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Thinking About *Bacillus subtilis* as a Multicellular Organism

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Summary

Initial attempts to use colony morphogenesis as a tool to investigate bacterial multicellularity were limited by the fact that laboratory strains often have lost many of their developmental properties. Recent advances in elucidating the molecular mechanisms underlying colony morphogenesis have been made possible through the use of undomesticated strains. In particular, *Bacillus subtilis* has proven to be a remarkable model system to study colony morphogenesis because of it well-characterized developmental features. Genetic screens that analyze mutants defective in colony morphology have led to the discovery of an intricate regulatory network that controls the production of an extracellular matrix. This matrix is essential for the development of complex colony architecture characterized by aerial projections that serve as preferential sites for sporulation. While much progress has been made, the challenge for future studies will be to determine the underlying mechanisms that regulate development such that differentiation occurs in a spatially and temporally organized manner.

Introduction

Implicit in our title is a tribute to James A. Shapiro who nearly twenty years ago proposed multicellularity as a general bacterial trait [1]. Shapiro's proposal in 1988 was based largely on observing colony morphogenesis, the subject we wish to broach here. Thus, we deem it appropriate to begin with a reminder of how the concept of bacterial multicellularity evolved from being considered an adaptive strategy of a few bacterial taxa, e.g. the Myxobacteria and the Actinomycetes, to today's pervading view that nearly all bacteria are capable of multicellular behaviors [2]. Shapiro's proposal did not persuade most microbial geneticists to embrace multicellularity as a basic tenet in bacteriology for they still held on strongly to the single-cell, pure-culture tradition stemming from Koch's postulates. Yet in 1877, published back-to-back with Koch's landmark paper identifying *Bacillus anthracis* as the etiological agent of anthrax [3], is Ferdinand Cohn's description of the multicellular nature of *Bacillus subtilis* cultures [4]. In beautiful and detailed hand drawings, Cohn depicts the multicellular aggregates he observed (Figure 1).

A remarkable change in the intellectual backdrop vis-à-vis bacterial multicellularity has occurred in the past ten years. Greatly inspired by the presentations and discussions at two international conferences organized by Shapiro and Martin Dworkin in 1991 and 1993, microbial geneticists in much larger numbers began to study multicellularity in diverse bacteria [5]. The finding that cell-cell communication mechanisms, a.k.a. "quorum sensing", were present in virtually all species had an enormous influence in the change in thinking [6]. All of

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this change led Shapiro to title his 1998 review on the subject: “Thinking about bacterial populations as multicellular organisms.” Fast forward ten years and ask the questions: Have we learned much more about bacterial multicellularity through studies of colony morphogenesis in the last ten years? Has the field moved forward significantly since Shapiro described *dev* mutations based on his studies of *E. coli* K-12 colonies [7]? Our answer to both questions is a categorical yes. Twenty years ago Shapiro had all the right ideas and tools to tackle multicellularity but his choice of strain was, in retrospect, misguided. He opted to carry out his genetic studies in M7124, “a strain that has been used as a standard bacteriophage and plasmid host for 18 years” [8].

We now know that growth of bacteria under laboratory conditions tends to select for strains that lose many of their multicellular attributes, a phenomenon we refer to as “domestication” [9]. As is apparent from the images presented in Figure 2, the robustness of colony morphology can be dramatically diminished in laboratory strains when compared to strains that have not been so extensively manipulated. Liquid cultures are routinely inoculated and then incubated with shaking. Liquid samples are then plated to isolate individual colonies. And the entire process is repeated day in and day out. Oftentimes, we specifically select for colonies whose constituent cells can be easily dispersed - directly asking for the loss of multicellularity! The realization that “undomesticated” or “wild” strains should be analyzed has been a key intellectual leap in the last decade. This has led to significant advancements in our knowledge of the regulatory mechanisms that mediate a bacterium’s transition from a unicellular existence to becoming a multicellular organism. Great strides have been made in studying such mechanisms in *Salmonella typhimurium* [10], *Pseudomonas aeruginosa* [11] and *Vibrio cholerae* [12], to mention but a few. Here we focus on the gram-positive spore forming model bacterium, *Bacillus subtilis*. Advances in understanding the multicellular behaviors of this organism have been particularly rapid in large part because of the enormous knowledge base already available [13].

B. subtilis morphogenesis

B. subtilis populations growing on top of an agar surface can result in the development of colonies[1] with elaborate architecture (Figure 3). It should be noted that the resulting morphology varies enormously depending on the strain and the environmental conditions utilized. Small variations in medium composition, incubation temperature, and agar content of the solid medium can have profound effects on colony architecture [14,15]. When using conditions such as those that led to the morphogenesis presented in Figure 3 the edges of the colony are characterized by aerial projections whose tips serve as the sites where sporulation is first observed [9]. This immediately makes it apparent that these colonies exhibit a marked degree of spatio-temporal organization with regards to the key differentiation process of sporulation. The initial determination that the tips of the aerial projections served as preferential sites for sporulation came from the analysis of strains harboring a fusion of the promoter of the late-sporulation-specific gene *sspE* to the reporter *lacZ* [9]. In addition to the localization of sporulating cells within the colony using P_{sspE}-*lacZ*, expression of sporulation genes in *B. subtilis* colonies has been visualized using fluorescence. Recently, Veening *et al.* obtained time-lapse video microscopy using sporulation-specific promoters fused to the gene encoding the Green Fluorescent Protein in developing colonies [15]. Unfortunately, in both of these approaches the magnification levels that were utilized did not allow visulization of individual cells so it is still not known if all of the cells in the areas where sporulation is observed are indeed undergoing sporulation. In addition, several other developmental processes extensively

[1]*Sense strictu* these are not colonies in that they are not formed from a single cell. In practice, a few microliters of cells in suspension are deposited on the agar surface. The diameter of the drop placed defines the diameter of the central area of the “colony”. If a single cell is deposited and allowed to grow, the final colony architecture is similar but lacks the structures observed at the center of these “spot colonies.”

studied in *B. subtilis*, namely competence, motility, and cannibalism, have not been examined to determine if they exhibit spatiotemporal organization within a colony.

Different Strains - Different Morphogenesis

Several studies have shown that the complex architecture displayed in colonies varies greatly depending on the strain used [9,15,16]. Different isolates obtained from the environment as well as different strains used in industry, such as *B. subtilis* natto (extensively used in Japan for the production of a food product derived from fermented soybeans), display a wide range of colony morphologies [17]. It is likely that variations in gene content as well as differences in the regulatory circuits involved account for the distinct phenotypes observed. In this regard, it is interesting to note that micro-array based comparative genomic hybridization analyses show that there is considerable genetic heterogeneity among members of the *B. subtilis* species [18].

The strain utilized in many of the studies of colony development, *B. subtilis* NCIB3610, is very closely related to the widely used (and consequently domesticated) laboratory strain *B. subtilis* 168 (both are contrasted in Figure 2) [9]. Therefore, in theory it should be possible to eventually identify all the genetic differences that account for their unique morphologies. This is a work in progress. Thus far, several genes have been identified as mutant in *B. subtilis* 168 and shown to contribute to morphogenesis in *B. subtilis* NCIB3610. For example, the lipopeptide surfactin is required for the morphogenesis of aerial projections that form along colony edges [19]. In addition, supplying exogenous surfactin restores normal morphology to mutants of *B. subtilis* NCIB3610 unable to make surfactin. Interestingly, surfactin is not produced by the domesticated *B. subtilis* 168 strains due to a frameshift mutation in *sfp*, a gene whose product is necessary for surfactin biosynthesis [9]. However, introduction of the wild-type allele of *sfp* into *B. subtilis* 168 does not lead to a strain able to make colonies whose architecture resembles that of *B. subtilis* NCIB3610.

Whether a strain secretes the exopolymer poly-γ-DL-glutamic acid (γ-PGA) or not also can have effects on colony morphology. Strains that secrete plentiful amounts of γ-PGA tend to give rise to mucoid colonies [16]. Domestication may play a role in reducing or abolishing the amount of γ-PGA produced by laboratory strains. *B. subtilis* 168 does not secrete γ-PGA and harbors mutations in the regulator of swarming *swrA* and in the promoter of the regulator *degQ* [16]. Both of these regulators appear to be required for γ-PGA biosynthesis because restoring these two mutated genes with their corresponding wild-type alleles led to a restoration of γ-PGA secretion. The resultant strain displayed enhanced cell-to-surface adhesion but did not yield colonies displaying a wrinkled morphology like that of *B. subtilis* NCIB3610 [16]. In addition, when the genes for γ-PGA biosynthesis were mutated in *B. subtilis* NCIB3610 there was no noticeable change in colony morphology [20]. Thus, while γ-PGA production can influence morphogenesis, it does not appear to play a role in the development of the aerial projections that are indicative of spatiotemporal organization and adumbrate multicellularity.

Genes Involved in Colony Morphogenesis

Our understanding of the molecular mechanisms underlying colony morphogenesis has grown enormously as a direct consequence of genetic analyses involving mutants that display altered morphologies. In this regard, this process has not differed dramatically from any other in which genetic approaches have been applied - if one has a robust phenotype, one can devise strategies to obtain mutants with altered phenotypes. Diverse genetic screens have been designed to identify *B. subtilis* genes involved in colony morphogenesis [21,22]. It is now apparent that there is one genetic circuit involved in switching a cell from a unicellular and motile state to becoming a producer of an extracellular matrix (Figure 4). Once some cells in a population commence extracelluar matrix production the population as a whole can share the resource and

be encased in a matrix that may provide protection and an increased potential for spatiotemporal organization [20]. The main products that compose the *B. subtilis* extracellular matrix are an exopolysaccharide, whose synthesis is directed by the products of the *epsA-O* operon (henceforth simply *eps*), and TasA, a secreted protein encoded in the three gene operon *yqxM-sipW-tasA* (henceforth simply *yqxM*) [20,23]. The other two genes in this latter operon encode a dedicated signal peptidase (SipW) and a protein whose role appears to be the proper localization of TasA to the matrix (YqxM) [20,24]. When a strain is unable to produce the extracellular matrix, the colony morphology is completely flat [20,23].

The master regulator controlling the switch in cell fates is the repressor SinR [25]. By binding to operators in the *eps* and *yqxM* promoter regions SinR prevents their transcription [23]. SinR indirectly promotes motility through a mechanism that remains poorly understood [26,27]. When conditions become propitious for the switch in cell fate to occur, the SinR repressor is antagonized by SinI [25].

What are the signals that lead to the production of active SinI? This is an area that still needs much investigation. Certain media lead to much more robust colony architecture than others. For example, the colonies shown in Figure 3 grew in a medium rich in glycerol and having glutamate as the main nitrogen source. Such conditions result in matrix production and thus wrinkled colonies. In contrast, colonies grown in LB tend to have very flat morphologies indicative of little or no matrix synthesis. Yet exactly what nutritional signals are key for matrix production remain unknown. However, genetic studies have yielded clues about how the synthesis of SinI is regulated. The central regulator of sporulation, Spo0A, acts as an activator of *sinI* transcription [28]. Not surprisingly, *spo0A* mutants develop flat colonies and non-adherent cells [9,29]. The Spo0A-repressed repressor AbrB is also likely involved in the circuitry but exactly how is not yet known [29]. Spo0A is known to become activated by phosphorylation both directly by some kinases and indirectly via a phosphorelay that receives phosphates from several other kinases [30]. At this point it is not known which kinases are involved in the activation of Spo0A in such a manner that *sinI* is transcribed. Two other gene products, YlbF and YmcA, have been implicated in SinI activity [21]. Because YlbF has been proposed to act as a modulator of protein stability in the competence pathway [31] it is believed that these two proteins act similarly with SinI.

Several other gene products have been shown to play some role in the development of complex colony architecture [21,22]. Among these are CcpA [32] and LuxS [17]. However, whether they feed into the SinR-dependent pathway regulating matrix synthesis remains unknown. The transcription factor DegU (the response regulator of the DegS DegU two-component system) has also been shown to influence colony morphology [33]. Low levels of DegU~P increase the wrinkling in colonies presumably indicating increased matrix production. In contrast, high DegU~P levels increase exoprotease production. The production of exoproteases in the mature biofilm was suggested to have a role in the escape of the cells from the extracellular matrix.

Conclusions and Future Directions: Do different cell types co-exist within a colony?

It has been proposed that differentiation occurs within colonies and that the microenvironments generated in the multicellular community affect where different cell types are localized [34]. *B. subtilis* colonies provide the ideal system in which to analyze differentiation because several cell types have already been shown to display population heterogeneity in liquid shaken cultures. Noise in gene expression, coupled with positive feedback loops, has been proposed as key in the formation of bistable populations with respect to the ON-OFF state of at least three different global regulators: Spo0A, σ^D, and ComK [35,36]. Thus, one might predict that different subpopulations would co-exist within a colony. The challenge now is to determine if

this is the case by visualizing individual cells and their patterns of gene expression in colonies. It will be very interesting to determine if indeed different cell types co-exist, if they are spatially and temporally organized as was suggested by the localization of sporulating cells, and if cell-cell communication plays a role in defining when and where each cell type exists.

Acknowledgements

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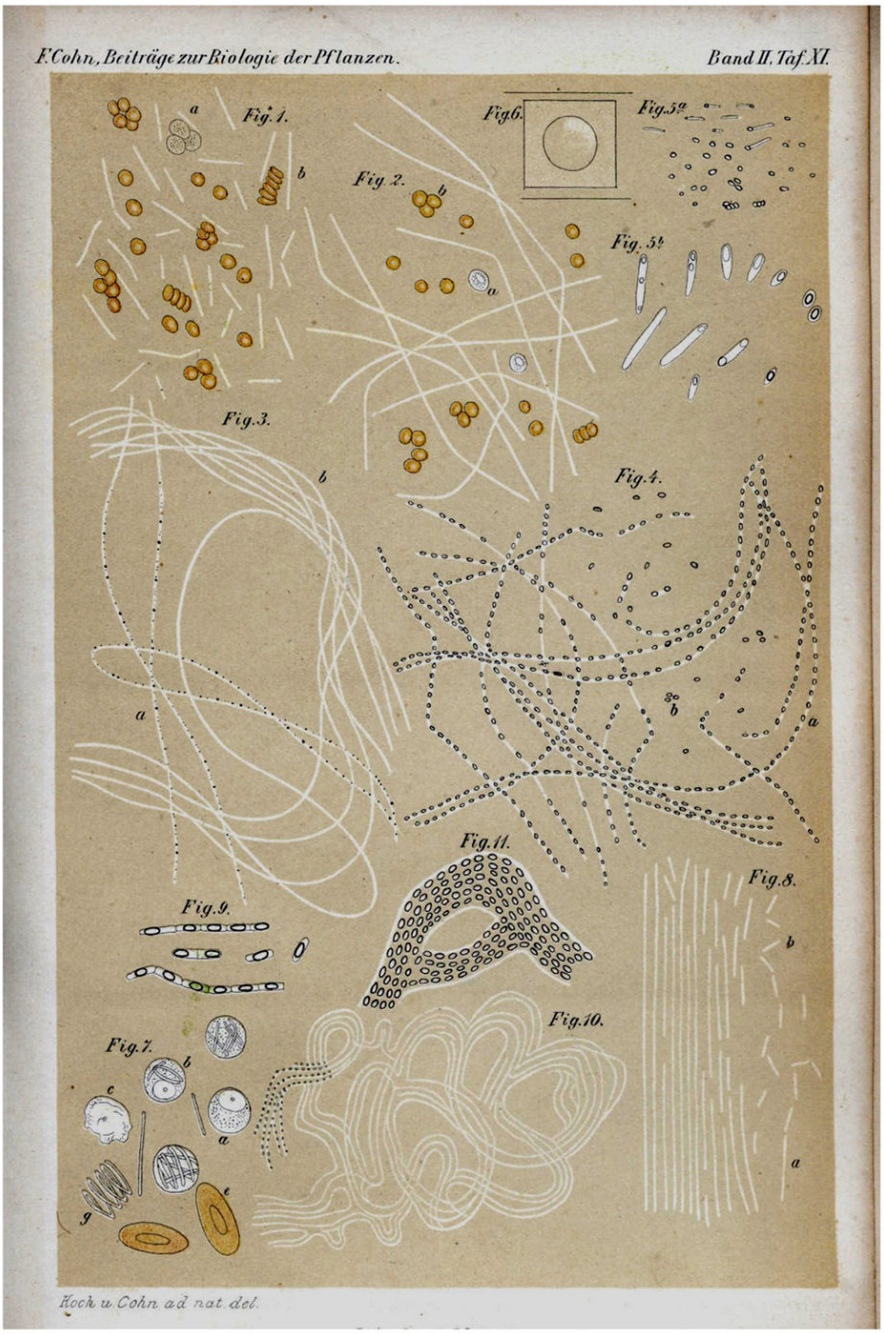

Figure 1.
Color plate containing the eleven figures accompanying Koch's (figs 1–7) and Cohn's (figs 8–11) papers from 1877 [3,4]. Original volume provided courtesy of the Farlow Botanical Library, Harvard University.

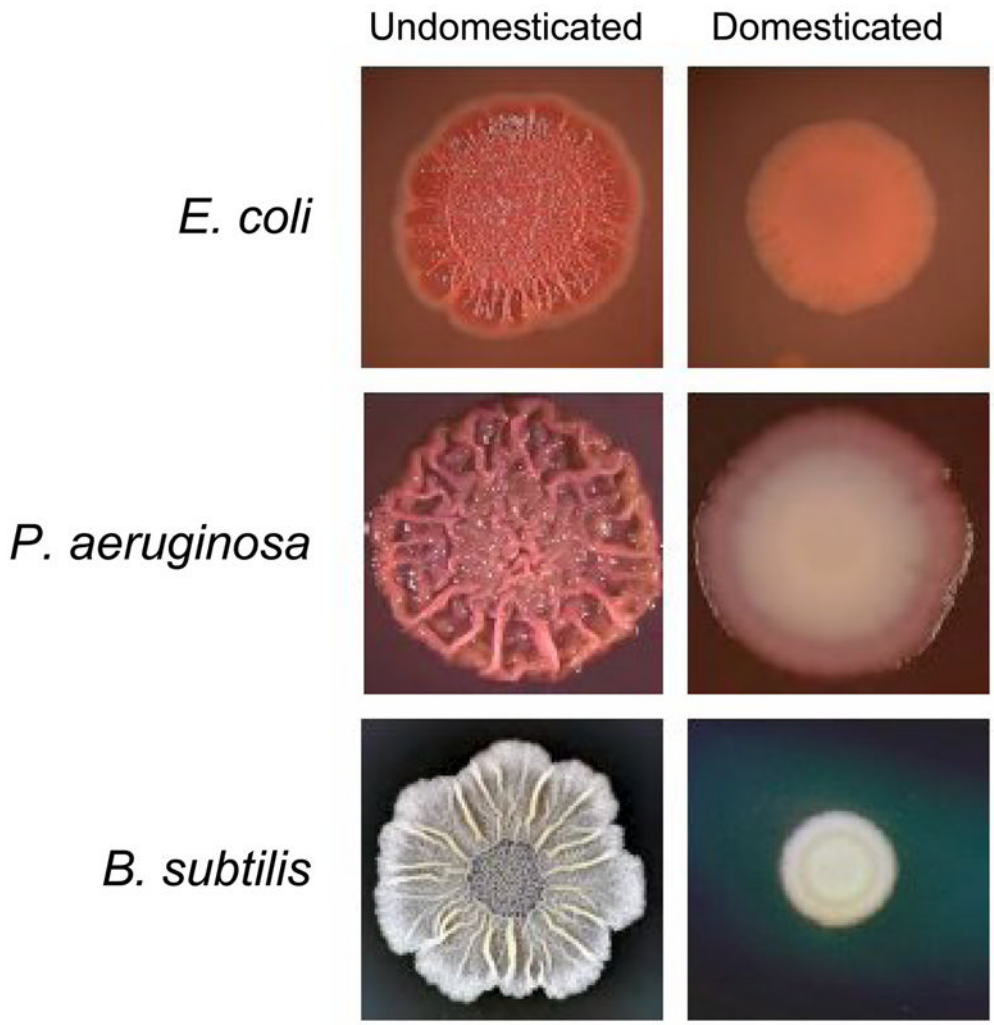

Figure 2.
Examples of the differences in colony morphology between undomesticated and domesticated strains of three bacterial species.

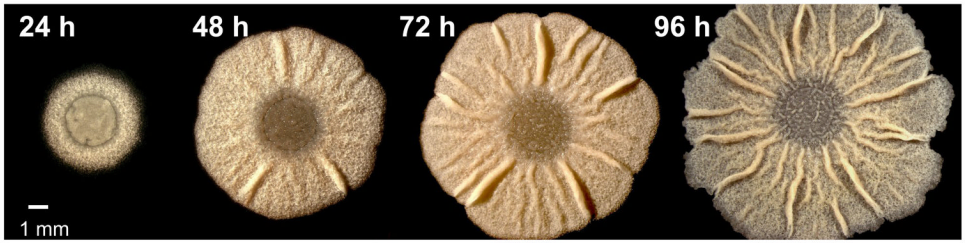

Figure 3.
Time course of *B. subtilis* colony morphogenesis.

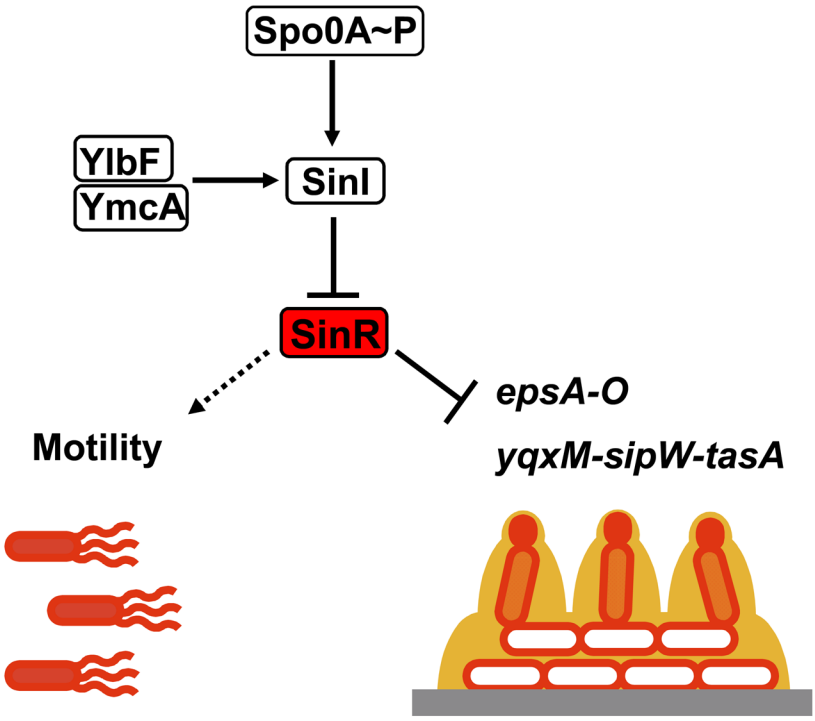

Figure 4.
Diagram outlining the genetic circuitry regulating the transition between motile cells and matrix-producing cells.