production. From Farrand, 1993. Five steps are indicated: 1. Agrobacterial cells enter a susceptible plant. 2. The T-DNA is transferred from the bacteria to plant cell nuclei. 3. Tumor grows, secreting opine (Op). 4. Opine taken up by the bacteria induces production of HSL. 5. The HSL induces conjugation between bacteria and plasmid transfer.

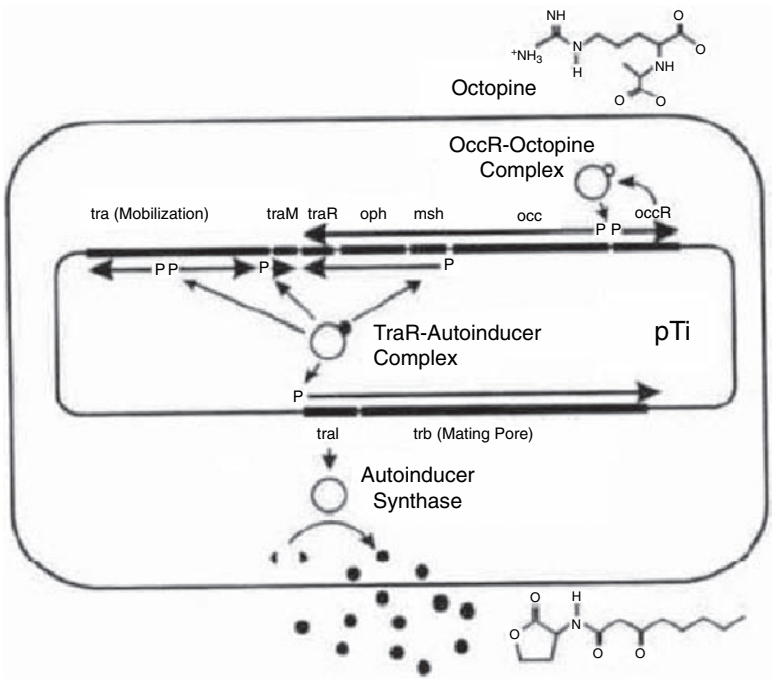

Fig. 5. Quorum-dependent regulation of octopine-type Ti plasmid *tra* and *trb* genes. The octopine-OccR complex elevates transcription of *traR*. The *traR*-autoinducer complex causes a further elevation in *traR* and *traI* expression as well as induces other *tra* and *trb* genes. Modified from Winans et al., 1999.

and plasmid transfer enhance the spread of the appropriate catabolic activity among the bacteria. A high density of donor cells enhances conjugation, and the role of this HSL is to assess the cell density, activate conjugation if appropriate, and spread the plasmid so that the specificity of opine produced by the plant tumor matches the ability of the *Agrobacterium* within the tumor to metabolize it.

Conjugation and plasmid transfer is controlled by a pair of LuxI-LuxR homologs designated *traI* and *traR* (see Fig. 5; Hwang et al., 1994; Piper et al., 1993). An HSL-TraR complex activates expression of transfer (*tra* and *trb*) genes (Piper et al., 1993). One lux box in the *tra* region is located between two divergently transcribed plasmid transfer genes, *traA* and *traC*. In sum, *Agrobacterium* receives chemical signals from its plant host and sends genetic signals to the plant and also to other agrobacterial cells.

Rhizobium

Bacterial signaling is needed to promote nitrogen-fixation within leguminous plants. Members of the genus *Rhizobium* engage in a symbiotic

relationship with peas, soybeans and other legumes to assimilate gaseous nitrogen and incorporate it into cellular material. Fixation is carried out only by a few specialized kinds of bacteria, which include the symbiotic Rhizobia, and these are highly specific for their plant host. Their specificity arises in part through the nature of their cell-to-cell signals.

Rhizobium in the soil invades tiny hairs on the roots of the legume, penetrating into the root tissues. There, the bacteria change their shape and size, becoming rounder and bigger, as they differentiate into bacteroids. Meanwhile, the root cells multiply and give rise to specialized nodules that house and feed the bacteroids. In this mutually beneficial association, bacteroids supply their host with a readily assimilated form of nitrogen (ammonia) and the plant supplies the bacteria with food (carbohydrates).

These developmental changes in both microbe and host result from a reciprocal molecular conversation. Legumes secrete flavonoids into the rhizosphere. When an appropriate flavonoid is recognized by the bacterium, it responds by synthesizing a lipochitooligosaccharide (LCO) signal, known as a Nod factor. Nod factors are major determinants of host specificity. Nod factors have a similar basic structure composed of a chitooligosaccharide (a linear chain of β-1,4-linked *N*-acetylglucosamines) linked to an acyl chain.

This core is modified in a way that is characteristic for each Rhizobium species. Variations include the degree of chitooligosaccharide polymerization (n in the figure), the nature of the fatty acid at R1, and modifications located at one or both ends of the oligosaccharide (R2–R7; Long and Staskawicz, 1993; Mergaert et al., 1997).

The appropriate Nod factor initiates the nodulation program in the plant, triggering the cell divisions that form a nodule, within which the *Rhizobium* differentiates into bacteroids that fix N_2. Thus, a two-way conversation between the bacterium and its plant host instructs them both in the successive steps that build a nodule.

Fruiting Body Development

Myxobacteria have adopted multicellularity as their strategy for survival. When they begin to exhaust their available food supply, they construct fruiting bodies (Reichenbach, 1993). Each fruiting body contains about 100,000 cells differentiated as asexual spores. Multicellular sporulation is thought to improve their long-term survival by enhancing spore dispersion and by providing a high cell density for cooperative feeding when the spores germinate (Reichenbach, 1984). In their vegetative phase, they also

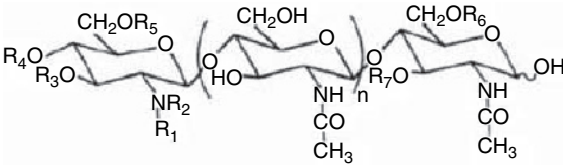

Fig. 6. Generic *Agrobacterium* Nod factor, a modified lipochitooligosaccharide that is synthesized by the bacterium. R1 is a fatty acid; R2 is hydrogen or methyl; R3, R4, or R5 are hydrogen or carbamoyl; R6 or R7 are various glycosyl modifications (Mergaert et al., 1997). The R6 branch may be sulfated (Schwedock et al., 1994). The degree of polymerization, n, may be 3, 4, 5, or 6.

feed cooperatively. The figure (7) shows *Myxococcus xanthus*, the "golden" myxobacterium, one of many species of myxobacteria, all of which are phylogenetically related. *Myxococcus* and *Stigmatella*, which has a stalk and multiple sporangioles, have the same size circular genome of 9.5 Mb and common initial steps of their morphological development (Reichenbach and Dworkin, 1981; Shimkets and Woese, 1992).

Using a capacity to move on the surface of other cells, *M. xanthus* builds a fruiting body as shown in Figure 7.

Starting from a uniform sheet of cells (Figure 7, panel 1) a punctate distribution of small asymmetric aggregates appears within a few hours (panel 2). More and more cells enter some of these early aggregates, and after about 10^5 cells (panel 4) have entered, a mound becomes a steep-sided hemisphere. Then the cells differentiate from long rods into spherical spores. Spores have thick walls, are metabolically dormant, resistant to radiation and dessication, and long-lived (White, 1993). A mature fruiting body is entirely filled with close-packed spores (panel 6).

To carry out their program of morphological development, the cells communicate with each other by emitting and responding to extracellular chemical signals. Three signals have been chemically identified: A, E, and C, which are passed between genetically identical cells to coordinate their development. Mutants that are unable to produce the A, C or E signals prematurely arrest the assembly of fruiting bodies (Hagen et al., 1978; Toal et al., 1995); each stops at a stage that corresponds to the time at which the signal is needed (see Figure 8).

These morphologic stages are linked with the developmental expression of sets of genes. Transcriptional fusions of developmentally regulated promoters to *lacZ*, the structural gene for β-galactosidase, report the activity of each promoter as expression of the enzyme. More than nine-tenths of the transcriptional *lacZ* fusion mutants modify development in a limited way, but they are not necessary for its completion

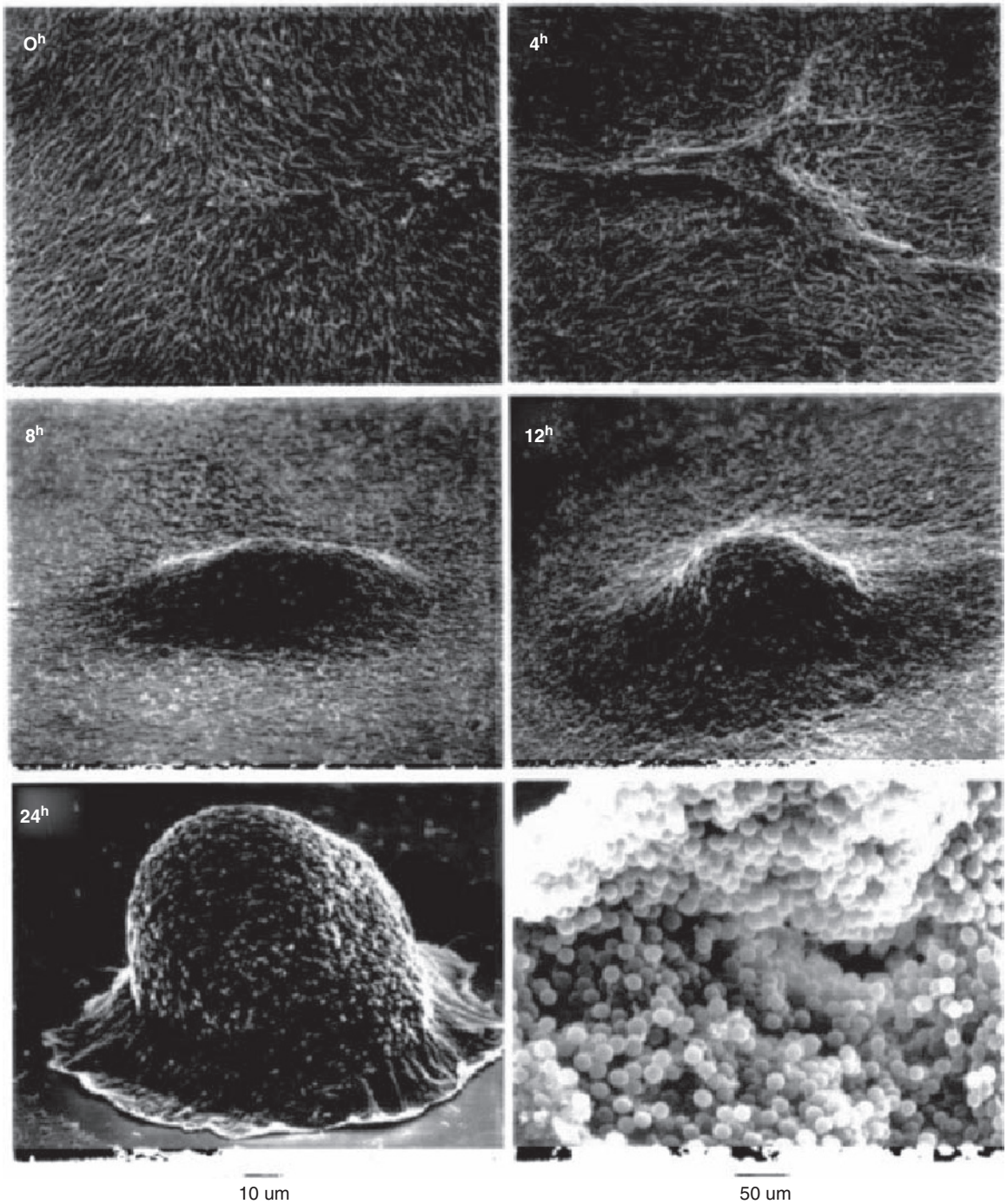

Fig. 7. Fruiting body development in *M. xanthus*. Development was initiated at 0 hours by replacing nutrient medium with a buffer devoid of a usable carbon or nitrogen source. The lower right frame shows a fruiting body that has split open, revealing spores inside. This frame is three times the magnification of the others. Scanning electron microscopy by J. Kuner. From Kuner and Kaiser, 1982.

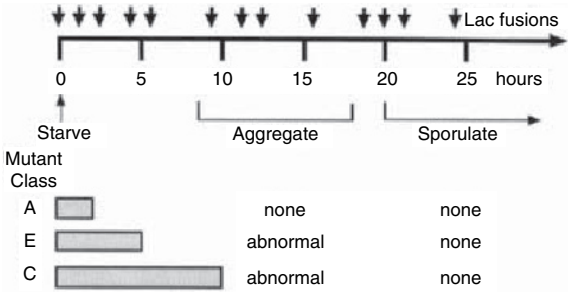

Fig. 8. Three classes of signal-defective developmental mutants of *M. xanthus*. Vertical arrowheads point to the time at which one of the lacZ fusions to a developmentally regulated promoter begins to be expressed. These fusions are reporters of normal development. The horizontal bars indicate the period of normal expression of reporters for each of the indicated mutants. Rightward from the end of the bar, development is defective; expression of the reporters is greatly reduced. The columns to the right show the morphological phenotype with respect to aggregation and sporulation of the indicated mutants.

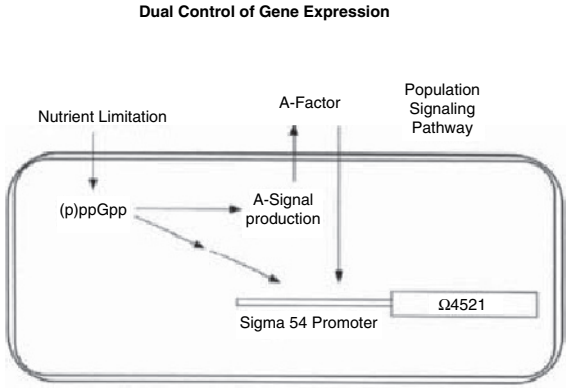

Fig. 9. Dual control of gene expression. As nutrient levels decrease and the protein synthetic capacity of the cell falls, the (p)ppGpp levels increase. Production of A-factor is induced by that increase, and it is released into the medium. Each starved cell releases a fixed amount of the set of A-factor amino acids, which pool in the fluid surrounding the cells. In responding to A-factor each cell perceives the pooled concentration of these amino acids in its vicinity. Promoters of genes that are A-factor and starvation dependent, like Tn5 lac *Ω4521*, receive one input from the cell's (p)ppGpp level and a second input from A-factor; both inputs are necessary for *Ω4521* expression.

(Kroos et al., 1986). Hence the fusions serve as reporters for the various regulatory stages of development without interrupting the overall flow of that program.

Mutants defective in producing extracellular signal A arrest at 1 to 2 hours of development, as a flat film of cells with no sign of focal aggregation (much like panel 1 of Figure 7). A-signal mutants are capable of sensing starvation, however (Singer and Kaiser, 1995), and express several early reporters (Figure 8). Mutants defective in producing extracellular signal E are blocked at 3 to 5 hours, and C mutants arrest after about 6 hours, partially aggregated and having expressed more genes.

A-Signaling

A-signal-production-defective mutants have been found in three genes. One, *asgA*, encodes a protein with a two-component receiver domain followed by a histidine protein kinase domain (Davis et al., 1995; Li and Plamann, 1996; Plamann et al., 1994) and is thought to function as a phosphorelay that, in response to starvation, is required for generation of extracellular A-factor. A-factor is a set of 6 amino acids (trp, pro, phe, tyr, leu and ile), peptides containing these 6 amino acids, or proteases capable of releasing these amino acids from *M. xanthus* cells (Kuspa et al., 1992a; Plamann and Kaplan, 1999; Plamann et al., 1992).

Myxococcus releases small quantities of these amino acids about 2 hours into development, then proceeds to take them back, Figure 9. This release and uptake helps *Myxococcus* choose between two alternative responses to nutrient limitation—on the one hand, entering stationary phase with very slow growth or on the other, fruiting body development with differentiation of spores. At least 30 new proteins are made during fruiting body development (Inouye et al., 1979), so a capacity to synthesize protein must be retained well into the sporulation phase. Accordingly, the cells must make their choice before any nutrient essential for protein synthesis has been totally depleted.

Starvation for any amino acid or starvation for carbon, energy, or phosphorous (Manoil and Kaiser, 1980) but neither starvation for purine or pyrimidine induces fruiting body development (Kimsey and Kaiser, 1991). The set of effective inducing conditions implicates the availability of a complete set of amino-acylated tRNAs. In *Myxococcus*, as in other bacteria, the absence or shortage of any one of the charged tRNAs leads a ribosome, sensing with a codon "hungry" for its cognate amino-acylated tRNA, to synthesize guanosine tetra- (and penta-) phosphate [(p)ppGpp] by condensing ATP and GTP. A rise in this highly phosphorylated nucleotide sets off a stringent response that stops the synthesis of new ribosomes and of the other major polymers of the cell, including DNA, phospholipids, and peptidoglycan (Cashel et al., 1996). Stringent conditions do allow certain genes to be expressed and selected proteins to be synthesized, insofar as activated amino acids are available. In this way, the genes for new fruiting body proteins are expressed and translated.

Accumulation of (p)ppGpp is both necessary and sufficient for fruiting body development. On

the one hand, ectopic production of (p)ppGpp in *M. xanthus* initiates early developmentally specific gene expression (Singer and Kaiser, 1995). The *E. coli relA* gene was introduced into *M. xanthus* for this purpose; its introduction was followed by production of the *E. coli relA* protein and (p)ppGpp accumulation without any prior starvation. Moreover, the rise in (p)ppGpp also induces production of A-factor (Singer and Kaiser, 1995). On the other hand, *M. xanthus* has its own *relA* gene, and either a point mutation or a deletion mutation in that gene blocks starvation-initiated development at the flat biofilm stage of Figure 7 frame 1 (Harris et al., 1998). In fact, these *relA* mutants arrest before expression of any of the developmentally regulated reporters.

A-SIGNALING AND DUAL CONTROL Each starved cell that has accumulated ppGpp releases a fixed amount of the set of A-factor amino acids. Being soluble, these amino acids then pool in the fluid surrounding the cells. In responding to A-factor, each cell perceives the pooled concentration of these amino acids in its vicinity (Kuspa et al., 1992b). Promoters of genes that are A-factor and starvation dependent, like Tn5 lac *Ω4521*, receive one input from the cell's (p)ppGpp level and a second input from A-factor. Both inputs are necessary for *Ω4521* expression (Kuspa et al., 1986). The promoter for *Ω4521* recognizes σ 54 rather than the more common σ 70 (Keseler and Kaiser, 1995). All known σ 54 promoters require an upstream activator protein to initiate open-complex formation and then transcription (Wedel and Kustu, 1995). An activator protein (Gorski and Kaiser, 1998) and the σ-54 holo enzyme are two spatially distinct input sites for controlling transcription of *Ω4521* (see Figure 9). The (p)ppGpp level indicates the level of nutrient currently available, since this nucleotide tracks nutrient changes. A-factor recalls the level of starvation 2 hours previously. The expression of *Ω4521* requires both inputs; very low expression occurs when either input is absent. In this way, the A-factor pool summarizes the votes of all the cells. Thus A-factor belongs to the class of extracellular signals called quorum sensors (Kaiser, 1996). Given the concentration window for an A-factor response, the reliability of the judgment whether to enter stationary phase or to initiate fruiting body development is increased when the whole population of cells has made it.

C-Signal

Primary functions of C-signaling are to bring cells into selected asymmetric foci until they have mounded into hemispheres of about 10^5 cells and to trigger the cells inside a completed hemisphere to differentiate into spores, by transforming the individual motile rod cells into nonmotile spherical dormant cells with tough coats. Null mutants of *csgA*, the gene encoding C-factor protein, form small irregular aggregates (Figure 7, frame 2) and stop developing at this stage. They also do not sporulate; the sporulation frequency of cells with csgA$^+$ is 10^5 fold higher than the mutant. Sporulation requires C-factor to initiate the cell-shape change and for expression of spore genes.

C-factor was purified from whole cells, and a 17-kDa protein was obtained (Kim and Kaiser, 1990d). Antibodies to C-factor reveal its exposure on the cell surface (Shimkets and Rafiee, 1990). Neither an aggregation reporter like the Tn5lac insertion *Ω4499* nor a sporulation reporter like *Ω4435* is expressed in the absence of C-factor. However, both are expressed if purified C-factor is added to *csgA* mutant cells (Kim and Kaiser, 1991). Higher levels of C-factor are needed for sporulation than for aggregation. Both the aggregation and the sporulation reporter were expressed when high levels of C-factor were added; neither was expressed when low levels were added, and only the aggregation reporter was expressed when intermediate levels were added. Whether the amount of C-factor is limited by promoter deletions or by adding variable amounts of purified C-factor, the same gradation of aggregation and sporulation was observed (Kim and Kaiser, 1991; Li et al., 1992).

The amount of C-factor in cell extracts rises between 8 and 18 hours of development, naturally staging aggregation before sporulation (Kim and Kaiser, 1990a). The rise in the concentration of C-factor per cell is a consequence of a positive feedback loop in the C-signaling circuit. Expression of the *csgA* gene is controlled by C-factor itself (Kim and Kaiser, 1991). Once C-signaling has started, *csgA* gene expression begins to rise. Because C-signaling depends on cell contact (Kim and Kaiser, 1991), that rise in C-factor concentration speeds up as aggregation proceeds.

C-Signal Transduction

The C-signal transduction circuit (Figure 10) branches twice, one branch for expression of *csgA* and another for aggregation and sporulation, so that one signal performs 3 tasks (Søgaard-Anderson et al., 1996b). C-factor on the surface of one cell interacts by contact with another cell (Kim and Kaiser, 1990b; Kim and Kaiser, 1990c; Kroos et al., 1988). End-to-end contact is believed to be necessary (Kim and Kaiser, 1990b; Sager and Kaiser, 1994; Wall and Kaiser, 1998). Whether C-factor signals as an enzyme or as a ligand has not been established as yet (Baker, 1994; Lee et al., 1995; Lee and Shimkets, 1994).

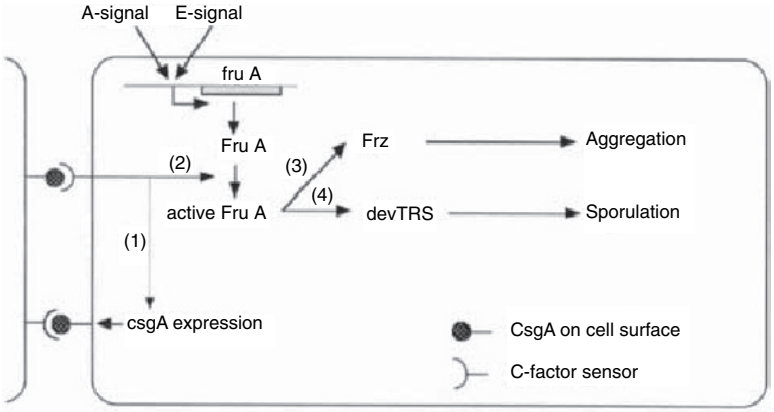

Fig. 10. C-signal transduction pathway. The structural gene *fruA* encodes a basic helix-turn-helix transcription factor. Steps (1), (2), (3), (4) are described in the text. The set of proteins Frz makes up a phosphorelay pathway and *dev* is an autoregulatory operon. The figure shows a pair of rod cells that are C-signaling to each other through their ends.

Following C-signal transmission, step (1) in Figure 10 causes the expression of *csgA* to rise; this is the positive feedback that increases the intensity of signaling once it has started. In step (2) of Figure 10, C-signaling activates FruA protein by a post-translational modification that is evident by its electrophoretic mobility (Ellehauge et al., 1998). The protein FruA is a DNA-binding response regulator with a helix-turn-helix (HTH) motif (Ellehauge et al., 1998; Ogawa et al., 1996). Activated FruA is necessary for aggregation and sporulation; there is no evidence that *csgA* expression is FruA-dependent. Synthesis of FruA protein depends on A-factor and E-factor, but not on C-factor (Figure 10).

The *frz* target of FruA is a phosphorelay, Figure 10 step (3), which modulates the frequency of reversal of direction (Blackhart and Zusman, 1985). In that relay, the FrzCD protein has a domain that resembles the carboxy-end of a methyl-accepting chemotaxis protein (McBride et al., 1989). However, FrzCD protein is not a membrane receptor; it is found in the cytoplasm and has no transmembrane or extracellular domain. Activated FruA sends a signal along the *frz* phosphorelay, as detected by the methylation of the FrzCD protein (Søgaard-Anderson and Kaiser, 1996a), and by a C-factor-induced increase in gliding speed and duration of gliding interval (Jelsbak and Søgaard-Andersen, 1999).

The methylation of FrzCD shifts during fruiting body development (Søgaard-Anderson and Kaiser, 1996a). Starting from a fully nonmethylated state early in development, the FrzCD protein gradually shifts to methylated states, and by 9 hours, the time of symmetrical mound building, all the FrzCD protein is methylated. Extracellular addition of purified C-factor to C-factor-less mutant cells directly and specifically induces the full methylation of their FrzCD protein, paralleling wild-type development (Søgaard-Anderson and Kaiser, 1996a). The cellular mechanism of aggregation, however, has not yet been established.

Both *fruA* and *frz* mutants were found in a screen of Tn5 insertion strains that arrested development in a state of partial aggregation, at the same stage as *csgA* mutants (Søgaard-Anderson et al., 1996b). Unlike *csgA* mutants, however, the targeted mutants are cell-autonomous; they are not rescued by addition of wild-type cells, or by addition of purified C-factor. Comparison of the properties of the *fruA* and *frz* mutants immediately showed that FruA is needed for aggregation and sporulation, but *frz* is only needed for aggregation.

The second target of activated FruA is the *dev* operon, Figure 10 step (4). Operon expression, as measured by the extent and time course of β-galactosidase expression from a Tn5 *Lac* transcriptional fusion to *devR*, depends on FruA in the same manner as it depends on C-factor (Ellehauge et al., 1998). The operon, in turn, is necessary for fruiting body sporulation; null mutants sporulate at 0.1% or less the frequency of wild type cells (Thony-Meyer and Kaiser, 1993). The morphological differentiation of myxospores occurs after aggregation is complete. In *Sigmatella aurantiaca*, another myxobacterium whose fruiting bodies have a more complex morphology (a stalk, branches and multiple cysts containing the spores), the aggregates pass through different intermediate shapes, including a myxococcus-like mound. Nevertheless myxospores do not form until the fruiting bodies are assuming their final branched shape (Qualls et al., 1978).

Activated FruA elevates the transcription of the *dev* operon. This operon has a switch-like quality, evident in the bimodality of operon expression in populations of developing cells (Russo-Marie et al., 1993). The two states may be consequences of the intense C-signaling in the densely packed cells that circulate inside nascent fruiting body aggregates and of little or no C-signaling in cells less densely arrayed in the periphery. Spores are found differentiated inside the fruiting body after its morphogenesis is complete, following intense C-signaling. Hence, the

chemical properties of A- and C-factors match the cell density at which they signal. For A-signaling, the cells are separate, the signal must diffuse between them in a generally aqueous environment, and the A-factor amino acids are water soluble. Quorum sensing A-factor counts the number of votes cast by those cells recommending fruiting body development. For transmitting the cell-bound C-signal, the cells must touch each other. This condition and the requirement for a high level of C-signaling to switch *dev* on test whether the cells have reached the high density characteristic of a nascent fruiting body, ensuring that it has the proper morphology.

Pheromone-Inducible Plasmid Transfer in *Enterococcus faecalis*

Enterococcus faecalis, a nonmotile Gram-positive species, exhibits a highly specific cell interaction to initiate plasmid transfer. Potential recipient cells excrete short peptides that stimulate a mating response by donor cells, which carry the corresponding plasmid. The peptides signal donor cells to synthesize surface adhesins, and mating mixtures form large aggregates of donors and recipients. The cells specifically adhere to one another. The interactions are summarized in the scheme of Figure 11.

At the center of the scheme, a plasmid-free recipient synthesizes two different pheromones, called cA and cB and shown as triangles and squares. The cell carrying plasmid pA (on the left) responds to cA and the cell carrying plasmid pB (on the right) responds to cB. Upon exposure to pheromone, a signal is transduced to the resident plasmid that results in expression of a plasmid gene encoding a surface adhesin (AS) on the donor cells. The adhesin has the capacity to bind to a normal constituent of the cell walls, probably lipoteichoic acid (LTA), the major wall antigen of Gram-positive cells. Once triggered by pheromone, donor cells will bind recipients and other donor cells as well. Chemotaxis toward the pheromones by donor cells is not evident. Within the resulting aggregates, which may be large, plasmid is transferred from donors to recipients by conjugation (see Conjugation in the Introduction).

Several pheromones have been isolated, purified, sequenced and synthesized (Table 1). Typically they are hydrophobic octapeptides. These pheromones are active at concentrations below 5×10^{-11} M, and as few as two molecules per donor cell may be sufficient to induce a mating response (Mori et al., 1988). Their specificity is high: pheromone cAD1 is unable to induce the clumping of cells which carry plasmid pPD1 even at 10^{-6} M, ten thousand times the threshold concentration for pheromone, and pheromone cPD1 is unable to induce the clumping of cells which carry plasmid pAD1. The plasmid also encodes formation and secretion of a competitively inhibitory peptide, which blocks the action of a corresponding pheromone. Several inhibitors have been purified, and they have the same length as their corresponding pheromones, as well as some identical residues. Inhibitors and their sequences also are shown in Table 1.

The plasmid-encoded adhesins, or AS, for pAD1 and pPD1 are large proteins with a proline-rich C-terminal region for cell-wall association, followed by a membrane anchor. The adhesins have been visualized by electron microscopy as dense, hair-like structures on the bacterial cell wall, protruding about 20 nm from

Table 1. *E. faecalis* pheromone amino acid sequences.

Pheromone or inhibitor	Peptide structure
cAD1	LFSLVLAG
iAD1	LFVVTLVG
cPD1	FLVMFLSG
iPD1	ALILTLVS
cCF10	LVTLVFV
iCF10	AITLIFI
cAM373	AIFILAS
iAM373	SIFTLVA
cOB1	VAVLVLGA

Structure of *Enterococcus faecalis* pheromones. The structures are based on work from the laboratories of M. Mori, J. Nakayama, A. Suzucki, and D. Clewell (Clewell, 1999).
L = leucine; F = phenylalanine; S = serine; V = valine; A = alanine; G = glycine; T = threonine; M = methionine; I = isoleucine.

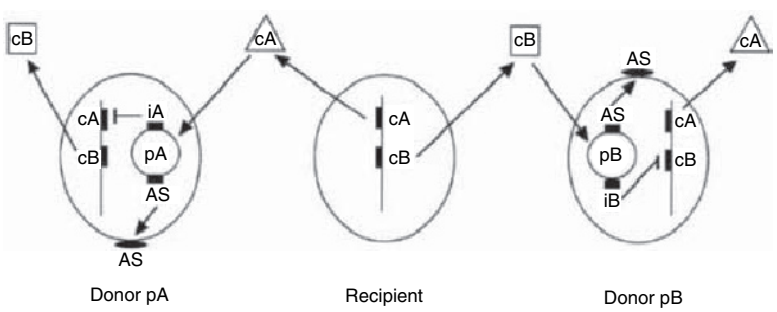

Fig. 11. Conjugal pheromones used by *Enterococcus faecalis*. The cell in the middle is plasmid free, while the cells at either side carry conjugative plasmids pA and pB, respectively. The cA and cB are two different pheromones; iA and iB are their corresponding inhibitor peptides, and AS is aggregation substance.

its surface (Galli et al., 1989). The adhesin molecules are unevenly distributed over the surface of cells, apparently attached only to the cell wall that was present when AS induction by the pheromone began. Such behavior suggests a convenient way for donor cells to dissociate from mating aggregates after plasmid transfer is complete. Growth and cell division would give rise to new donor cells free of the adhesin. These new donors would tend to dissociate from the clumps.

The target for the adhesin on *E. faecalis* cells is thought to be lipoteichoic acid (LTA) because LTA-deficient mutants lose the ability to conjugate with active donor strains. Moreover, a reversal of the normal donor-recipient orientation of adhesin-LTA allowed practically normal conjugation and plasmid transfer (Dunny, 1990).

In the genome sequence of *E. faecalis*, which has been completely determined, three of the pheromones appear in one or another of the recipient protein signal sequences (Clewell, 1999), and a single cell produces at least five different pheromones. The use of signal peptides, which are often released from cells, suggests that the entire pheromone-induced conjugation response may have evolved when potential donors gained a capacity to detect potential recipients by the signal peptides they release. At least 11 different plasmids encode pheromone responses, and the genes that specify their aggregation substances are homologous for 10 of them. Moreover, at least two of these plasmids, pAD1 and pCF10, have a similar organization of pheromone-induced genes and an identity of more than 85% for their structural genes encoding aggregation substance (Galli and Wirth, 1991). The whole set of plasmids may have evolved from a common ancestor plasmid. In conclusion, *E. faecalis* uses sequence-specific peptides to induce the agglutination of plasmid donors and recipients. The plasmid is transferred from donor to recipient within the aggregate; the aggregate then disperses.

Swarming

Swarming cells spread from a point of inoculation radially outward over a solid surface such as agar. Swarming motility, which is cooperative, organized and relatively rapid, is observed in many Gram-negative and some Gram-positive bacteria. Motility depends on interactions between cells that have appendages. However, both flagella and type IV pili promote swarming, whereas other pili do not. Flagella and pili are thought to propel a cell by different means; nevertheless the two cause remarkably similar spreading patterns. Comparison of the two modes may offer insight into the cell-cell interactions that generate swarm behavior. Swarming is inducible in flagellated cells but constitutive in cells with type IV pili.

Swarming Based on Flagella

Among Gram-negative bacteria, swarming with flagella has been found in the genera *Aeromonas*, *Escherichia*, *Proteus*, *Pseudomonas*, *Rhodospirillum*, *Salmonella*, *Serratia*, *Vibrio*, and *Yersinnia* (Harshey, 1994a). Swarming also has been observed in *Bacillus* and *Clostridium*, which are Gram-positive (Harshey, 1994a). All these swarming bacteria possess flagella, which they use for swimming, but swarming requires that the swimming cells change size and physiology. The shape modifications adapt cells to life on a surface, instead of swimming in a liquid environment. The differentiation involves flagella and other gene products that control cell division. In *Vibrio parahaemolyticus*, 40 genes or more are expressed for the swarming state. The differentiation inhibits septation and produces an elongated multinucleated cell (up to 80 μm long in *Proteus*) with a two to fifty-fold higher surface density of flagella, depending on the organism (Harshey, 1994a). *E. coli*, *Proteus*, *Salmonella typhimurium*, and *Serratia marcesens* use the same type of flagellar organelle for swimming and swarming. The structures of the flagella of *E. coli* and *S. typhimurium* are well known, and they are essentially identical (Macnab, 1996).

V. parahaemolyticus is the only swarmer to have distinct organelles for swimming and swarming (Harshey, 1994a). It possesses hundreds of lateral flagella, while undifferentiated swimming cells have but a single polar flagellum. A proton-motive force powers the lateral flagella, while a sodium-motive force drives the polar flagellum (Atsumi et al., 1992). These distinctions have been turned to experimental advantage in the study of swarming.

Signaling (using the chemotaxis phosphorelay) but not chemotactic behavior has been shown to be essential for the differentiation of swarm cells in *E. coli* (Burkart et al., 1998). A quadruple mutant that lacks all four inner membrane chemoreceptor-transducers (Tar, Tsr, Tap, and Trg) fails to swarm, but mutants lacking any one of the four continue to swarm (Burkart et al., 1998). Swarming is rescued in the quadruple mutant by restoring either Tsr or Tar, which happen to be the most abundant chemoreceptors in *E. coli*. However, neither the presence of saturating chemoeffector concentrations or mutations that destroy ligand binding to Tsr or Tar interferes with swarming or with hyperflagellation. In addition, mutants with defects in *cheA*, *B*, *R*, *W*, *Y*, or *Z* elongate, but none

hyperflagellate or swarm (Harshey and Matsuyama, 1994b). Apparently communication of the receptors with the *cheA* kinase is essential. For swarming, it appears that the chemoreceptors signal through the chemotaxis pathway and induce swarmer-cell differentiation, but the receptors are responding to other unknown signals rather than their well-known chemoeffectors (Burkart et al., 1998).

Interference with either the *fla* genes or the functioning of the polar motor results in constitutive expression of lateral flagella in *V. parahaemolyticus* (Kawagishi et al., 1996; McCarter et al., 1988). Transposon insertion within the *lonS* gene of *V. parahaemolyticus*, which encodes an ATP-dependent protease homologous to the *E. coli lon* gene, causes expression of *lafX*, one of the genes high in the pathway of differentiation and cell filamentation—even in the absence of UV irradiation (Stewart et al., 1997). Targets of *lonS* might include a transcriptional activator of *laf* genes and an inhibitor of cell division (Stewart et al., 1997). FlhDC in *P. mirabilis*, an operon known to regulate transcription of the genes of the flagellar hierarchy, has been suggested to control swarmmer differentiation as well since it plays a role in the inhibition of cell division (Furness et al., 1997). The operon also affects cell division in *E. coli* (Pruss and Matsumura, 1996). Transcription of the *flhDC* operon rises during differentiation and decays as the cells dedifferentiate back into swimmers (Furness et al., 1997).

The natural trigger to differentiate *V. parahaemolyticus* swarm cells may be the high medium viscosity which is found on a surface and places a mechanical load on the polar flagellum, thereby signaling and inducing the cell to differentiate (McCarter et al., 1988). A high viscosity medium also triggers hyperflagellation in *Se. marcesans* (Alberti and Harshey, 1990) and *P. mirabilis* (Allison et al., 1993). Swarmer cells of *P. mirabilis* transferred to liquid medium return rapidly to normal vegetative morphology; cells in the center of a swarm colony on a plate also return. Both suggest that swarmer cells must be continuously signaled to maintain the differentiated state (Harshey, 1994a; Shinoda and Okamoto, 1977).

Isolated swarmer cells barely move, suggesting that cells must be close together to swarm, and that flagella from neighboring cells coalesce into shared bundles. Swarm cells of *P. mirabilis* do align closely along their long axis, forming two-dimensional rafts that migrate by coordinate flagellar action. A mutant, *ccmA*, that has an abnormal curved shape loses the capacity to swarm but not to swim, as if, due to its shape, it could not align with other *ccmA* cells (Hay et al., 1997).

Swarming Based on Type IV Pili

Pili have been implicated in the swarming of a wide variety of Gram-negative bacteria, many of which are pathogenic (Henrichsen, 1975). These include *Acinetobacter calcoaceticus*, *Dichelobacter nodosus*, *Eikenella corrodens*, *Kingella denitrificans*, *Moraxella bovis*, *Myxococcus xanthus*, *Neisseria gonorrhoeae*, *N. meningitidis*, *Pseudomonas aeruginosa*, Synechocystis PCC6803 and enteropathogenic *Escherichia coli* (Henrichsen, 1983; Kaiser, 1979; Manning and Meyer, 1997; Strom and Lory, 1993). Remarkably, the swarming pili of all these organisms belong to the structural class known as type IV. Type IV pili (abbreviated Tfp) are distinguished from other pili by their strictly polar origins on cells, by N-terminal sequence conservation of the pilin monomer units, by sequence conservation of a core set of proteins for pilus assembly, and by supporting bacterial motility (Ottow, 1975; Strom and Lory, 1993). A broad collection of reviews on Tfp can be found in Manning and Meyer, 1997, and a recent review on Tfp and motility in Wall and Kaiser, 1999a.

Pilus-Dependent Swarming in Myxobacteria

Myxobacteria move by gliding, which occurs at a solid-liquid, solid-air, or solid-solid interface. Gliding consists of smooth motions in the interfacial plane that are directed along the long axis of the cell, with occasional reversals of direction (Hodgkin and Kaiser, 1979a).

Myxococcus xanthus has two independent gene systems named A and S, which control swarming behavior based on gliding (Hodgkin and Kaiser, 1979b). Mutations in any gene of either system only inactivate that system alone, but cells can still swarm by means of their remaining system, albeit less efficiently. However, no A^- S^- double mutant cell can move more than its length, nor can it swarm. More than fifty A^- S^- double mutants have been constructed which are defective in different A and S genes, and all fail to swarm. While the wild type cells (A^+ S^+) produce large, flat, spreading colonies with a serrate edge, the A^- S^- mutants produce small domed-shaped colonies with sharp, non-serrate edges. Ten mutants have been mapped to the *mglA* (mutual gliding) gene, which encodes a small Ras-like G-protein. This gene is required to complement both A^- and S^- motility defects (Hartzell, 1997; Hodgkin and Kaiser, 1979a).

Pili are essential for S-motility, but not for A-motility (Kaiser, 1979; Wu and Kaiser, 1995). Although wild type cells (A^+ S^+) swarm by combining the A and S swarm patterns, any A^-

S^+ mutant cells swarm by the S swarm pattern, and the role of pili is thereby revealed. Pili are usually found in tufts of 2 to 8 fibers, and always at the poles. The S-motility is lost when pili are shaved off cells by violent shearing action, but reappears as the pili grow back (Rosenbluh and Eisenbach, 1992). The S-motile cells cannot move at all when they are far apart. Nevertheless, individual cells that are separated from their nearest neighbor by less than a cell length do move (Kaiser and Crosby, 1983). Because pili tend to break when cells are washed, it is difficult to determine the length of pili on cells isolated from colonies; nevertheless many pili are roughly the length of cells. The rate at which an S-motile swarm expands depends on the cell density, showing strong dependence on cell interactions (Kaiser and Crosby, 1983). The swarming rate of S-motile cells is highest when the average cell-cell distance is less than about 1.5 μm. The bodies of cells do not have to touch to interact (Kaiser and Crosby, 1983). Spreading is evident in the change in morphology of a young S-motile colony after 2 hours (Figure 12). The colony has spearhead-like clusters of 50 or more cells but almost no single cells.

Non-swarming *M. xanthus* strains, either A^- S^- or *mglA* mutants, are unable to form fruiting bodies (see *M. xanthus* A- and C-signaling). Almost all A^- S^+ and A^+ S^- single mutants delay fruiting body aggregation, reflecting a need for the type IV pilus system (Cheng, Wee, Wu and Kaiser, unpublished). Abnormal fruiting body development is a consequence of the defective swarming when either system is absent (Hodgkin and Kaiser, 1979b; Wu et al., 1998).

UV-irradiation, chemical mutagenesis, or transposon insertion was used to create a library of about 160 independent S-motility mutants to explore the role of TfP in swarming. The S-motile (A^- S^+) colonies are flat and their spreading edges are smoothly serrated, whereas A^- S^- mutants of an A^- S^+ strain have smooth, dome-shaped colonies with sharp non-serrate edges. This library of S-motility mutants is divided into four groups named *pil*, *tgl*, *dsp*, and *frz*.

The largest group of S-motility genes is the *pil* genes. Over 100 mutations have been mapped within a contiguous *pil* gene cluster that contains 17 genes and is shown in Figure 13 (Wall et al., 1999b; Wu and Kaiser, 1995; Wu et al., 1998; Wu et al., 1997b). Products of fourteen of these genes

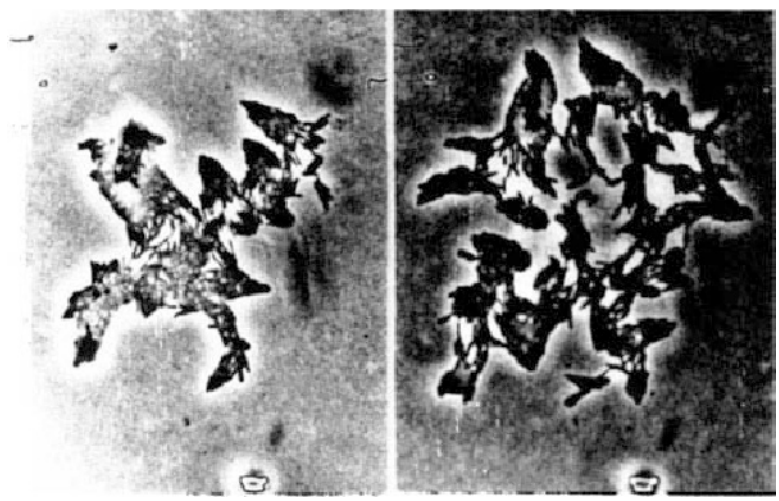

Fig. 12. Expansion of an S-motile microcolony. The two panels show the same colony at 0 (left) and 2 hours (right) at 33°C. Magnification scale: 1 cm is 15 μm. From Kaiser and Crosby, 1983.

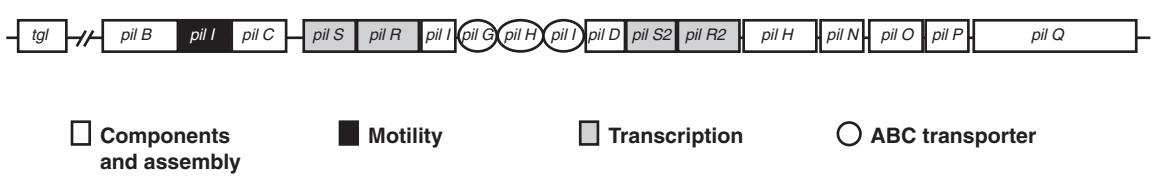

Fig. 13. *M. xanthus* Pil gene cluster and the function of each gene in type IV pilus assembly, motility, and control of gene expression. From Wall and Kaiser, 1999a.

are about 30% identical and 50% similar to proteins in *P. aeruginosa* and *N. gonorrhoeae* that encode their type IV pilin and related assembly proteins. The *Myxococcus pilG*, *pilH* and *pilI* genes have no homologs among other Tfp systems. The *pilH* gene encodes a member of the ABC transporter family with an ATP-binding cassette (Wu et al., 1998). In-frame deletions, transposon insertions and other null mutants reveal the functions of all 17 *pil* genes except *pilD*, for which deletion appears to be lethal. Null mutation in 14 of the 17 genes, including *pilG*, *pilH* and *pilI*, abolishes S-motility. These mutations, with the exception of *pilT*, also abolish the production of pili. Pili remain in a *pilT* mutant; this gene appears to be necessary for pilus function as opposed to pilus assembly. Mutant screening and sequencing left and right of the *pil* cluster shown in Figure 13 revealed no other *pil* homologs or S-motility genes in that vicinity. Clearly, Tfp is essential for S-motile swarming.

The *tgl* mutants lack S-motility and pili, like the *pil* mutants. They are distinguished from the *pil* mutants by their map position (distant from the *pil* cluster) and by their capacity to be stimulated, a process of phenotypic rescue (Hodgkin and Kaiser, 1977; Rodriguez-Soto and Kaiser, 1997a; Rodriguez-Soto and Kaiser, 1997b). It is the only stimulatable gene in the S system, although five stimulatable genes are known in the A system (Hodgkin and Kaiser, 1977; Rodriguez and Spormann, 1999). The *tgl* gene contains a type II signal sequence, suggesting it encodes a lipoprotein. Six tandem but degenerate tetratrico peptide repeats (TPR) follow the signal sequence (Rodriguez-Soto and Kaiser, 1997a). Tetratrico peptide repeats motifs are associated with proteins that interact with other proteins (Das et al., 1998). The Tgl protein may be an assembly factor for the pili, since it is not part of the pilus fiber (Rodriguez-Soto and Kaiser, 1997b).

The third group of S^- mutants is the *dsp*. All 21 *dsp* mutants map in one cluster. Transductional linkage within this group suggests an approximately 10 kb *dsp* region. Unlike wild type cells, the *dsp* mutants grow as *disp*ersed cultures in liquid medium; they neither clump nor aggregate, even though they have pili. Autoaggregation of cells in suspension is common for bacteria that have Tfp (Bieber et al., 1998; Knutton et al., 1999; Manning and Meyer, 1997; Wu et al., 1997b). Like *pilT* mutants, all *dsp* mutants retain pili, yet lack S-motility. The *dsp* mutants are depleted in extracellular fibrils, but do not totally lack them (Chang and Dworkin, 1994; Dana and Shimkets, 1993). Fibrils are peritrichous filaments composed of approximately equal amounts of polysaccharide and protein, and help to hold cells together. In addition to a cohesive function, it has been suggested that fibrils transfer signals, perhaps by ADP-ribosylation (Dworkin, 1999). A chaperone of the HSP70 family encoded by the *sglK* gene is necessary for production of one of the protein components of fibrils, and *sglK* mutants are S^- (Weimer et al., 1998). However, the relationship between S-motility and fibrils could be indirect, because *esg* mutants, which are also defective in fibril production, retain S-motility (Ramaswamy et al., 1997).

The final group of S-motility genes is called *frizzy*. These genes control the frequency of reversal of gliding direction (Blackhart and Zusman, 1985), and possibly speed (Spormann and Kaiser, 1999). The *frz* genes are homologous to the enteric bacterial chemotaxis genes for swimming (McBride et al., 1989). When a *frz* mutation is combined with an A^- mutation, the *frz* genes behave like S-genes (Fontes and Kaiser, 1999). Swarming of A^- *frz* double mutants is greatly diminished. However, it is not eliminated, resembling A^- *dsp* mutants.

It may be useful to think of S-motile swarming as the product of a pathway shown in Figure 14. Blocks at any stage in the pathway would create an S^- mutant. The first stage (pilin synthesis) includes control of *pilA* expression by PilS/PilR, a two-component regulatory system (Wu and Kaiser, 1997a), membrane insertion of a pilin monomer, and finally processing by the bifunctional PilD peptidase/N-terminal methylase. Processed subunits are then polymerized into a pilus fiber. The pili contact other cells and other pili, providing cell interactions that may also involve the fibrils and proteins bound to fibrils. In the last step, pilT and frz proteins may help to control movement behavior. The pili, and possibly the fibrils, are thus essential for social swarming, but their role is not yet clear (Kaiser, 1979; Wu et al., 1998).

Twitching Motility Twitching describes a type of surface translocation that, like gliding, does not depend on flagella, and in which the movements of individual cells appear as small, intermittent jerks (Henrichsen, 1972). Like gliding, twitching depends on humid agar but unlike gliding, twitching is jerky, not smooth (Henrichsen, 1972; Henrichsen, 1975; Henrichsen, 1983). Henrichsen surveyed some 1,000 bacterial

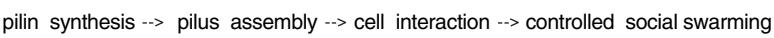

Fig. 14. Steps on the dependent pathway to social swarming in *M. xanthus*.

strains representing more than 50 species, classifying their surface translocations as twitching or gliding. About 20 of his species were found to twitch, and some he demonstrated to have polar pili. All strains of the same species with polar pili exhibited twitching. Moreover, twitching was not found in variants of the strains that had lost their polar pili by mutation, or in strains with pili that were not type IV. Henrichsen concluded from this study that twitching depends on polar pili. Subsequent genetic studies of *P. aeruginosa* and *N. gonorrhoeae* showed that their twitching depends on their Tfp (Strom and Lory, 1993). Behavior, sequence conservation of Tfp genes among *P. aeruginosa*, *N. gonorrhoeae*, and *M. xanthus*, and strong similarities of mutant phenotype clearly connect twitching and social gliding (Mattick et al., 1996; Wall et al., 1999b; Wu and Kaiser, 1995).

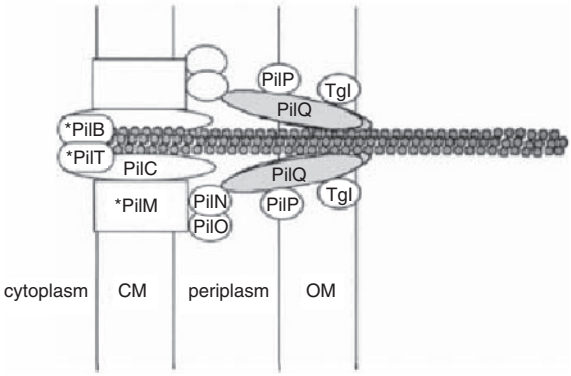

Fig. 15. Model of the type IV pilus in *M. xanthus*, based on genetic studies and on location of the individual proteins. From Wall and Kaiser, 1999a.

Type IV Pilus Fiber Structure

The structure of the pilus fiber and its mode of assembly (and possibly disassembly) are relevant to pilus function in twitching and gliding, as well as to the cell interactions necessary for swarming. The Tfp fiber has a three-layered helical structure of coiled α-helices surrounded by a β-sheet. These two inner layers are covered with the C-terminal regions of adjacent monomers, based on a 2.6 Å resolution X-ray crystal structure of dimers of pilin protein from *N. gonorrhoeae* (Forest and Tainer, 1997; Parge et al., 1995). The *N. gonorrhoeae* fiber has 5 pilin monomers per helical turn, a rise of about 4 nm per monomer, and an outer diameter of about 6 nm. The helix parameters and diameter agree with fiber diffraction data from *P. aeruginosa* Tfp, which suggests that the proposed structure is representative of the Tfp in many organisms. The N-terminal amino acid sequence, which is highly conserved from one bacterial species to another, forms the innermost coil of staggered, hydrophobically packed, parallel α-helices. Hydrophobic packing and the flexibility of α-helices may allow pili to bend and to adopt twisted or bundled conformations. The middle layer of β-sheet is continuous from one pilin monomer to the next in the sense that any crosssection of the fiber would cut through 25 β-strands. The β-sheet hydrogen bonding may provide much of the mechanical stability required for a fiber whose length of up to 4 microns approaches 600 times its diameter of 6 nm. It is generally believed that the pilus is assembled from its base, since there is no channel in the center of the fiber and one would be needed for assembly from the tip. Moreover, a pool of pilin is found in the cytoplasmic membrane, ready to drive assembly. No pool of pilin in the cytoplasm would be required for assembly from the tip, as found in the assembly of flagella from flagellin.

Based on the structure of the pilus fiber, on the genetic studies, and on the localization of pilus proteins, a model of the pilus and its assembly system is presented in Figure 15. This structural model is neutral with respect to theories of pilus retraction by disassembly and reassembly of the fiber as suggested by Bradley and others (Bradley, 1980).

In *conclusion*, swarming is observed only when the cell density is high. The similarities in spreading behavior between flagellar swarming and type IV pilus swarming could arise because both types of cells move in groups, both involve elongate cells which tend to move parallel to their long axis, and both types of cells maintain close contact with other cells.

Heterocyst Differentiation in Cyanobacteria

Cyanobacteria carry out photosynthesis as well as nitrogen fixation. In photosynthesis, atmospheric CO_2 is reduced using water as the primary electron donor and O_2 is generated. This generation of O_2 is a problem for nitrogen fixation because nitrogenase, the enzyme that catalyzes reduction of N_2, is sensitive to O_2. Perhaps, to solve this problem, cyanobacteria of the genus *Anabaena* differentiate specialized cells called heterocysts to house their nitrogenase. The heterocysts have thick walls, which prevent O_2 from penetrating; nitrogenase is thereby permitted to function inside. *Anabaena* forms chains of photosynthesizing vegetative cells, punctuated with an occasional heterocyst.

If *Anabaena* is grown in a medium that provides ample fixed nitrogen as NH_4^+, nitrogen

fixation is not required and no heterocysts are formed, only vegetative cells. But when cells that have been grown with ample NH_4^+ are washed, then resuspended in medium free of fixed nitrogen, they develop heterocysts during the next 24 hours. Since the vegetative generation time under these conditions is also about 24 hours, heterocyst differentiation can be thought of as growing a new cell with a different wall and a somewhat different set of enzymes. The RNA hybridization data suggest as many as 1,000 protein differences between vegetative cells and heterocysts (Buikema and Haselkorn, 1993).

When nitrogen fixation is needed, about one cell in 10 becomes a heterocyst. Nitrogen fixation and respiration in the heterocyst require a supply of reductant and of carbon from the adjacent, photosynthesizing, vegetative cells. Reductant and carbon are provided in the form of maltose, sucrose, or other disaccharides. In return, the heterocyst releases fixed nitrogen in the form of glutamine to its vegetative neighbors.

The intercellular exchange of metabolites illustrates the metabolic interdependence of vegetative cells and heterocysts. These cells are also developmentally interdependent in that the heterocysts differentiate at fairly regular spatial intervals (Wilcox et al., 1973). Chain-breaking experiments show that many more cells have the potential to become than normally become heterocysts (Wilcox et al., 1973). Because all the cells in a chain have received the same environmental cue, the implication is that some kind of local cell interaction underlies the regular spatial pattern of heterocysts. To appreciate this pattern, consider that all cells in a chain of vegetative cells grow and divide, causing the chain to elongate internally. To maintain a fixed ratio of heterocysts to vegetative cells, new heterocysts must differentiate in proportion to the new vegetative cells that form. Moreover, a regulatory system that appropriately selects particular vegetative cells to become heterocysts would seem to be necessary so that fixed nitrogen (glutamine) will be equally available to all the growing cells. Since the levels of hundreds of proteins differ between heterocysts and vegetative cells, the process needs coordination. It is hard to escape the inference that a cell interaction triggers one daughter cell to become a proheterocyst and its sister to remain a vegetative cell.

Each new heterocyst forms very near the center of a segment of vegetative cells. This maintains a stable 1/10 ratio and is clearly the most efficient way to exchange fixed carbon and fixed nitrogen between vegetative cells and heterocysts. But what are the cell interactions and how do they generate the pattern? A promising start to answering this question comes with the discovery of the gene *patS* (Yoon and Golden, 1998).

Overexpression of *patS* blocks heterocyst differentiation, while a *patS* null mutant has an increased frequency of heterocysts clustered abnormally along the chain of cells. This contrast of phenotypes suggests that *patS* encodes an inhibitor of heterocyst differentiation. Moreover, *patS* is expressed in proheterocysts but not in vegetative cells. The *patS* gene can encode a peptide of 17 amino acids, and a synthetic peptide corresponding to its C-terminal pentapeptide has the capacity to inhibit heterocyst development. Yoon and Golden propose that heterocysts synthesize and secrete a *patS* peptide that prevents neighboring vegetative cells from becoming heterocysts.

Sporulation of *Bacillus* Subtilis

Bacillus subtilis usually divides by binary fission, but when the cells are starved for carbon, nitrogen, or phosphorous they differentiate a new type of cell, an endospore. The spore can survive for years without food and retains an ability to germinate and to produce rapidly growing and dividing vegetative cells when food is again available. The developing cell undergoes a rigid sequence of morphological and biochemical changes over a period of about 8 hours as it makes the spore. A program involving the expression of more than 100 genes governs progression through the morphological and biochemical stages. The transcriptional program is controlled by the sequential activation of different subunits of RNA polymerase.

The earliest morphological event in that developmental program is a redirection of septum formation from its usual midcell position to a sub-polar one. An asymmetric septum partitions one copy of the chromosome to the larger mother cell and one copy to the smaller forespore. However, the two cells are held together by a common cell wall. These two cells, each with its own nucleus and cytoplasm, are in a position to interact with each other, and they do. Their interactions can be considered to couple gene expression to morphology. This example will focus on the best understood interaction between the two daughters as they codevelop. There is evidence for more interactions in the sporulation program as well (Kroos et al., 1999; Sonenshein, 1999; Stragier and Losick, 1996).

A different cascade of σ factors is active within each compartment; in the mother cell σ^E is replaced by σ^K, while in the developing spore σ^F is replaced by σ^G. Moreover all four of these sporulation-specific σ factors are inactive at the moment of their synthesis. The σ^E and σ^K factors are synthesized in the mother cell as inactive precursor proteins, while in the developing spore σ^F and σ^G are held in an inactive state by associ-

ation with inhibitory proteins. In particular, σ^K is synthesized as a larger but inactive precursor, pro-σ^K. Activation of σ^K in the mother cell occurs by proteolytic removal of its 20 amino acid N-terminal (Kroos et al., 1989). During sporulation, the activation of σ factors is carefully regulated. Pro-σ^K associates with both the mother cell membrane and the outermost membrane surrounding the forespore after engulfment. The processing enzyme, thought to be the product of *spoIVFB*, is also made in the mother cell and is located in the same membrane as pro-σ^K (Kroos et al., 1999). However, cleavage in the mother cell is not constitutive, but depends on a protein located in the inner membrane of the forespore, the spoIVB protein (Cutting et al., 1991). Concentration or timing changes of this protein, which is synthesized under control of σ^G in the forespore, changes the timing of pro-σ^K, which strongly supports a regulatory role for spoIVB in that processing (Stragier and Losick, 1996). Two other membrane proteins, believed to form a complex with it, block the activity of the spoIVFB protease. This complex is thought to be the target of the spoIVB protein from the forespore. This interaction relieves the inhibition of the protease, and pro-σ^K is processed in response to the forespore signal. Thus, during *B. subtilis* sporulation, the mother cell and forespore interact with each other to activate a stage-specific sigma factor.

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