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THINKING ABOUT BACTERIAL POPULATIONS AS MULTICELLULAR ORGANISMS

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ABSTRACT

It has been a decade since multicellularity was proposed as a general bacterial trait. Intercellular communication and multicellular coordination are now known to be widespread among prokaryotes and to affect multiple phenotypes. Many different classes of signaling molecules have been identified in both Gram-positive and Gram-negative species. Bacteria have sophisticated signal transduction networks for integrating intercellular signals with other information to make decisions about gene expression and cellular differentiation. Coordinated multicellular behavior can be observed in a variety of situations, including development of *E. coli* and *B. subtilis* colonies, swarming by *Proteus* and *Serratia*, and spatially organized interspecific metabolic cooperation in anaerobic bioreactor granules. Bacteria benefit from multicellular cooperation by using cellular division of labor, accessing resources that cannot effectively be utilized by single cells, collectively defending against antagonists, and optimizing population survival by differentiating into distinct cell types.

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INTRODUCTION: A DECADE OF CHANGES

Ten years ago, *Scientific American* published an article entitled "Bacteria as Multicellular Organisms" (141). It was based largely on the observation of pattern and organized cellular differentiation in the colonies of many bacterial species, including *E. coli*. While stimulating interest in the idea that bacteria may be more interactive than generally realized, that 1988 article did not convince most microbiologists that multicellularity should be considered a basic tenet in our thinking about how bacteria operate. Multicellularity was still widely considered a specialized adaptive strategy of particular groups, such as the *Myxobacteria* or *Actinomycetes*. The historical tradition of single-cell, pure-culture microbiology derived from Koch's postulates and medical bacteriology still held sway, despite an old and well-established alternative interactive, multicellular tradition based on environmental microbiology (42).

Today, the intellectual landscape is dramatically different. Intercellular communication and concerted multicellular activities are now generally accepted to be common among bacteria (62, 81, 184). The change in thinking is due mainly to the discovery that "quorum-sensing" signal molecules, in particular the *N*-acyl homoserine lactones (AHLs) in Gram-negative species, are used throughout the eubacterial kingdom to regulate the expression of a wide variety of phenotypes (40, 54, 55, 90, 129). The past decade has also witnessed the discovery of new phenomena, such as autoaggregation of chemotactic bacteria (20, 24, 25, 185) and coordinated behaviors in complex colony morphogenesis (12, 14, 53, 104, 109). The molecular basis of intercellular coordination is being clarified in multicellular taxa such as the *Myxobacteria* (152) and *Streptomycetes* (33), and homologies have been discovered between intercellular and unicellular regulatory circuits (177). Our knowledge of biochemical and thermodynamic coordination within microbial consortia has grown, particularly during anaerobic biotransformations (e.g. 47, 130). And new optical and

molecular technologies reveal pattern and physiologically significant spatial organization among bacterial populations (e.g. 52).

This review emphasizes the conceptual basis for thinking about bacterial populations as multicellular organisms. I present my view of a few core ideas underlying an integrated view of bacterial multicellularity and then provide selected examples to illustrate them. A more detailed discussion of particular aspects can be found in the recent book dedicated to this theme (148).

CORE CONCEPTS OF BACTERIAL MULTICELLULARITY

Briefly stated, the core concepts of bacterial multicellularity may be summarized as follows:

1. Bacterial cells have communication and decision-making capabilities that enable them to coordinate growth, movement, and biochemical activities.

2. Examples of communication and coordinated behaviors are widespread (possibly ubiquitous) among bacterial taxa and are not limited to a few groups with a specialized multicellular vocation.

3. Bacterial populations derive adaptive benefits from multicellular cooperation and their ability to integrate the diverse activities of different cells. These benefits include (but are not limited to):

   (*a*) More efficient proliferation resulting from a cellular division of labor;

   (*b*) Access to resources and niches that cannot be utilized by isolated cells;

   (*c*) Collective defense against antagonists that eliminate isolated cells; and

   (*d*) Optimization of population survival by differentiation into distinct cell types.

INTERCELLULAR COMMUNICATION

Diverse Classes of Signal Molecules

Intercellular communication is the basis of coordinated multicellular function. Because the AHL field has been so well reviewed (40, 54, 55, 62, 62a, 129, 168), it is not discussed in detail here. But it is worthwhile pointing out several basic characteristics of AHL signaling. Because short-chain AHLs cross bacterial membranes freely, their concentration reflects total production and, consequently, total density of the producing population (82). Thus, a major function

of at least some AHL molecules is to provide a quorum-sensing function (54). The canonical quorum-sensing function is assumed to assure an individual cell of a critical population density before undertaking expression of specialized functions. AHL production is determined by genetic loci homologous to *Vibrio fischeri luxI* , and AHL concentration is sensed by proteins homologous to *V. fischeri* luxR. Significantly, modules of luxI–luxR homologues operate in many different bacterial control circuits governing a wide range of phenotypes, and the same AHL molecule can regulate distinct functions in different bacterial species (40, 54, 55, 62, 62a, 129, 168). Thus, *AHL/luxI/luxR* signaling systems are standard molecular routines that can be incorporated into regulatory circuits whenever multicellular control is adaptively useful. Some AHLs stimulate their own production and can establish positive autoinducer feedback loops, in effect serving as more than simple quorum sensors (40).

AHLs are far from unique as intercellular signals between bacteria. Density- and growth-phase phenomena are common, and their study has led to the identification of many additional classes of signaling molecules (Table 1).

The γ-butyrolactones of *Streptomyces* species may be considered analogues of the homoserine lactones (16), but other signaling molecules are quite distinct, including some ordinarily classed as toxins (antibiotics and bacteriocins). Overdose of a particular signal can be lethal, as with mammalian hormones like insulin. Interestingly, a *Rhizobium leguminosarum* bacteriocin has recently been found to be an AHL (133). In Gram-positive bacteria, antimicrobial peptides are signaling molecules as well, often stimulating their own production (Table 1; 90).

The oligopeptides are a widely utilized class of intercellular signals in Gram-positive bacteria (Table 1; 90), just as they are in eukaryotes. Oligopeptide pheromones can be considered the first class of prokaryotic communication molecule to be detected (in the 1960s) as competence factors in *S. pneumoniae* and *B. subtilis* (32, 46, 171, 172), but their structures were only recently determined (71, 102, 113, 156, 176). The tremendous diversity of oligopeptides makes them especially suitable when a high degree of discrimination is required, as in the use of distinct oligopeptide mating pheromones and inhibitors involved in regulating the transfer of each of many conjugative plasmids in gram-positive cocci (35, 124).

There are important differences between the properties of oligopeptides and AHLs, especially the fact that oligopeptides do not transfer freely across bacterial membranes, as do short-chain AHLs (82). In general, Gram-positive oligopeptide signals are synthesized from larger precursor proteins, transported outside the cell by ATP-binding-cassette (ABC) transporters, and detected by surface receptors that belong to two-component protein kinase regulatory systems (90). Because some receptors in Gram-positive bacteria show homology

Table 1 Signaling molecules other than *N*-acyl homoserine lactones

Bacterial species	Signal	Molecular class	Phenotype affected	Citation
Myxococcus xanthus	A factor	Amino acid (mixture)	Fruiting body development	83
Escherichia coli	Aspartate, glutamate	Amino acid	Chemotactic autoaggregation	24, 25
Streptomyces griseus	A-factor	γ-butyrolactone	Sporulation, streptomycin production	16, 75
Streptomyces virginiae	VB-A	γ-butyrolactone	Virginiamycin production	75
Streptomyces sp. *FR1-5*	IM-2	γ-butyrolactone	Staphylomycin production	16, 75
Streptomyces sp. Y-86,36923	Butalactin	γ-butyrolactone	Antibiotic production	16
Streptomyces viridochromogenes	Factor I	γ-butyrolactone	Anthracycline production	16
Streptomyces alboniger	Pamamycin-C	Macrolide	Aerial mycelium formation	75
Streptoverticillium sp.	Carbazomycinal	β-lactam derivatives	Aerial mycelium formation	75
Nocardia sp.	B-factor	Adenosine derivative	Rifamycin production	75
Cylindrospermum licheniforme	(Unnamed)	Fused lactam, thioketon rings	Akinete formation	75
Stigmatella aurantica	Lipid pheromone	2,5,8-trimethyl-8-hydroxy-nonan-4-one	Fruiting body formation	77, 161
Myxococcus xanthus	E-factor	Branched-chain fatty acids	Fruiting body formation	169, 184
Staphylococcus aureus	Rap	Octapeptide	Toxic exoprotein, virulence factor secretion	78
Bacillus subtilis	ComX	Decapeptide, modified tryptophan	Transformation competence	102
Bacillus subtilis	CSF	Pentapeptide	Transformation competence, sporulation	156
Bacillus subtilis	Sporulation factor	Oligopeptide	Sporulation	176
Streptococcus pneumoniae	ComC	Heptadecapeptide	Transformation competence	71
Lactococcus lactis	Nisin	Oligopeptide	Nisin (lantibiotic) production	90
Lactobacillus plantarum	Bacteriocin inducing factor	26 amino acid oligopeptide	Plantaricin (class II antimicrobial)	90
Lactobacillus sake	Bacteriocin inducing factor	Oligopeptide	Sakacin (class II antimicrobial)	90
Carnobacterium piscicola	Bacteriocin inducing factors	24 and 49 amino acid oligopeptides	Carnobacteriocin (class II antimicrobial)	90
Enterococcus faecalis	Sex pheromones	Oligopeptides, plasmid-specific	Agglutination, plasmid transfer	35, 124
Enterococcus faecalis	Sex pheromone inhibitors	Oligopeptides, plasmid-specific	Inhibit sex pheromone binding	35, 124
Myxococcus xanthus	C factor	Protein	Fruiting body formation	88, 152

to oligopeptide uptake systems from enteric bacteria, it will not be surprising to discover oligopeptide signals in the Gram-negatives as well.

Not all the small diffusible molecules used for interbacterial communication belong to special classes of dedicated signals. Amino acids also serve as communication molecules in the initiation of fruiting body formation in *Myxococcus xanthus* (83, 88, 152) and during autoaggregation in chemotactic *E. coli* (24, 25). In *M. xanthus*, the extracellular A signal is a mixture of several amino acids generated by surface protease activity (83). When the mixture reaches a density threshold of 10 μM at $\geq 3 \times 10^8$ cells per ml, it activates a cellular

signal transduction system leading to expression of early functions needed for fruiting body development. By serving as a signal to ensure that development only initiates above a critical cell density, A signal fills the canonical quorum-sensing function. The role of amino acids in chemotactic autoaggregation is different. Cells of chemotactic *E. coli* can be observed to form punctate aggregates in fluid medium, often generating striking spatial patterns of spots, lines, and circles (24, 25). Autoaggregation can be blocked by analogues or mutations blocking Tar chemotaxis receptor (24), and excretion of aspartate and glutamate, powerful chemoattractants, occurs in stressed cultures (25). Both amino acids thus signal *E. coli* cells to change their density under conditions when it may be advantageous to do so.

Our knowledge of intercellular signaling by larger proteins is still limited to a few examples (Table 1). The best characterized is the C factor of *M. xanthus* (88, 152). Although we do have a great deal of information about pili and type IV transport systems in interbacterial DNA exchange (36), the role of complex surface organelles such as pili, macromolecular transport complexes, and extracellular fibrils in bacteria-bacteria signaling remains largely to be explored in all but a few bacteria. Pili are required for social motility, fruiting body development, and spore formation in *M. xanthus* (80, 186). Similarly, *M. xanthus* mutants defective in the formation of extracellular fibrils are blocked in cohesion in liquid suspension and are defective in motility, predation, and all stages of fruiting body formation (6, 9, 10). These deficits can be corrected by addition of purified fibrils to a fibril-less mutant culture (30). Piliation is also an important determinant of *N. gonorrhoeae* aggregation and colony morphology (167). In scanning electron micrographs, networks of pili can be seen to connect the gonococcal cells (170). There has long been known to be a correlation between *N. gonorrhoeae* colony morphology and virulence (87). It is ironic that our understanding of the strictly prokaryotic roles of complex surface organelles in cell-cell communication lags behind our understanding of their roles in communication between prokarytoic and eukaryotic cells during symbiosis and pathogenesis (7, 48). Such elaborate systems must surely also be utilized to coordinate the activities of distinct bacterial cells.

Given our growing knowledge of the sophisticated ways that nodulating bacteria use specific exopolysaccharides to alter plant cell behavior (7, 39, 96), we can also expect to find these highly diversified chemical structures used for interbacterial communication. Exopolymers are certainly an important component of many multicellular populations, such as *P. putida* and *E. coli* colonies, where they are readily visualized by scanning electron microscopy (SEM) (139, 140), and they play a critical role in collective motility.

Signal Response Systems: Interpreting Chemical Messages in an Informationally Rich Environment

From the perspective of the core multicellularity concepts outlined above, it is to be expected that intracellular systems responding to intercellular signals will be molecular computing networks allowing each cell to make appropriate decisions and adjust its activity to coordinate with other cells in the group. This expectation is very much in line with current thinking about the decision-making capabilities of cells in higher organisms (5, 22). Beppu (16) has pointed out parallels between the use of protein kinases by *Streptomyces griseus* in the γ-butyrolactone response and in eukaryotic signal transduction systems. The *Myxobacteria* and *Bacillus* communities have documented two good examples of complex signal-processing networks that respond to intercellular cues.

Fruiting body development and sporulation in *M. xanthus* involve a cascade of signaling events (152). Commitment to fruiting body formation must occur under suitable conditions of cell density and spatial organization (88, 125, 126). Transfer of C-factor signal requires cell motility to achieve the proper alignment of the signaling bacteria (127) and reinforces this alignment by modulating the activity of the intracellular Frz network, which controls the reversal of *M. xanthus* cell movement (153, 177). The C-factor–Frz interaction appears to generate the periodic rippling that precedes formation of fruiting bodies (127) and is thought to accomplish two important goals: building up cell density and aligning cells for subsequent morphogenetic movements. Genetic studies show that the C-factor affects other signal transduction components as well as the Frz system and also positively stimulates its own production (154).

Both *B. subtilis* competence and sporulation depend on population density and extracellular factors (63, 64, 155, 176). There are two extracellular factors stimulating competence: the ComX pheromone, a modified decapeptide (102), and competence stimulating factor (CSF), a pentapeptide (156). There are at least two extracellular sporulation factors as well (64), and one of these is CSF, which serves both as a competence and sporulation factor (156). Commitment to competence and sporulation involve major cellular changes at the end of exponential growth in response to external, internal, and intercellular conditions (63, 157). They represent mutually exclusive cellular differentiation outcomes. Besides extracellular signals, factors determining the competence/sporulation decision include nutritional deprivation, glucose, TCA cycle activity, and status of the genome with respect to cell-cycle and DNA damage. The key competence regulator is the ComK transcription factor, and the key sporulation regulator is phosphorylated Spo0A transcription factor. Common elements lead to activation of both factors, such as Spo0K permease, the CSF receptor. The ability of

an individual cell to integrate all the signals and decide between competence and sporulation requires the operation of a highly interconnected regulatory network including negative feedback between the competence and sporulation pathways, a four-step phosphorelay leading to Spo0A phosphorylation, and positive feedback loops on the final expression of ComK and Spo0A∼P (63). This elaborate molecular network is the kind of distributed computing system described by Bray (22).

A strong prediction of the multicellular view is that the complexities of the *M. xanthus* and *B. subtilis* signal response systems will prove to be typical rather than exceptional among bacteria. This prediction is being realized with the AHL molecules. Further sophistication in the cellular responses to AHLs has become evident with the discovery of the use of multiple AHLs to influence a particular phenotype, such as bioluminescence (40) or virulence (183), and of sequential cascades of AHL signaling linked to signal transduction functions such as RpoS in *P. aeruginosa* (92, 112, 134). AHL signaling must involve more than quorum-sensing in cases where exogenous AHLs are not sufficient to stimulate exoprotein production in low-density cultures of *E. carotovora* (107) and *P. aeruginosa* (31).

COORDINATED MULTICELLULAR BEHAVIORS—A GENERAL BACTERIAL TRAIT

We often forget that the well-agitated suspension culture is largely a laboratory construct. Many microbiologists no longer remember that most bacteria proliferate and survive attached to surfaces (182). When we examine surface cultures, we find that bacteria differentiate biochemically and morphologically, and they interact in ways that produce spatially organized populations. It is worth pointing out that colony development and collective motility phenomena in bacteria (e.g. 41, 72, 151) hold valuable lessons for understanding the formation and development of biofilms, perhaps the most widespread multicellular prokaryotic structures in nature (38, 115). It has recently been reported that an AHL plays an essential role in the spatial organization of *P. aeruginosa* biofilms in the laboratory (38a).

E. coli Colony Development

Cell-cell interactions in an *E. coli* microcolony begin after the first cell division. The two daughters elongate alongside each other unless one of the sibling bacteria is attracted by a third nearby bacterium (150). Attractions and fusions of microcolonies are invariably observed, even when the chemotactic sensory system is absent (144, 150). The standard rule for *E. coli* microcolonies is to maximize cell-to-cell contact, i.e. population density, rather than individual

cell access to substrate. Nonetheless, cells on agar divide just as rapidly as cells in comparable well-aerated liquid medium (142). Thus, *E. coli* has evolved to reproduce efficiently in a multicellular context.

Multicellular, density-dependent aspects of initial colony spreading can be observed most readily with colonies inoculated as small spots from a micropipette (140). The cells at the edge of a spot inoculum are initially disordered but align themselves during the first two hours of growth after inoculation (150). Before the inoculated spot expands, it fills in and cells around the periphery pile up to create a multilayered mound (140). Once this mound has formed, active expansion over the substrate begins (146). This structure includes exopolymers visible in micrographs (140, 146) and develops into a distinct peripheral zone demarcated by a deep groove, which expands with the colony (140). The groove appears to define the region of active spreading over the substrate, consistent with geometrical interpretations of colony growth dynamics (117). Using small fragments of glass wool as probes, it can be confirmed that colony spreading involves the development of multicellular structure rather than autonomous cell divisions at the periphery. When a spreading colony encounters such a fiber, motile cells at the periphery swim into the liquid around the fiber, coat it, and form a younger population. With short fibers (<100 microns), the coating population does not have time to develop the structure needed for spreading and stays in a small zone along the fiber, which is engulfed by the advancing colony (146).

After 24 hours or more of development, *E. coli* colonies display considerable spatial organization. Organized cellular differentiation was first detected as concentric patterns of beta-galactosidase staining (136, 137). Some of these patterns resulted from expression of stable *lacZ* fusions, including those to *polA* (143, 146), but other patterns reflected the formation of concentric zones of derepression and replication of Mu*dlac* elements under control of the Mu*cts62* repressor (145, 149). Concentric patterning was also visible in surface contours of colonies lacking genetic modifications (143–145). SEM examination revealed zones within colonies characterized by cells of distinct sizes, shapes, and patterns of multicellular arrangement (140). Vertical sections through colonies revealed stratification into layers of cells with different protein contents, many of which appeared to be nonviable (144). Such zones containing dead bacteria are analogous to the stalks of Myxobacterial fruiting bodies created by cell lysis (152). Programmed cell death clearly plays a role in colony and fruiting body morphogenesis, and bacterial examples of this phenomenon are rapidly accumulating (4, 29, 51, 187). All these data indicate an unanticipated capacity for cellular differentiation and creation of discrete zones of differentiated cells ("tissues") in *E. coli* K12 colonies. The *E. coli* results parallel the earliest systematic observations of colony development showing spatially organized cellular differentiation (95).

B. subtilis Colony Development

Morphogenetic studies of *B. subtilis* colonies were pioneered by physicists interested in pattern formation (12, 14, 53, 104). By altering nutrient levels and agar concentrations, they observed transitions between distinct colony morphologies corresponding to forms described in inorganic material. At lowest nutrient levels and highest agar concentrations, *B. subtilis* colonies assumed a fractal shape typical of structures generated by diffusion-limited aggregation (DLA) (12, 14, 53, 104, 110). DLA shapes occur when the physical processes of nutrient diffusion govern bacterial growth, i.e. when the bacteria are starved, incapable of active motility, and have lost capabilities to control morphogenesis. As nutrient concentrations increase (but still at high agar concentrations), colonies assume the so-called Eden configuration and expand by creating arrays of highly elongated cells encased in exopolymer around the periphery (12, 104, 175). At intermediate nutrient and agar concentrations, dendritic patterns develop that increase colony surface area to permit more efficient uptake of nutrients. Inside the dendrites, groups of moderately elongated, highly motile cells are found encased in an envelope of exopolymer (15). The dendrites advance as the cell groups push on the exopolymer envelope. At certain agar and nutrient concentrations, transitions occur from highly branching to more compact colonies. These transitions have been modeled as resulting from activation of long-range negative chemotaxis functions (13). The physics-inspired approach thus implicates both group motility, with an important role for exopolymers, and intercellular chemotactic signaling.

Besides fascinating colony shapes, patterns of differential *lacZ* expression from synthetic gene fusions analogous to those in *E. coli* colonies have recently been observed in *B. subtilis* (108, 109, 128). When colonies carrying the fusion constructs are placed on agar capable of inducing different colony morphologies, the beta-galactosidase patterns are observed to be "nested" inside the overall colony shape, suggesting a connection between the control of colony expansion and *lacZ* fusion expression (108).

Proteus and Serratia Swarming

B. subtilis motility in colonies is related to a collective process observed in both Gram-positive and Gram-negative species, called swarming (72). Swarming involves rapid migration over a surface by groups of elongated, hyperflagellated "swarmer" cells encased in exopolymers (11, 166, 179). Length and hyperflagellation distinguish swarmer cells from "swimmer" cells, which resemble motile *E. coli* and are capable only of swimming in fluid medium, not of migrating over agar surfaces (74). Starting with the book Hauser published over a century ago (70), swarming has been studied most intensively in *Proteus mirabilis* and *Proteus vulgaris*. Recent studies have also focused genetic and

molecular techniques on *Serratia marcescens* (106, 111) and *Serratia liquefaciens* (43, 44, 57, 58).

The key aspect of swarming motility is its collective nature. Isolated swarmer cells do not migrate over agar, only groups or "rafts" of aligned swarmers do (165). Raft size is proportional to the hardness of the agar because swarming is more difficult at higher agar concentrations (119, 163, 164). Swarmers are also encased in exopolysaccharide as they migrate (159). The acidic capsular polysaccharide produced by *Proteus mirabilis* plays a key role in swarming mobility; mutants that lack it are inhibited in their migration (67). The inherent multicellularity of *Proteus* swarm colony morphogenesis provides part of the explanation for the formation of symmetrical colonies with periodically spaced terraces (18). Terracing results from cycles of alternating swarming and consolidation phases (11). Swarming periodicity is not based on cycles of nutrient exhaustion and chemotactic migration (119). Instead, it is possible to explain the clock-like behavior of swarming *Proteus* colonies by variation in a multicellular parameter, the age-weighted swarmer cell population density (45).

Genetic studies with *Serratia liquefaciens* have revealed dual genetic control of swarming in that species (57, 58). Swarmer cell differentiation is triggered by ectopic expression of the FlhDC motility/chemotaxis regulators (43). A second level of swarming control in *Serratia* involves AHL signaling. Mutation of the *swrI* homologue of *luxI* leads to a swarm-defective phenotype (44). The AHL does not play any role in swarmer cell differentiation but instead stimulates production of a surfactant essential for *Serratia* swarming (58). *Serratia* swarm colonies produce cyclic peptide surfactants critical to motility (105), and the *swrI* mutant phenotype can be reversed by the addition of detergents (PW Lindum, U Anthoni, G Christoffersen, L Eberl, S Molin, M Givskov, manuscript in preparation).

Granule Development in Anaerobic Bioreactors

In nature and industry, most biotransformations are carried out by microbial consortia, not by monocultures (100, 130). Such consortia have definite physical organizations, most commonly biofilms or granular aggregates. A paradigm of spatially organized consortia demonstrating self-organization is the class of biotransforming granules that form in upflow anaerobic sludge blanket (UASB) reactors (132). UASB reactors are the most widely used form of high-rate reactor for anaerobic biological wastewater treatment to achieve the biodegradation of organic substances to CH_4 and CO_2. Ancillary objectives include the breakdown of toxic pollutants, such as halogenated hydrocarbons (21).

In the UASB granules, several different groups of bacteria carry out sequential metabolic processes (132, 160): (*a*) conversion of xenobiotics to biodegradable molecules, as in dehalogenation; (*b*) hydrolysis of polymers to small molecules;

(*c*) fermentation of small molecules to H_2, CO_2, acetate, and short-chain volatile fatty acids (mainly propionate and butyrate); (*d*) oxidation of volatile fatty acids to acetate and H_2/CO_2; and (*e*) conversion of acetate, H_2, and CO_2 to methane by aceticlastic methanogens.

The organisms that carry out these diverse processes are organized in granules of about 0.1–5 mm in diameter. The size and composition of the granules depend on the wastewater composition and conditions such as temperature (3, 99, 160). Much of the physical integrity of the granules is due to large amounts of exopolymers, particularly proteins and polysaccharides (65).

The microbial flora of these granules is rich in the aceticlastic methanogens, including *Methanosarcina* spp. (66, 132). *Methanosarcina* can transition between a multicellular aggregate and a disaggregated single-celled form (37). In addition, many propionate and butyrate-fermenting *Syntrophobacter* spp. and *Syntrophomonas* spp. have been identified in microcolonies in intimate association with the methanogens (3, 132, 160). The spatial organization of the bacteria is critical for thermodynamic reasons. The partial pressure of hydrogen must be kept low to ensure efficient fermentation of the volatile fatty acids (130, 131). Many interspecific syntrophic reactions are only energetically beneficial if hydrogen transfers occur over distances of a few microns or less (160). Because of the need for such close proximity, random cell–cell associations would lower metabolic efficiency. On this basis, signaling mechanisms to organize the syntrophic species can be predicted. Larger-scale organization is observed in the distribution of distinct species (99) and of distinct metabolic processes (3) within the UASB granules. By introducing new species, the metabolic capabilities and substrate range of the granules can be extended. Dechlorination ability was acquired by a bioreactor seeded with *Desulfomonile tiedjei*, and the *Desulfomonile* cells were seen by immunofluorescence to be incorporated into the granules (2).

ADAPTIVE BENEFITS FROM MULTICELLULAR COOPERATION

More Efficient Proliferation from Cellular Division of Labor

In a 1988 *Scientific American* article, nitrogen-fixing heterocyst formation in filamentous photosynthetic cyanobacteria was cited to illustrate how two cell types could cooperate in a monospecific multicellular population (1, 141). Likewise, the ability of *Proteus* to spread rapidly over an agar surface depends on swarmer cell differentiation, and the velocity of spreading is directly related to the rate of biomass production (119). Monocultures also display

larger-scale functional differentiation of various cell groups, such as the stalks and sporangia in Myxobacterial fruiting bodies (152) and substrate and aerial mycelia in *Streptomyces* (33).

Microbiologists know myriad cases where biochemical cycles involve the participation of multiple different species, and the example of *Methanobacillus omelianskii*, supposedly a single organism that is actually a symbiotic association of two different species (23), is a classic reminder of the limitations of the pure culture approach (42). Laboratory studies document increased biodegradation by mixed cultures (e.g. 47). Syntrophic processes mediated by microbial guilds or consortia underlie the mineral cycles and thermodynamics of biogeochemistry (100). As with the UASB granules, we need to find out how capabilities for communication and spatial organization are used interspecifically. Interspecific plasmid transfer and communication of symbiotic and pathogenic organisms with their eukaryotic hosts demonstrate that many bacteria can coordinate their behaviors with other species (7, 48). In some cases, such as the type IV transport systems related to plasmid transfer mechanisms, the parallel is explicit between prokaryote-eukaryote and interspecific communication among bacteria (7, 181). An interesting variant on interspecific signaling is the excretion of chemoattractants by predatory *M. xanthus* to entrap prey *E. coli* (151).

Access to Resources and Niches That Require a Critical Mass and Cannot Effectively Be Utilized by Isolated Cells

In common laboratory media, bacteria are supplied with simple growth substrates readily utilized by individual cells. In nature, many bacteria break down complex organic polymers, requiring the concerted action of many cells. The predatory *Mxyobacteria* utilize a "wolf pack" strategy to attack and lyse their prey organisms by liberating digestive extracellular enzymes and absorbing the cell contents (122). Using *M. xanthus* and casein as a model substrate, substrate utilization was found to be dependent on population density (121). Interestingly, groups of *M. xanthus* cells can migrate chemotactically but individual cells cannot (151). *M. xanthus* grazes on cyanobacteria in ponds (27). However, the aqueous environment can dilute both the lytic exoenzymes and liberated nutrients. Thus, the predators construct spherical colonies and trap prey organisms in pockets where lysis and feeding can occur efficiently (27), showing that multicellular behavior to permit resource utilization includes the capacity for morphogenesis of organized macroscopic structures.

Other bacteria illustrate the role of population density in resource utlization. The phytopathogen *Erwinia carotovora* synthesizes exoenzymes for plant cell wall degradation, but does so under control of an AHL quorum-sensing system (79, 116). The circuit apparently ensures that the bacteria will only invest their

cellular capital in digestive exoenzyme production once there are sufficient cells for effective attack on the plant structure. A similar strategy apparently accounts for the AHL control of synthesis of elastase, toxins, and other virulence factors by the opportunistic pathogen *Pseudomonas aeruginosa* (92, 112). An extra rationale for quorum-sensing by pathogens is to restrain synthesis of virulence factors that, if synthesized constitutively, could be detected by host defense systems at subeffective concentrations and lead to the destruction of low-density populations (62a, 162).

Collective Defense Against Antagonists That Eliminate Isolated Cells

Many agents can effectively kill isolated bacterial cells in suspension but are ineffective against dense or organized populations of the same bacteria. One example is catalase protection against oxidative damage. There is no difference in the survival to hydrogen peroxide challenge of *cat* (catalase-defective) and *cat*$^+$ (catalase-positive) *E. coli* strains when tested in dilute suspension, but in thick suspensions or in microcolonies on an agar surface, the *cat*$^+$ strain shows much greater resistance (98). Appropriately, catalase expression is RpoS-dependent (91). Another example is a penicillin-resistant biofilm on a pacemaker composed of penicillin-sensitive *S. aureus* bacteria; the biofilm fed a recurring septicemia that could be cleared but never eliminated by antibiotic therapy (103). It appears to be a general rule that organization in biofilms and colonies provides enhanced resistance to a wide range of antibacterials (38, 89, 180).

Multicellular defense also has an aggressive aspect. Many bacteria produce antibiotics, generally under the control of intercellular communication and quorum-sensing systems. In the *Actinomycetes*, antibiotic synthesis is frequently regulated by γ-butyrolactone signaling molecules and is coupled with morphological differentiation (Table 1). Similarly, the synthesis of carbapenem by *Erwinia carotovora* to eliminate competitors for nutrients liberated by degraded plants is regulated by a two-stage AHL hierarchy (107). In the Gram-positive bacteria, a number of antimicrobial peptides are also subject to autoinduction (Table 1) (90).

Optimization of Population Survival by Differentiation into Distinct Cell Types

Inevitably, bacterial populations encounter new circumstances in which they will have to survive diverse physical, chemical, nutritional, and biological challenges. From an ecological point of view, the population is the key biological entity. Its survival depends upon having the right cells when confronted by phage attack, antibiotics, desiccation, or the need to utilize novel growth

substrates. Populations have several distinct mechanisms for creating new cell types.

SPORULATION AND FORMATION OF DORMANT CELLS The most obvious diversification is formation of spores or other resistant, dormant forms. Sporulation is subject to intercellular signaling and multicellular regulation in all cases that have been closely investigated, including *B. subtilis* (63), *M. xanthus* (152), and *Streptomyces coelicolor* (33). Moreover, spore formation is often connected with elaborate processes of multicellular morphogenesis. Thus, spore formation can be considered a function of the entire interactive population. Among nonsporulating bacteria, dormant forms can survive for long periods under difficult conditions. In *E. coli*, survival in stationary phase involves several factors, including the RpoS sigma factor (91), which is subject to quorum-sensing control in *P. aeruginosa* (92) and possibly also in *E. coli* (76). An interesting corollary to multicellular control of dormancy is the suggestion that exit from dormancy involves intercellular signaling and "wake-up" pheromones, both in isolated cells (84, 85) and in biofilm populations (8). This may explain why many dormant cells are difficult to culture from dilute suspension but not from denser inocula. An extracellular protein needed for resuscitation of dormant *Micrococcus luteus* cells has been reported (86).

EXCHANGE OF GENETIC INFORMATION Besides sporulation, *Bacillus subtilis* populations can develop subpopulations competent for DNA uptake with potential to incorporate new genetic information and thereby novel proliferation and survival abilities (155). Development of competence is a multicellular process involving intercellular signaling and elaborate signal processing. In *B. subtilis*, competent cells can take up DNA from any source (155). A different kind of competence occurs in *Neisseria gonorrhoeae*. Competence is constitutive (19) and is restricted to the uptake of DNA fragments carrying an *N. gonorrohoea*–specific sequence tag (60). *Neisseria* populations use DNA exchange for the purpose of stimulating recombination (gene conversion) events that alter the primary structures of their surface proteins (including pili) (167). Such phase and antigenic variations in surface protein structure allow *Neisseria* populations to modulate aggregation and virulence properties and to evade immune surveillance.

DNA exchange mechanisms are ecologically significant. Plasmids, phages, transposons, and other mobile genetic elements play major roles in the evolution of antibiotic resistance (17, 26, 178), pathogenicity determinants (34, 48), and new catabolic pathways (174). Taking our knowledge of genetic exchange to its logical conclusion, Sonea & Panisset (158) developed the radically multicellular concept of the distributed prokaryotic genome. They argued that there is one

large bacterial genome encoding more functions than can be accommodated in any individual cell, but that every bacterial species has access to the whole through mobile elements. Thus, bacteria can be tailor-made for new ecological niches (cf. 93).

MUTATION DNA restructuring frequently involves the same mobile elements that participate in intercellular genetic exchange (17, 26, 138, 147). Even purely intracellular processes of genetic change serve to diversify the parental population. For survival of the individual bacterium, loss of surface receptors is almost always not beneficial, but the reliable appearance of receptorless mutants means the population as a whole will survive phage attack. Similar logic applies to bacteria with impaired ribosome efficiency plus streptomycin resistance (61). Many natural bacterial populations, such as pathogens, have a mutator phenotype, suggesting advantages to elevated rates of spontaneous mutation in highly variable environments (94).

The phenomenon of adaptive mutation indicates that bacteria can increase their mutational activity in response to starvation and other stress conditions (49, 123, 147). Enhanced mutagenesis under selection or starvation is almost always studied in dense, postlogarithmic populations wherein intercellular signaling is most intense: lawns on selective plates (28, 135), papillae forming on aging colonies (68, 69), and saturated liquid cultures (101). For example, in the *lac33* frameshift reversion system, there is a role for F'*lacpro* transfer functions that are activated by the conditions prevailing in dense, highly aerobic surface populations (56, 114, 118).

Several suggested mechanisms for adaptive mutation can reasonably be considered in a multicellular context. One is the proposal that some cells in stressed populations undergo extensive DNA changes, do not survive, but donate fragments of their rearranged genomes to other cells, which then go on to proliferate as the experimentally detected adaptive "mutants" on selective media (73, 120). The view that signal transduction networks stimulate the action of natural genetic engineering systems in response to starvation or other stresses (147) has been strengthened by the observation that the growth phase regulators, RpoS and HNS, have opposite effects on the *araB-lacZ* fusion system (59). The RpoS requirement for fusion formation suggests potential quorum-sensing involvement (76), even though an *E.coli* AHL signal has not been detected (P Williams, GSAB Stewart, personal communication).

The recent observation that many revertants of the *lac33* frameshift contain additional unselected mutations provides experimental support for a hypermutable state under selective conditions (50, 173). Hall's original proposal for a hypermutable state (in which individual cells undergoing elevated random mutagenesis perish unless they produce the right mutation for the current selective

conditions) has a multicellular logic (69). A single hypermutable cell has little probability of survival, but a population containing many hypermutable bacteria can multiply the chances of success by orders of magnitude and thus has a clear advantage, when in trouble, to sacrifice a subpopulation to the hypermutable state.

Critical tests of a possible multicellular dimension to intracellular processes of genetic change must come from experiments on the regulatory mechanisms underlying adaptive mutation. A key focus will be the roles, if any, of intercellular communication molecules.

CONCLUDING COMMENTS: IS THERE A CONFLICT BETWEEN CELLULAR AND MULTICELLULAR VIEWS OF BACTERIAL POPULATIONS?

Through studies of collective behaviors and intercellular signaling molecules, we are beginning to appreciate the extensive capacities for communication and coordination that enhance bacterial power to operate in the biosphere. Collectively and coordinately, bacteria act far more efficiently than they could as autonomous agents. Any effort to provide a formal definition of bacterial multicellularity or draw boundaries between single-celled and multi-celled perspectives would be counterproductive. The key to bacterial multicellularity resides in the ability of each individual cell to receive, interpret, and respond to information from its neighbors. In other words, recognizing bacterial multicellularity deepens our appreciation of the information-processing capabilities of individual bacterial cells. Meaningful information transfer between components and the system as a whole is integral to the notion of organism. I predict the concept of organism will increasingly be seen as a fundamental idea throughout science, which in all fields is moving away from Cartesian reductionism toward a more connectionist, interactive view of natural phenomena. Exploring this more organic view of nature will require detailed investigation of self-organizing complex systems. Bacteria provide some of the best experimental material available (145, 148). Thus, thinking about bacterial populations as multicellular organisms may help put microbiology at the top of the scientific agenda in the 21st Century.

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