Division of Labor in Biofilms: the Ecology of Cell Differentiation

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ABSTRACT The dense aggregation of cells on a surface, as seen in biofilms, inevitably results in both environmental and cellular heterogeneity. For example, nutrient gradients can trigger cells to differentiate into various phenotypic states. Not only do cells adapt physiologically to the local environmental conditions, but they also differentiate into cell types that interact with each other. This allows for task differentiation and, hence, the division of labor. In this article, we focus on cell differentiation and the division of labor in three bacterial species: Myxococcus xanthus, Bacillus subtilis, and Pseudomonas aeruginosa. During biofilm formation each of these species differentiates into distinct cell types, in some cases leading to cooperative interactions. The division of labor and the cooperative interactions between cell types are assumed to yield an emergent ecological benefit. Yet in most cases the ecological benefits have yet to be elucidated. A notable exception is M. xanthus, in which cell differentiation within fruiting bodies facilitates the dispersal of spores. We argue that the ecological benefits of the division of labor might best be understood when we consider the dynamic nature of both biofilm formation and degradation.

INTRODUCTION

One of the most remarkable features of the evolutionary process is its capacity to construct. In billions of years a primordial soup of organic compounds evolved to the theater of life extant today. This ability to construct is best illustrated by a number of transitions that have occurred during the natural history of our planet, such as the evolution of the first prebiotic cells, eukaryotes, multicellularity, and eusociality (1, 2). These transitions all bear a number of striking similarities (2). First, construction evolves through cooperation (3–6). That is, new organizational layers come about through the cooperative interaction of biological units that previously functioned independently. For example, organelles evolved from microbes that engaged in mutualistic interactions through endosymbiosis, and multicellularity evolved from cells that cooperate by sticking together, either via incomplete cell division or through aggregation (7). In addition to cooperation, a second aspect characterizes major evolutionary transitions: the division of labor (8, 9). A precise definition of the division of labor will be given below, but one can loosely speak of division of labor when individuals—during their cooperative interactions—specialize in performing different “tasks.” Perhaps the most striking example comes from multicellular development. Multicellular organisms consist of many specialized cell types (e.g., muscle cells, neurons, epithelia, etc.). Despite being genetically identical, these cells have differentiated and thereby organized themselves in different physiological and morphological structures (e.g., organs) that together make up the individual.

In this article we focus on the division of labor within bacterial biofilms. In contrast to multicellular organisms, the division of labor among bacterial cells in biofilms is less self-evident and thus the subject of some debate. This is partly because many classical evolutionary concepts, such as individuality, are mainly inspired
by metazoan life and are therefore not readily applicable
to microorganisms (1, 10, 11). For example, views differ
as to whether bacterial biofilms are a primordial form
of multicellular development or, rather, an aggregate
of individuals (12–14).

To get a common understanding of the concepts that
we use throughout this article, we first discuss the theo-
retical and conceptual basis of cooperation, phenotypic
heterogeneity, and the division of labor. This is
particularly important because the conceptual grounds
of the division of labor are strongly embedded in both
evolutionary and ecological theory. This part first ends
with a few well-examined case studies of multicellularity
and the division of labor in microbes. In the second part
of the article, we discuss the division of labor in biofilms
with a particular emphasis on Myxococcus xanthus,
Bacillus subtilis, and Pseudomonas aeruginosa.

COOPERATION, SPECIALIZATION,
AND THE DIVISION OF LABOR

Cooperation: an Alignment of Fitness Interests

The division of labor requires a cooperative interaction
between specialized individuals. Cooperation is defined
as a phenotypic behavior that is costly to perform for
an individual but benefits its interaction partner. In a
well-mixed population that consists of cooperative and
noncooperative individuals, one expects that the latter
have a selective benefit because they receive the benefits
of cooperation without paying the costs (3). For exam-
ple, imagine that there is a population of bacterial cells
that produce a siderophore to scavenge iron (15, 16). If
it is costly to produce the siderophore, a mutant that
stops producing it and still benefits from that produced
by others is expected to have a selective advantage
(Fig. 1A). It is therefore challenging to explain why co-
operation is not exploited. Since the division of labor
cannot evolve or be maintained in the presence of such
exploitation, we first have to explain the premise of co-
operation. There are multiple mechanisms that can ex-
plain the evolution of cooperation, which all result in the
emergence of assortative interactions: cooperators are
more likely to interact with each other than defectors are
to interact with cooperators (3, 17).

Perhaps the simplest mechanism to explain the evolu-
tion of cooperation is spatial segregation (18–21). When
cooperative genotypes grow separately from noncooper-
ative genotypes, exploitation is impossible. Under those
conditions cooperation will evolve because groups of
cooperative individuals perform better than groups of
noncooperative individuals. In multicellular organisms
that go through a single-cell bottleneck (e.g., metazoans),
cells are genetically identical and cooperation can easily
evolve because there is no risk of exploitation (5). By the
same token, we expect that cooperation more readily
evolves in monoclonal biofilms (or clonal pockets in-
side nonclonal biofilms), because there are fewer genetic
variants that could exploit cooperation. In this article,
we limit our discussion to monoclonal biofilms. This
does not imply that cooperation and, hence, the division
of labor cannot occur between genetically distinct indi-
viduals or even species. There are multiple mechanisms
that can explain the evolution of cooperation between
nonrelated individuals, and endosymbiosis is perhaps
the most remarkable example of such mutualistic inter-
action (3, 22–24).

Phenotypic Heterogeneity

A crucial aspect of the division of labor is the speciali-
zation of cells to perform different tasks. Phenotypic
variation can result from various proximate mechani-
sms: plastic responses to local environmental condi-
tions, noise in gene expression, epigenetic variation, or
genetic variation. In monoclonal biofilms, most pheno-
typic variation results from nongenetic differences. Here,
we refer to this variation as phenotypic heterogeneity.
In developmental biology, phenotypic heterogeneity is
typically studied by reaction norms, in which the phe-
notypic response of an individual is plotted against a
gradient of environmental conditions to which the in-
dividual is exposed (25, 26). Figure 1B shows a number
of reaction norms. When an individual is nonrespon-
sive, the reaction norm is flat (reaction norm 1); this
phenomenon is also called developmental robustness or
canalization. When an individual is plastic, it can either
respond in a linear fashion to changes in the environ-
ment or in a nonlinear way (reaction norms 2 and 3). When
individuals strongly specialize to perform differ-
ent tasks, such as when they divide labor, one expects
multiple alternative phenotypic states (reaction norm
4). The presence of discrete phenotypic states is also
known as polyphenism (as opposed to polymorphism, in
which alternative phenotypic states result from genetic
variation).

A number of features characterize reaction norms
underlying the division of labor. First, as mentioned
above, one expects that cells express a small number of
relatively discrete phenotypic states that represent the
alternative cell types (27). Cell types can develop in re-
response to discrete environmental signals or via a regu-
larly amplification of continuous environmental signals.

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using positive or double-negative feedback loops (28, 29). Regulatory feedback loops can also affect a cell’s commitment to a cell type. That is, such loops can result in bistable regulatory switches in which the environmental conditions that trigger the differentiation event are different from those that are necessary to revert to the original phenotypic state (e.g., hysteresis [30, 31]). When a cell’s commitment is irreversible, we speak of terminal differentiation. In the second half of the article we discuss a number of bistable regulatory switches in B. subtilis.

A second property of reaction norms underlying the division of labor is that different phenotypic states are mutually exclusive: a cell expresses either one of the alternative cell types. In the reaction norm of Figure 1B we only show a one-dimensional phenotypic response, but one can imagine that there are multiple dimensions; each dimension would correspond to an alternative cell type.

In conclusion, the division of labor is characterized by mutually exclusive and discrete phenotypic states that specialize in complementary tasks. It is important to note that various regulatory mechanisms can underlie the presence of these phenotypic states. Moreover, the presence of mutually exclusive phenotypic states does not necessarily imply that cells divide labor. There are many lifestyle switches that can result in the same reaction norms. For example, for certain bacteria the onset of biofilm formation is defined when some cells switch from a motile planktonic lifestyle to a surface-attached aggregative lifestyle. This switch is accompanied by two mutually exclusive and discrete phenotypic states: motile cells and matrix-producing cells (32). Thus, like cooperation, phenotypic specialization is a requirement for the division of labor but by itself is not sufficient to prove that there is division of labor.

Division of Labor

It is important to make a distinction between phenotypic specialization on the one hand and the division of labor on the other hand. Phenotypic specialization is a cell-level property, and the division of labor is a colony-level property. Even though the division of labor by definition requires the presence of different cell types, the presence of different cell types does not imply that cells divide labor. In a biofilm, cell specialization might simply be an adaptive response to the local environmental conditions to which cells are exposed and thereby does not involve cooperative interactions between different cell types. The only way to disentangle phenotypic specialization and the division of labor is by examining the fitness consequences of differentiation at both the cellular and colony levels (Fig. 1C) (by colony level we do not strictly mean the whole colony, but rather the level at which cells interact as a group). When cells divide labor, a colony that consists of multiple cell types performs better than a colony that consists solely of any one of them. It is important to note that this advantage is not a mere consequence of cells adapting to local environmental conditions, but rather an emergent property from the interaction between cells. For example, the division of labor allows cells to carry out specific tasks, thereby avoiding the regulatory or metabolic burden of switching between different tasks or expressing them simultaneously. This is, of course, only possible when cells specialize in complementary tasks and share the associated benefits. Thus, one can recognize the division of labor from the emergent fitness benefits that occur at the colony level due to the cooperative interaction of specialized cell types, in which the various cell types are somehow interdependent (33).

An alternative way, albeit indirect, to recognize the division of labor is to examine the cell-level consequences of cell differentiation. When cell differentiation reduces the fitness of a cell, its evolutionary origin can only be explained by colony-level fitness benefits that result from the division of labor (Fig. 1C) (when excluding alternatives like nonadaptive phenotypic heterogeneity or bet-hedging, see discussion below). Perhaps the most compelling example of this can be found in metazoans. Some cells in metazoans are destined to become gametes, whereas others terminally differentiate into somatic cells. Terminal differentiation can never be viewed as a cell-level adaptation because cells that become somatic do not contribute to reproduction and therefore have a relative fitness of zero. This is particularly apparent for cells that undergo so-called programmed cell death (34–37). The type of division of labor in which only a fraction of cells contribute to reproduction is called reproductive division of labor. Terminal differentiation is also present in some microbes (38). We discuss several examples of this below.

Although terminal differentiation excludes alternative hypotheses that can explain cell differentiation, it does not explain the benefits that are associated with the division of labor. These benefits are nevertheless responsible for the remarkable evolutionary success of the division of labor. For many biological systems a detailed understanding of the emergent properties that result in the interaction between cell types is lacking. In the second part of the article we argue that such understanding can often be acquired through ecology. In the following
BACTERIAL MULTICELLULARITY AND THE DIVISION OF LABOR

Although multicellular eukaryotes are well known for their remarkable organismic adaptations, multicellularity evolved about two billion years earlier in bacteria (39, 40). Here we focus on two instances of bacterial multicellularity: filamentous multicellularity in cyanobacteria and aerial hyphae in actinobacteria (5). These clear examples of division of labor are used as a stepping stone toward discussing different cell fates within biofilms.

Cyanobacteria present a beautiful example of bacterial differentiation. Some cyanobacteria form filaments that can express up to four different cell types (41–43): photosynthetic cells, heterocysts, akinetes, and hormogonia. The first two cell types are known to divide labor. Heterocysts fix nitrogen using the enzyme nitrogenase. Since nitrogenase is sensitive to oxygen, nitrogen fixation is incompatible with photosynthesis. Consequently, cells cannot fix nitrogen and carbon at the same time. Because cells need both carbon and nitrogen, the strong phenotypic trade-off resulted in the evolution of two specialized cell types—heterocysts and photosynthetic cells—that share their resources, as opposed to a generalist that inefficiently fixes both nitrogen and carbon (see Fig. 2 for the role of phenotypic trade-offs on the division of labor). In cyanobacteria that divide labor, such as Anabaena, large filaments of photosynthetic cells are typically interspersed by a smaller number of heterocysts (44, 45). Heterocyst development is triggered by nitrogen deprivation (46); a positive regulatory feedback loop subsequently ensures a cell’s developmental commitment (47). At the same time, lateral inhibition—via a signaling peptide (PatS)—prevents neighboring cells from differentiating into heterocysts (Fig. 3A), resulting in a semi-regular spacing of heterocysts along the filament (48–51). Heterocysts are terminally differentiated and cannot divide, so in addition to metabolic cooperation there is also reproductive division of labor (52, 53). In contrast to Anabaena species, the filamentous cyanobacteria Plectonema boryanum has evolved an alternative strategy: rather than separating nitrogen and carbon fixation in space, it separates the processes in time by switching back and forth between nitrogen and carbon fixation (54). The advantage of temporal, instead of spatial, differentiation is that it does not require cooperation between cells. It is therefore plausible that the regulatory mechanisms for temporal cell differentiation evolved first and were later coopted in some species during the evolution of spatial division of labor, which presumably is more efficient (55). Phenotypic trade-offs, like the one described here, also underlie the division of labor in other species (56, 57).

The developmental pattern of many actinobacteria is another example of bacterial multicellularity where division of labor is clear. Most cells are part of a vegetative mycelium, consisting of branching hyphae, and others form aerial hyphae that produce spores. Although many details of the regulation of cell differentiation in actinomycetes are yet to be discovered, there are some good indications for the division of labor, especially, during aerial hyphae formation in Streptomyces coelicolor (Fig. 3B). Upon starvation, aerial hyphae develop from the vegetative mycelium and grow into the air by locally breaking the water tension (58, 59). The aerial hyphae go through a tightly regulated developmental cascade that results in an apical sporogenic cell consisting of prespore compartments and a subapical stem cell. In each of the prespore compartments a spore matures (59). In the case of aerial hyphae formation, there are no apparent phenotypic trade-offs that could explain the interaction between specialist phenotypes as described above for cyanobacteria. However, the different cell types do cooperate. For example, the vegetative mycelium secretes

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FIGURE 1 Conceptual and theoretical basis for the division of labor. (A) Growth of cooperative and noncooperative cells when mixed (left) or segregated (right). When mixed, the noncooperative genotype performs better than the cooperative phenotype; it benefits from cooperation without paying the costs. When segregated, the cooperative genotype performs better. (B) Reaction norms. Different colored lines and associated numbers show different types of reaction norms as indicated on the right. (C) Fitness consequences of cell differentiation at the individual level (i.e., cell) and group level (i.e., colony or part of the colony). When cell differentiation is not beneficial at either level, phenotypic heterogeneity is nonadaptive. When it is only beneficial at the cell level, there is cellular specialization. When it is only beneficial at the colony level, there is division of labor. When it is beneficial at both levels, one cannot directly determine the function of cell differentiation. doi:10.1128/microbiolspec.MB-0002-2014.f1
proteases that presumably help break down the substrate mycelium, thereby providing nutrients for sporulation (60, 61). In addition, a significant fraction of the vegetative mycelium undergoes cell lysis (61–64). The nutrients that are liberated from these dead cells are thought to benefit aerial hyphae formation (61, 64). Since cell lysis cannot be a local adaptation, this phenomenon can only be explained by division of labor that results in a colony-level advantage (assuming that cell lysis is an evolutionarily selected trait). In conclusion, these examples of bacterial multicellularity exhibit cell differentiation and cooperative interactions between cell types, indicating that division of labor does exist. These examples can therefore be used to evaluate cell differentiation in bacterial biofilms. In the next section, we discuss the phenotypic heterogeneity that emerges during biofilm formation and evaluate if there are indications, like those seen in the examples above, that cells divide labor in bacterial biofilms.

**DIVISION OF LABOR IN BIOFILMS**

Surface-attached biofilms are heterogeneous by nature. The accumulation of cells on surfaces inevitably results in gradients of nutrient sources, electron acceptors, waste products or any other products that are generated by cells (65–67). Not surprisingly, cells respond to these gradients by physiological adaptation (67). One would...
therefore expect that biofilm formation invariably results in the phenotypic heterogeneity of its constituent cells. At the same time, the environmental gradients also afford an organizing potential (68). The chemical gradients—akin to morphogen gradients in eukaryotic development—confer positional information to cells, which allows them to spatially organize themselves via cell differentiation (69, 70). This could subsequently facilitate cooperative interactions between cell types and, hence, the division of labor. Here, we discuss whether cell differentiation processes in biofilms might indeed be explained by division of labor, as opposed to being simply the result of local physiological adaptation. In particular, we focus on biofilm formation in M. xanthus, B. subtilis, and P. aeruginosa.

**M. xanthus Multicellularity**

Myxobacteria are social bacteria that survive in groups during both nutrient-rich growth and starvation. These bacteria are predatory and secrete antibacterial compounds. Cells then grow on nutrients obtained as they break down macromolecules that are released after prey bacteria are killed (71). When nutrient levels decrease, thousands of cells aggregate into mounds within which cells differentiate to form heat- and desiccation-resistant spores, often producing remarkable aerial structures (fruiting bodies) (72, 73). Despite the fact that myxobacterial aggregates were not referred to as biofilms historically, they are an excellent example of robust bacterial biofilms during both nutrient-rich and -replete conditions (14, 74–76). In contrast to the filamentous multicellularity we described for cyanobacteria and actinobacteria, biofilm and fruiting body formation in myxobacteria result from cell aggregation (i.e., colonial multicellularity; see Fig. 3C). As a consequence, there is no unicellular bottleneck, and multiple genotypes could partake in its development. As we describe below, this has allowed for extensive studies analyzing fruiting body development in “chimeric” populations where strains harboring different mutations are mixed.

When starved, M. xanthus cells undergo an elaborate developmental program that culminates in at least three different cell fates: spores, peripheral rods, and cells that will lyse (72, 73). Sporulation only occurs within
fruiting bodies, and cells that will sporulate differentially express certain genes required for sporulation (77) and show different protein profiles than nonsporulating cells, termed “peripheral rods” (73, 78). Peripheral rods are a discrete subpopulation of cells that remain outside of the fruiting bodies. This subpopulation is proposed to function as persister cells which do not undergo cell division but are likely ready to respond to any sudden increase in nutrients (73, 79). In addition to exhibiting a different protein profile than spores and presporulating cells, peripheral rods also lack the presence of lipid bodies that are found in cells that are destined to become spores (73, 80). As cells undergo the morphological changes required to become spores, the lipid bodies are consumed. Therefore, it has been proposed that the lipid bodies provide the energy required for cells to sporulate (80). There is also a significant portion of the cells that lyse during fruiting body formation. However, the overall number of lysed cells, the timing of lysis, and the proposed mechanism of lysis varies depending on the strain studied and conditions that are used (73). Historically, cell lysis was proposed to provide nutrients that allow sporulation. In addition, more recent results suggest that cell lysis may also play a role in the aggregation of cells (73, 78).

Since M. xanthus cells must coordinate their behavior during vegetative motility, predation, and fruiting body formation, cells must be able to communicate with each other. Indeed, an M. xanthus cell interacts with its neighbors using both contact-dependent mechanisms and secreted signals (76). Amazingly, M. xanthus cells that come in contact with each other exchange outer membrane proteins and lipids in a regulated manner that results in phenotypic changes in cells (76). When nutrients are sparse, a mixture of extracellular amino acids and peptides (termed the A-signal) is important for the onset of fruiting body formation by ensuring that cellular aggregation only starts when there is a critical mass of starving cells (81, 82). Subsequent to A-signaling, a second contact-dependent signal becomes important. The so-called C-signal is a processed form of the CsgA protein (p17), and C-signaling functions in a threshold-dependent manner to regulate different behaviors (72, 83). C-signaling is required for aggregation, and aggregation stimulates C-signaling. Therefore, there is a positive feedback loop that ensures the continuation of fruiting body formation once it has started (84). The level of C-signal that a cell senses may be an important determinant of its fate as a spore, peripheral rod, or lysed cell (72, 73, 76). In other myxobacterial species, such as Chondromyces apiculatus and Stigmatella aurantiaca, nonsporulating cells in the fruiting body can function as stalks that presumably aid in the process of spore dispersal (85). For convenience, below we refer to the nonsporulating cells inside an M. xanthus fruiting body as “stalk” cells as well.

Using Chimeric Fruiting Bodies to Determine the Role of Different M. xanthus Cell Types

Since cell lysis in stalk cells is under regulatory control (78, 86), the behavior of stalk cells cannot be explained by local adaptation. Instead, cell lysis is expected to yield benefits at the colony level, much like the lysis of vegetative mycelium in Streptomyces discussed above. However, in contrast to Streptomyces, the fitness consequences and interaction of different cell types can be examined directly by studying chimeras: fruiting bodies formed by multiple genotypes (87). Numerous studies of M. xanthus have examined chimeras consisting of genetically engineered mutants, wild isolates, and laboratory-evolved genotypes (87–94). This plethora of experiments resulted in some unique insights. For example, fruiting body formation depends on cooperation. When A-signal or C-signal mutants were mixed with a wild-type strain, the total spore production of a fruiting body dropped and the mutants produced a disproportionately high share of spores in comparison to the wild type (90). In other words, the mutants behave like developmental cheaters: by avoiding the costs of signal production they were able to increase the production of spores. Similar results were obtained when mixing a wild-type strain with its evolved descendants. Velicer and colleagues (95) evolved M. xanthus for approximately 1,000 generations under “asocial” conditions. The evolved genotypes were highly aberrant in social behaviors such as fruiting body formation. However, when mixed with their “social” ancestor, they couldpartake in fruiting body formation and had a competitive advantage by producing a disproportionately high fraction of spores at the expense of the overall spore production (90). These studies show that stalk cells express cooperative traits that contribute to the total spore production of a fruiting body and can be exploited by other genotypes. Consequently, fruiting body formation can be seen as a developmental process in which the division of labor between spores and stalk cells results in an effective dispersal organ.

**B. subtilis** Differentiation

As explained above, when cells divide labor, phenotypic differentiation is characterized by discrete phenotypic states (i.e., cell types) that are mutually exclusive. These
phenotypic states can be recognized from the multimodal distribution of gene expression, concerning the genes that encode for the respective phenotypes. For *B. subtilis*, bimodal distributions in gene expression have been associated with a number of (not necessarily mutually exclusive) phenotypes that appear during biofilm formation (96–98): motility, surfactin production, matrix production, protease production, and sporulation. These phenotypes are typically referred to as cell types. While matrix-producing cells are the only cells that are essential for the formation of biofilms, all of these cell types can be found within a biofilm. Motile cells have an upregulated expression of the *fla* *che* operon, which is required for the biosynthesis of flagella (99). The expression of this operon does not necessarily mean that cells within the biofilm are actually motile, because once cells begin to express the *epsA-*O operon (which encodes enzymes that produce the exopolysaccharide component of matrix), there is a feedback where the EpsE glycosyltransferase protein physically binds to and inhibits FliG, a component of the flagellar motor. This interaction inhibits flagella rotation, thereby inhibiting motility in cells that have begun to produce extracellular matrix (100).

Surfactin-producing cells produce the surfactant surfactin (101–103), which also functions as a communicative signal that triggers matrix production (104, 105) and an antimicrobial (106, 107). Matrix-producing cells express the *epsA*-*O* and *tapA-sipW-tasA* operons, which results in the production of, respectively, extracellular polysaccharides (EPS) and the structural protein TasA (108–110). TasA assembles into amyloid-like fibers that attach to cell walls via an accessory protein, TapA (111, 112). Another protein that contributes to the extracellular matrix is BslA, which is important for surface hydrophobicity (113–116). However, unlike the *epsA-*O and *tapA* operons, *bslA* expression occurs in all of the cells within the population (116). In addition, matrix-producing cells also produce antimicrobial toxins (Skf and Sdp) that kill other species or those sibling cells which do not express the immunity genes (i.e., cannibalism) (117–120). Protease-producing cells secrete bacillopeptidase and subtilisin, two proteolytic enzymes, which are encoded by the *bpr* and *aprE* genes (98, 121, 122). Finally, spores are stress-resistant dormant cells that are formed during the developmental process of endosporulation (84, 123–125).

**Regulation of Differentiation in *B. subtilis***

In general, cell differentiation in *B. subtilis* is triggered in response to some environmental signals that—via sensor kinases—initiate a phosphorylation cascade. The phosphorylation cascade integrates the environmental information by funneling the regulatory input toward the phosphorylation of one (or a few) downstream regulatory protein(s). This key regulatory protein, which is typically subject to a positive or double-negative regulatory feedback loop, converts the continuous environmental information into a discrete Boolean switch (i.e., bistable or binary switch) that controls if a phenotype is either expressed or not. In the case of *B. subtilis*, a few cell types have been shown to be controlled by bistable regulatory switches (30, 126). Figure 4A shows a simplified scheme of a part of the regulatory pathways that underlie these bistable switches (for details see references 127, 128, and references therein).

One of the key regulatory proteins controlling biofilm formation is Spo0A, which is a transcriptional regulator involved in the regulation of motility, matrix production, protease production, and sporulation (97, 128, 129). There is a graded response to the level of phosphorylated Spo0A-P (130, 131). When levels of Spo0A-P are low, cells are motile (132). In response to intermediate levels of Spo0A-P, cells produce matrix (i.e., EPS and TasA) and—dependent on the phosphorylation of another regulatory gene (DegU)—secrete proteases (96, 122, 130). At high levels of Spo0A-P, cells initiate sporulation.

The phosphorylation state of Spo0A can be modulated by five histidine kinases, four of which appear to be important for biofilm formation (133, 134). These kinases sense a variety of environmental signals, including self-generated products like surfactin and matrix (105, 135–138). Once phosphorylated, Spo0A-P indirectly represses the expression of *sinR* (139–141). SinR represses the *epsA-*O and *tapA-sipW-tasA* operon by competing for the binding sites of an activating protein, RemA (142). Spo0A-P also represses AbrB, which, like SinR, is a repressor of the *epsA*-*O* and *tapA-sipW-tasA* operon as well as of *bslA* (113, 143, 144). Both SinR and AbrB are part of a double-negative feedback loop. The repression of SinR derepresses the expression of *slrR* (145–147). SlrR subsequently sequesters SinR by forming a SinR-SlrR complex, which further relieves SinR-mediated repression of the matrix genes, and slrR and also represses motility genes. The sequestering of SinR by SlrR therefore results in a bistable switch. Consequently, matrix production and motility become two mutually exclusive cell types (128, 148). At high levels of Spo0A-P the repression of SinR weakens (not shown in Fig. 4A), which downregulates matrix production and simultaneously triggers the sporulation process (126, 149).
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**A** Signal-response regulation

- \( S_{\text{KinA-E}} \)
- \( S_{\text{DepS}} \)
- \( \text{DegU-P} \)
- Extracellular protease-production

**B** Pattern formation

**C** Feedback

Surfactant
Quorum-sensing signals
Cannibalistic toxins
Matrix

Cellular contingency
Environmental conditionality
Besides translating environmental signals to binary phenotypic responses (29), regulatory feedback loops can also amplify stochastic fluctuations (i.e., noise) in the levels or activities of regulatory components (28, 96, 150, 151). As a consequence, cells can differentiate into different cell types, despite being exposed to the same environmental conditions (152). Matrix production, protease production, and sporulation are subject to probabilistic cell differentiation (122, 148, 153), which is generally viewed as a product of evolutionary adaptation (28, 151). The probability of differentiation can be manipulated by changing the level of noise or the regulatory circuit that underlies differentiation (154–157). The aforementioned switch toward matrix production, via the double-negative feedback loop between SinR and SlrR, is a well-studied example of probabilistic cell differentiation. Norman and colleagues (148) showed—using a highly controlled microfluidics device—that the differentiation toward matrix production is, at least in part, stochastic. However, once differentiated, the time spent as a matrix producer is tightly controlled, such that cells are committed to matrix production for a number of generations (148, 158, 159). As suggested by Norman and colleagues, this commitment may allow for the cooperation between the progeny of a differentiated cell (148).

**B. subtilis Division of Labor**

Knowing the regulatory mechanisms and their consequences, we are left with the question of what such probabilistic cell differentiation tells us about the division of labor in biofilms. If cells indeed respond differently to the same environmental conditions, cell differentiation cannot possibly be explained by local physiological adaptation, because supposedly there is only one “optimal” phenotype. Assuming that the phenotypic heterogeneity is adaptive, there are only two alternative explanations for the presence of probabilistic differentiation: bet-hedging and the division of labor. Bet-hedging is an adaptive strategy to cope with unpredictable environmental fluctuations (160). When there are relatively infrequent, unpredictable, and strong environmental changes, a cell can get a fitness advantage by producing a mixture of progeny that is phenotypically diverse (96, 150, 161, 162). In this way, a cell ensures that at least a fraction of its progeny is adapted to the unforeseen environmental changes. A commonly used example of bet-hedging is bacterial persistence in which a small fraction (10^{-5} to 10^{-6}) of cells differentiate into a slow-growing state and become resistant against environmental stressors such as antibiotics (163). Since only a small fraction of cells differentiate into persister cells, a bet-hedging genotype hardly pays a cost for producing them, while, at the same time, it does ensure its survival in the event of a sudden antibiotic influx. Bet-hedging would only evolve when environmental conditions change in an unpredictable way and do not allow for a direct phenotypic response (164–167). Although it is unknown how predictable the environmental changes are during biofilm formation, we would argue that they are relatively gradual and therefore predictable (see discussion below). Thus, probabilistic cell differentiation during biofilm formation might be better explained by the division of labor.

Although direct evidence for cooperative interactions between different cell types in *B. subtilis* is lacking, some properties of probabilistic cell differentiation in *B. subtilis* make the division of labor plausible. Contingent on the actual environmental conditions, the rates at which differentiation occurs are relatively high in comparison to persistence (148, 154, 155, 168). In addition, even though the onset of cell differentiation is sensitive to noise, the regulation thereafter is more deterministic, which hints at some form of coordination. Finally, many cell types secrete products into the environment, which allows for a direct interaction between differentiated and nondifferentiated cells. For example, matrix and protease producers all secrete products that are in principle available to their nondifferentiated siblings.

**FIGURE 4** Cell differentiation and pattern formation in *B. subtilis* biofilms. (A) Simplified scheme of the regulatory circuit that controls cell differentiation. Regulatory repression (red T-bars) or stimulation (green arrows) can involve both transcriptional regulation and (de)phosphorylation. The gray box shows the expected developmental transition in time throughout biofilm formation: motile cells differentiate to matrix-producing cells, which later sporulate. $S_{\text{KinA-E}}$ and $S_{\text{DegS}}$ are environmental signals that affect the sensory kinases KinA-E and DegS. (B) Pattern formation in cross-sections and top view of *B. subtilis* colony biofilms. Cell types shown in cross-sections are sporulating cells (artificially colored yellow or green), motile cells (blue), and matrix-producing cells (red). In the top view, sporulating cells are shown in green and colocalize with the biofilm wrinkles. (C) Feedback between cellular contingency and environmental conditionality. Images are adapted from references 144 and 169. doi:10.1128/microbiolspec.MB-0002-2014.f4

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Even though the bistable switches are sensitive to internal regulatory noise, cell differentiation is largely regulated by external factors because the kinases that phosphorylate Spo0A respond to specific signals. The conditionality of cell differentiation on the local environmental conditions leads to spatial pattern formation, in which certain cell types preferentially occur in specific regions of the biofilm (134, 169). Vlamakis and colleagues (169) showed that motile cells mainly occur on the edges and lower parts of *B. subtilis* biofilms. Matrix producers occur more in the center, and sporulating cells more on the top of biofilms (Fig. 4B). Other studies furthermore showed that spores are largely localized in the wrinkles of a biofilm and in structures that resemble fruiting bodies (103, 144). Cells not only respond to their environment, but also strongly shape their environment. For example, cells consume resources, such as nutrients and oxygen, and secrete surfactin, matrix (e.g., EPS, TasA, and BslA), communicative signals (e.g., surfactin), antimicrobial toxins (Skf and Sdp; see discussion below), and proteases. These products significantly affect the structure of a biofilm, which becomes immediately apparent from studying mutants (108, 115, 144). In addition, some of the products (e.g., the communicative signals) directly affect cell differentiation by triggering one of the sensor kinases (105, 136). As a consequence, there is feedback between the contingency of cell differentiation on environmental conditions and the subsequent influence of these cell types on their environment (i.e., environmental conditionality) (Fig. 4C). Although this feedback results in pattern formation, this by itself does not necessarily mean that different cell types are interacting in a cooperative manner (170).

Despite the detailed knowledge of cell differentiation in *B. subtilis*, relatively little is known about how the different cell types interact and what the fitness consequences are of their interaction. It is, however, plausible that they do cooperate, because many of the cell types presumably pay a cost for producing products that are secreted in the environment and benefit other cells. A recent study, for example, showed that EPS is costly to produce, while it facilitates colony spreading (262). EPS-producing cells could be exploited by EPS-deficient mutants, thereby showing that non-matrix-producing cells benefit from EPS produced by others. Furthermore, similar to developmental chimeras in *M. xanthus* (88), matrix-deficient mutants, *eps* and *tasA* or *eps tasA* and *bslA* (formerly *yuaB*), can complement each other when they are mixed and thereby form a biofilm that is indistinguishable from that of the wild type (109, 171). A recent study showed that *eps* and *tasA* mutants can also complement each other during plant root colonization: despite being unable to colonize the root by themselves, together they can (172). These chimera studies confirm that cells can interact by sharing products they secrete in the environment. Since all these studies are based on interactions between mutants, it is still unknown if similar interactions also occur between cell types in wild-type biofilms. However, it is likely that interactions do occur, given the prevalence of probabilistic cell differentiation (as discussed above).

Another interesting aspect of cell differentiation with respect to the division of labor is the production of antimicrobial toxins. Only matrix-producing cells produce toxins, which can kill sibling cells that have a low level of Spo0A—P and therefore do not express the necessary immunity genes (118). The nutrients that become available through cell lysis are consumed by the matrix-producing cells, which consequently delay sporulation (117, 119). Although antimicrobial toxins are more effective against other soil-dwelling bacteria than sibling cells (120), it is surprising that not all biofilm-inhabiting cells express the necessary immunity genes. Toxin-induced cell lysis is therefore often compared to programmed cell death and viewed as an altruistic trait that benefits matrix-producing cells (37, 119). Cells within *B. subtilis* biofilms also lyse in a manner that is independent of the Skf and Sdp toxins. Localized patterned cell death coupled with the production of extracellular matrix results in the complex wrinkling pattern observed in *B. subtilis* biofilms (173). Cell lysis is commonly observed during biofilm formation in other organisms. For example, in *Pseudomonas aeruginosa* cell lysis has been associated with biofilm dispersal (174, 175). In the next section we evaluate biofilm formation in *P. aeruginosa* and, in particular, the interaction between various subpopulations that appear during biofilm growth.

**P. aeruginosa Microcolony Division of Labor**

*P. aeruginosa* is an opportunistic pathogen with a broad host range and, like *B. subtilis* and *M. xanthus*, a common inhabitant of the soil (176). Biofilm formation in *P. aeruginosa* is typically studied in flow chambers. The biofilms that are formed in flow chambers are much smaller than the colony biofilms or pellicles studied in *B. subtilis* and are referred to as microcolonies (175, 177). Microcolonies are at most a few hundred micrometers thick and can easily be examined using scanning...
confocal laser microscopy, which allows for a detailed examination of the three-dimensional structure. This showed that the shape of microcolonies depends on the nutrient conditions (178). When cells are grown on citrate as the sole carbon source, colonies are flat. In contrast, when grown on glucose, colonies have a mushroom-like shape: there is a relatively narrow stalk at the bottom that is topped by a wider cap (Fig. 5).

Given their interesting morphology, mushroom-shaped microcolonies have been intensely studied. To understand their development, many studies have examined biofilms composed of two different strains (176, 178–184). Like in the case of M. xanthus, multiple predefined mutants were mixed to examine how these mixtures affect microcolony development. Although many of these studies were not intended to examine cooperation, which was the case for chimera studies in M. xanthus, they did provide a number of interesting insights. For example, Klausen and colleagues (178) showed that the mushroom-shaped structures resulted from the interaction between two subpopulations: motile and nonmotile cells (185). This was shown by studying chimeras of motile wild-type cells with twitching motility-deficient mutants (pilA), each labeled with a distinct fluorescent protein (Fig. 5A). Initially, the nonmotile cells formed small “stalk” colonies by localized clonal growth. After approximately 4 days, the motile cells moved on top of these stalk colonies via type IV pili-mediated twitching motility. This migration results in the formation of caps and, hence, the mushroom-shaped microcolonies (178). Perhaps through chemotaxis (186), motile cells might climb on top of the stalk cells to access more nutrients (182, 187). In citrate minimal medium the absence of mushroom-shaped microcolonies can be explained by the lack of nonmotile cells, and therefore no stalks can form (179). Finally, it is important to note that even though Klausen and colleagues (178) examined genetic chimeras, the same phenotypic subpopulations are present in the absence of genetic variation (i.e., phenotypic heterogeneity) (185).

**FIGURE 5** Pattern formation in *P. aeruginosa* microcolonies. (A) Fruiting bodies consisting of nonmotile stalk cells (blue) and motile cap cells (yellow). (B) Localization of eDNA in microcolonies (red). (C) Localization of rhamnolipid production (yellow). (D) Live (green) and dead (yellow/red) cells after EDTA treatment in a 4-day-old microcolony. (E) Schematic overview of mushroom-shaped microcolonies and the interaction between the stalk and cap cells through the production of the iron-scavenging siderophore pyoverdine. Images adapted from references 178, 190, 192, and 196. doi:10.1128/microbiolspec.MB-0002-2014.f5
Signaling During Microcolony Formation

There are a number of phenotypic differences between the stalk and cap subpopulations. On average, the stalk subpopulation has a higher cell density than the cap subpopulation and is less metabolically active (184, 188). In addition, stalk cells produce quorum-sensing signals, surfactants, and siderophores, while cap cells do not (183, 189–192). There are three important quorum-sensing systems that regulate microcolony formation: Las, Rhl, and Pqs. The Las and Rhl systems involve two homoserine lactone signals, and the Pqs system involves a quinolone. All three quorum-sensing signals are predominantly expressed in the stalk of the microcolony, which might be explained by the high cell density (183, 189, 192). For some growth conditions the Las system is essential for microcolony development (193), whereas for others it is not (194–196). These differences might be explained by the nutrient-dependent influence of quorum sensing on motility in P. aeruginosa biofilms (197). The Rhl system is required for rhamnolipid production (198). Given that the rhl genes are expressed more in the stalk cells, it is perhaps not surprising that rhamnolipids are also predominantly produced in the stalk (Fig. 5C) (199). Rhamnolipid surfactants are important for a number of biofilm-related properties. They affect the spacing between the microcolonies (199). They are necessary for early stages of microcolony formation and cap formation (181). Finally, depending on the culturing conditions, rhamnolipids play a role in the dispersal of cells at the end of microcolony formation (180, 200).

The quorum-sensing systems are also involved in the production of extracellular DNA (eDNA) during microcolony formation (196). eDNA is a major component of the extracellular matrix and is essential for both the establishment of microcolonies and their early development (201, 202). Treatment with DNase prevents colony establishment and triggers dispersal in young microcolonies (201). The eDNA consists of chromosomal DNA and originates from a small subpopulation of cells that undergoes cell lysis (196). The degree of cell lysis and, hence, the production of eDNA depends on the Pqs system (196, 203): high levels of quinolone signal result in high levels of cell lysis, while low levels of quinolone reduce the degree of cell lysis. Interestingly, eDNA production is regulated in time and space. eDNA is produced before cap formation by the stalk subpopulation and is primarily localized at the outermost edge of the stalk colony (Fig. 5B). It has been suggested that eDNA facilitates cap cells’ migration on top of the stalk subpopulation (182, 183). Treatment of stalk colonies with DNase prior to migration inhibits cap formation, as does lack of the Pqs system (182). In agreement with the localization of eDNA (Fig. 5B), Yang and colleagues (192) showed that pqsA, a gene that encodes for an essential component of the Pqs system (203), is expressed before cap formation in the outermost edge of the stalk. In addition, they showed that both the expression of pqsA and the production of eDNA depend on the iron concentration in the medium. At high iron concentrations pqsA expression was repressed and, consequently, mushroom-shaped microcolonies were not formed. The lack of structure made biofilms more susceptible to antibiotic treatment (192). Other studies have also shown that mushroom-shaped microcolonies are more resilient against antimicrobial and EDTA treatments (Fig. 5D) because of the different physiological states of stalk and cap cells (185, 188, 204, 205).

Sharing of Common Goods within the Biofilm

Given that cell lysis is part of the regulatory circuit of microcolony formation, it could be viewed as a cooperative trait. That is, it may be argued that a fraction of stalk cells sacrifice themselves to help cap cells migrating on top of them. By the same token, it may be argued that the production of quorum-sensing signals, siderophores, surfactants, and polysaccharides are cooperative traits. Many of these public goods are solely produced by the stalk cells. The question therefore arises as to whether they are somehow shared with the cap cells. That is, is there an interaction between the stalk and cap subpopulation? Yang and colleagues (183) showed in a number of elegant experiments that these subpopulations indeed interact. They focused on two particular common goods: Pqs signaling and pyoverdine production (siderophore). Both common goods are necessary for the formation of mushroom-shaped microcolonies (206). For each experiment, Yang and colleagues made a chimera consisting of two genotypes: one motile and one nonmotile (pilA). As described previously, the pilA genotype always occurs in the stalk subpopulation (178). In addition, either the motile or the nonmotile genotype is given an additional mutation that abolishes common good production: either a pqsA mutation that prevents quorum sensing or a pvdA mutation that prevents pyoverdine production. If the cap subpopulation depends on the common good produced by the stalk cells, the chimeras with defective stalk cells should be aberrant in normal microcolony formation, while this should not be the case for those of defective cap cells. The pilA/pqsA and pilA/pvdA chimeras both produce normal mushroom-shaped microcolonies, in which nonmotile pilA cells form the stalk and either pqsA or pvdA cells...
form the cap. This is not surprising because also in wild-
type microcolonies, cap cells do not express \textit{pqsA} or
\textit{pvdA}, so a mutation in the cap cells should not af-
fect their phenotype (191, 192). In contrast, both the
\textit{pilApqsA/wild-type} and \textit{pilApvdA/wild-type} chimeras
are defective in cap formation. This shows that cap-
formation indeed depends on the Pqs system and pyo-
verdine production of the stalk cells.

In the case of the Pqs system, the results are in agree-
ment with the studies discussed above. Pqs signaling
results in localized cell lysis and the release of DNA. This
eDNA is necessary for the cap cells to migrate on the
stalk cells. This was furthermore confirmed by the fact
that \textit{pilApqsA/wild-type} chimeras did produce caps in
the presence of exogenously added DNA (183). In the
case of pyoverdine production, the cap subpopulation is
dependent on the pyoverdine produced by stalk sub-
population (Fig. 5E). In \textit{pilA/pvdA} chimeras’ cap cells
express \textit{fpvA}, a gene that encodes the ferric-pyoverdine
uptake system, indicating that cap cells take up the
pyoverdine produced by the stalk cells. The interaction
between the stalk and cap cells was further confirmed by
the fact that \textit{pilA/fpvA} chimeras were also defective in
cap formation. In that case, stalk cells do produce pyo-
verdines, but \textit{fpvA}-mutant cap cells cannot access them
because they lack the uptake system.

All in all, Yang and colleagues (183) demonstrated that \textit{P. aeruginosa}
microcolony formation comes about through the interaction of heterogeneous subpopula-
tions. Mutants defective in type-IV pili formation, Pqs
signaling, or pyoverdine production cannot produce normal microcolonies alone. However, when mixed,
they produce mushroom-shaped microcolonies indis-
tinguishable from wild type. Although it is yet unclear
if there is an ecological advantage associated with cap
formation and if common good production is costly
(although in the case of Pqs-mediated cell lysis there
obviously is a fitness cost), this study shows that het-
erogeneous subpopulations inside a biofilm can engage
in a cooperative interaction.

**Dispersal of \textit{P. aeruginosa} Microcolonies**

Another interesting stage in \textit{P. aeruginosa} biofilm for-
mation occurs after approximately 7 days when cells
start to disperse (194). Like the onset of biofilm forma-
tion, this dispersal stage is subject to regulation and
therefore differs from other dispersal events (e.g., biofilm
sloughing) that primarily result from shear stress (184,
207, 208). Since mostly single cells disperse from the
microcolonies during this phase, this type of dispersal is
also called seeding dispersal. Seeding dispersal depends
on a number of environmental factors (209, 210) such as
nutrients (211), rhamnolipids (180), quorum-sensing
signals (200, 212), nitric oxide (213), and oxygen (214).
The phenotype of dispersing cells is more similar to those
of motile cells that initiate biofilm formation than it is to
the cells inhabiting a mature microcolony (194). In
general, the dispersal stage is characterized by a phe-
nomenon called “hollowing”; cells localized in the cen-
ter of the microcolony disperse, while those on the edges
remain statically attached to the surface (174, 194, 215).

Webb and colleagues (174) discovered that the occur-
rence of hollowing is consistently associated with lo-
calized cell death and lysis. Only a subpopulation of cells
in the center of the microcolony remains viable, becomes
motile, and, subsequently, disperses. Surprisingly, given
the consistent timing of hollowing, cell lysis was largely
mediated by the hyperinfectivity and lytic effects of an
otherwise nonlytic filamentous prophage. The induction
of the prophage is more common in the biofilm when
compared to planktotic cells and can, under some con-
ditions, be affected by quorum-sensing signaling (174,
200, 203, 212, 216).

Hollowing at the end of microcolony maturation is
often hypothesized to be an adaptive trait that facilitates
dispersal (175, 215, 217). In agreement with this hy-
pothesis, Rice and colleagues (218) showed that the
virulence of a wild-type strain was significantly higher
than that of a prophage-deficient mutant when infecting
mice. In other words, phage-mediated hollowing seems
to facilitate \textit{P. aeruginosa} virulence. In addition, it has
been shown that phage-mediated hollowing occurs in
natural isolates from cystic fibrosis patients (219). Not
only \textit{P. aeruginosa} shows the hollowing phenotype; in
\textit{Pseudoalteromonas tunicata} a similar phenomenon
occurs (220–222). \textit{P. tunicata} is a common inhabitant
of the marine environment, where it colonizes surfaces
of eukaryotic organisms. Similar to \textit{P. aeruginosa},
during hollowing in \textit{P. tunicata} the majority of cells in the
center of the microcolony lyse (220). The remaining vi-
able cells become motile and disperse. Dispersal is highly
reproducible and always occurs after the same period
of biofilm development. In contrast to \textit{P. aeruginosa}, cell
lysis in \textit{P. tunicata} is not induced by a prophage, but
through the expression of an autotoxin protein called
AlpP (220, 222). In the absence of this autotoxin, hol-
lowing did not occur, and the number of dispersing cells
was greatly reduced. Besides increasing the number of
dispersing cells, the dispersing cells that result from
AlpP-mediated hollowing have a higher metabolic ac-
tivity and express a wider range of phenotypes, which
might be beneficial for subsequent colonization (221).
Thus, colony hollowing in *P. aeruginosa* and *P. tunicata* illustrates that a nonadaptive cellular behavior (i.e., cell lysis) can facilitate a colony-level function (i.e., dispersal). In consideration of the division of labor, the interaction between cell types should therefore be evaluated with respect to the ecological functionality that emerges at the colony level (or at a lower organizational level; e.g., clonal pockets in multispecies biofilms).

Although one might be inclined, based on the previous examples, to assume that cell lysis evolved to facilitate dispersal, there is insufficient evidence for this assumption. In fact, similar to Skf and Sdp produced by *B. subtilis* (120), AlpP is effective in direct competition with other species, and its autotoxicity might just be a side effect (223–226). Likewise, the prophage-induced cell lysis in *P. aeruginosa* might simply result from the classic conflict between a phage and its host (13). In general, one should be cautious in giving an evolutionary interpretation without considering the ecological context under which traits have evolved (13, 226, 227).

### Dispersal Organs: Microcolonies and Fruiting Bodies

To interpret the patterns observed in *P. aeruginosa* microcolonies, microcolony formation is often loosely compared to the developmental process of fruiting body formation in *M. xanthus* (14, 74). There are a number of similarities. In short, both species initiate colony formation in response to nutrient depletion (72, 84, 228). Initially they form a monolayer of cells that aggregate into colonies via pilus-mediated movement and cell division (74, 229). Colony formation is, at least in part, regulated by a number of quorum-sensing signals and involves the secretion of extracellular matrix as well as localized cell lysis (82, 86, 189, 196). Eventually, both species form colonies with a mushroom-shaped structure, from which only a fraction of cells eventually disperse. Therefore, both microcolonies and fruiting bodies are often hypothesized to facilitate dispersal (84, 175).

### BENEFITS OF DIFFERENTIATION AND DIVISION OF LABOR

In the sections above, we described three organisms which have independently evolved to display phenotypic heterogeneity in biofilms. Furthermore, we showed that many of the evolved cell types interact inside these biofilms. Yet the question often remains: Why do cells differentiate? As pointed out by previous reviews (14, 187, 207, 230), any form of multicellular pattern formation—with or without developmental regulation—results from the feedback between cellular responsiveness and environmental conditionality (Fig. 4C) (69, 70, 170). To understand the significance of pattern formation, one should ask not only how cells respond, but also why they do so (13, 14, 231). As described in the beginning of this article, when cells differentiate to divide labor they are expected to cooperate with each another. This cooperative interaction should result in an emergent benefit (i.e., ecological functionality) at the colony level. One therefore needs to determine the fitness costs and consequences that are associated with the expression of a cell type: Is cell differentiation costly? Who benefits from cell differentiation? What is the emergent benefit from a cooperative interaction between different cell types? Studies in which chimeras of different genotypes are examined, such as those discussed above for *M. xanthus*, help answer these questions. In this way, a number of phenotypes that are associated with biofilm formation have been shown to be cooperative traits (16, 232–236). For example, the stalk cells in microcolonies of *P. aeruginosa* produce pyoverdine. A number of studies showed that pyoverdine production is costly for a cell (i.e., reduced cell division rate) and that, in unstructured environments, pyoverdine-deficient mutants can exploit pyoverdine-producing wild-type cells (15, 232, 237, 238). Aggregation, however, constrains pyoverdine diffusion. This might explain why pyoverdine-producing cells are not exploited in natural settings (239). Knowing that some cell types express cooperative traits is only the first step in understanding the interaction between different cell types. The second step, which is perhaps the most challenging, is to understand why different cell types cooperate, such as the apparent cooperation between stalk and cap cells in microcolonies. To characterize the potential ecological advantages that are associated with biofilm formation, one typically compares mature biofilms with planktonic cells. In this way, two major ecological advantages have been discovered (67, 228, 240, 241): (i) protection against environmental stress and (ii) metabolic cooperation. These benefits are based on a rather static comparison between biofilms and planktonic cells. We believe that the ecological functionality of cell types may be better understood when we appreciate the dynamic process of biofilm formation. Biofilms are a transient stage in the life cycle of bacteria. Considering this complete life cycle can help us in determining the potential functions and benefits that might emerge from the cooperative interaction between different cell types.
LIFE CYCLE BIOLOGY: THE ECOLOGY OF CELL DIFFERENTIATION

Most biofilms form a transient stage in the bacterium’s life cycle, as shown in M. xanthus, B. subtilis, and P. aeruginosa (208, 242). Consequently, the life cycle of bacteria can roughly be divided into two life phases which alternate over time: a unicellular life phase and a multicellular (biofilm) life phase. This biphasic life cycle is analogous to life cycles seen in multicellular organisms, in which the multicellular life phase is typically associated with resource acquisition, while the unicellular life phase is required for sexual reproduction or dispersal (170, 208). If we greatly simplify the life cycle of a biofilm-forming bacterium, there are two overarching environmental conditions that cells encounter: those that result in a transition toward biofilm formation and those that result in motility. In the first transition cells have to colonize a surface and build a biofilm. In the second transition the biofilm breaks down and cells disperse (Fig. 6).

When cells encounter a new surface, they benefit if they adhere to this surface before their competitors do, spread quickly, and prevent their competitors from colonizing the surface as well. The advantage derived from colonizing a surface first is also known as the “founder effect” and can simply result from the fact that colonization is only possible when cells can directly access the substrate. An and colleagues (243), for example, showed that the outcome of the competitive interaction between P. aeruginosa and Agrobacterium tumefaciens depends on the order in which these species colonize the surface. Once adhered to the surface, a colony can spread passively by division or actively by motility and surfactant production (244). Both B. subtilis and P. aeruginosa produce large amounts of surfactants during a process called swarming, in which hyper-flagellated cells spread over the surface (245–249). In the case of swarming in B. subtilis, the majority of surfactin is produced in the center of the swarm, from which it spreads and covers the whole colony (250, 251). Finally, cells are expected to make the surface uninhabitable for other soil-dwelling organisms. All in all, one can imagine that the division of labor can enhance the effectiveness of colonization by having cells specializing in the different tasks.

The breakdown of biofilms and subsequent cell dispersal also involve a number of tasks. To efficiently disperse, cells should anticipate adverse conditions, such that dispersal units (e.g., spores) develop in a timely fashion but not too early (259). For biofilm dispersal, cells should break down the biofilm matrix, develop dispersal units, and facilitate these units to actually disperse. Biofilm degradation provides nutrients to cells, which can increase the total number of dispersal units that eventually develop. However, having many dispersal units is only effective when they detach from the surface. As seen in the dispersal organs of M. xanthus and S. coelicolor, dispersal can be facilitated if cells break their surface tension with water and rise from the colony. Furthermore, the mere breakdown of matrix already facilitates dispersal by weakening the adherence that cells have to each other. As for colonization, the division of labor might greatly enhance the effectiveness of dispersal (260, 261). For example, imagine that a B. subtilis biofilm is confronted with a sudden drop in nutrient availability. There might be too few nutrients for all cells to sporulate on time. One can imagine that some cells specialize in producing proteases to break down the biofilm. Other cells might produce antimicrobial toxins that primarily kill cells that are unlikely to sporulate anyway. The lysed cells subsequently provide nutrients for the sporulating cells, which therefore can finish sporulation. In the end, this task specialization

FIGURE 6  Simplified schematic view of the life cycle of biofilm formation. The life cycle is divided into two life phases: the multicellular phase and the dispersal phase. Various environmental conditions influence the switch toward aggregation, typically mediated by a second messenger. When the second messenger passes a certain threshold, aggregation is initiated and, the other way around, when it drops below a certain threshold, cells revert to the dispersal phase. doi:10.1128/Microbiolspec.MB-0002-2014.16
might maximize the total number of spores that disperse. To examine if cells indeed divide labor, competition experiments are required, and one should study the effect of cell differentiation on total spore production. One could, for example, examine if total production is lower in the absence of cell lysis (i.e., when all cells are immune against Skf and Sdp).

**CONCLUSIONS**

Biofilms are remarkable examples of biological construction. In many bacteria, cells that can live independently decide otherwise by aggregating into biofilms. These biofilms are characterized by heterogeneity. The mere presence of a surface results in environmental gradients that trigger differentiation into various cell types. In this article we discussed cell types that are not simply intermediates of surface results in environmental gradients. The mere presence of a surface results in environmental gradients that trigger differentiation into various cell types. In the end, “organismality” and “individuality” cannot be defined as an all-or-none concept. Clear examples of intermediate levels of functional integration, in which different degrees of division of labor may be at play, have been studied. With this in mind, biofilms might be the ideal system for acquiring a more general understanding of the evolution of biological organization.

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Division of Labor in Biofilms: the Ecology of Cell Differentiation

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