Cellular interactions in single- and mixed-species biofilms of *Bacillus subtilis*

DISSERTATION

To fulfill the requirements for the degree of

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by

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OVERVIEW OF MANUSCRIPTS

This is a cumulative dissertation that comprises the following manuscripts.

Author abbreviations:

Ramses Gallegos-Monterrosa (RGM)
Ákos T. Kovács (ATK)
Balázs Bálint (BB)
Eisha Mhatre (EM)
Gergely Maróti (GM)
Ivana Seccareccia (IS)
Markus Nett (MN)
Péter Bihari (PB)
Pierre Stallforth (PS)
Robert Barnett (RB)
Sebastian Götze (SG)
Sonja Köppenhöfer (SKö)
Stefanie Kankel (SKa)
Tino Barchewitz (TB)

- **From environmental signals to regulators: modulation of biofilm development in Gram-positive bacteria.**

  Authors: Eisha Mhatre, Ramses Gallegos-Monterrosa, and Ákos T. Kovács.

  Publication: This manuscript was published in Journal of Basic Microbiology (2014), Volume 54, Issue 7, pages 616-632.

  Presented as: Chapter 1.

Conceived the paper	EM (40%), RGM (40%), ATK (20%)
Wrote the manuscript	EM (40%), RGM (40%), ATK (20%)

- **Specific *Bacillus subtilis* 168 variants form biofilms on nutrient-rich medium.**

  Authors: Ramses Gallegos-Monterrosa, Eisha Mhatre, and Ákos T. Kovács.

  Publication: This manuscript was published in Microbiology (2016), Volume 162, Issue 11, pages 1922-1932.

  Presented as: Chapter 2.

Conceived the project	ATK (100%)
Designed the experiments	RGM (40%), EM (40%), ATK (20%)
Constructed bacterial strains	RGM (70%), EM (30%)
Performed the experiments	RGM (60%), EM (40%)
Analyzed experimental data	RGM (60%), EM (30%), ATK (10%)
Wrote the manuscript	RGM (80%), EM (10%), ATK (10%)

- ***Lysinibacillus fusiformis* M5 induces increased complexity in *Bacillus subtilis* 168 colony biofilms via hypoxanthine.**

  Authors: Ramses Gallegos-Monterrosa, Stefanie Kankel, Sebastian Götze, Robert Barnett, Pierre Stallforth, and Ákos T. Kovács.

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Conceived the project	RGM (40%), ATK (60%)
Designed the experiments	RGM (70%), ATK (30%)
Constructed bacterial strains	RGM (100%)
Performed the biological experiments	RGM (80%), SKa (20%)
Performed and analyzed chemical experiments	SG (60%), RB (10%), PS (30%)
Analyzed experimental data	RGM (80%), ATK (20%)
Wrote the manuscript	RGM (75%), PS (10%), ATK (15%)

- **Draft genome sequence of the soil isolate *Lysinibacillus fusiformis* M5, a potential hypoxanthine producer.**

  Authors: Ramses Gallegos-Monterrosa, Gergely Maróti, Balázs Bálint, and Ákos T. Kovács.

  Publication: This manuscript was published in Genome Announcements (2016), Volume 4, Issue 6, pages e01272-16.

  Presented as: Chapter 4.

Conceived the project	ATK (100%)
Performed experiments	RGM (100%)
Performed genome sequencing	BB (50%), GM (50%)
Wrote the manuscript	RGM (40%), BB (20%), GM (10%), ATK (30%)

- **Unraveling the predator-prey relationship of *Cupriavidus necator* and *Bacillus subtilis*.**

  Authors: Ivana Seccareccia, Ákos T. Kovács, Ramses Gallegos-Monterrosa, and Markus Nett.

  Publication: This manuscript was published in Microbiological Research (2016), Volume 192, pages 231-238.

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Conceived the project	MN (100%)
Designed the experiments	IS (50%), ATK (10%), MN (40%)
Constructed bacterial strains	RGM (100%)
Performed the experiments	IS (85%), RGM (15%)
Analyzed experimental data	IS (50%), ATK (10%), RGM (10%), MN (30%)
Wrote the manuscript	IS (50%), MN (50%)
Corrected the manuscript	ATK (50%), RGM (50%)

- **Variance of cell-cell communication networks governs adaptation to distinct life-styles in *Bacillus subtilis*.**

  Authors: Ramses Gallegos-Monterrosa, Tino Barchewitz, Sonja Köppenhöfer, Balázs Bálint, Péter Bihari, Gergely Maróti, and Ákos T. Kovács.

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Conceived the project	RGM (50%), ATK (50%)
Designed the experiments	RGM (60%), ATK (30%), BB (5%), GM (5%)
Constructed bacterial strains	RGM (50%), TB (30%), SKö (20%)
Performed the experiments	RGM (85%), TB (10%), SKö (5%)
Performed amplicon sequencing	BB (40%), PB (40%), GM (20%)
Analyzed experimental data	RGM (80%), ATK (20%)
Wrote the manuscript	RGM (80%), ATK (20%)

A brief description of each manuscript can be found in the Outline and Aim of the Dissertation.

SUMMARY

Microbiology, the study of the smallest living beings, has had a profound impact on human societies and interests. It has provided solutions for diseases that previously plagued mankind for millennia, and it has also changed the nature of entire industries by making their processes more understandable and easier to control. Traditionally, these important benefits have been obtained using liquid shaken cultures that contain cells from only one microbial species. However, this research model does not in fact reflect a common microbial lifestyle.

In nature, microorganisms commonly live in sessile communities called biofilms. These communities have high cell densities that promote the development of communications networks based on signaling molecules, and also allow for complex interactions to form among cells forming part of the biofilm. Furthermore, cells living in natural environments are often exposed to members of other species, which may become collaborative partners in the development on the biofilm, or compete for resources. The recent development of better molecular biology tools and more sophisticated microscopy techniques has made it possible for researchers to study these complex microbial interactions. Even more, the application of social theory and big-data informatic approaches to the study of large microbial populations has brought forward the novel field of sociomicrobiology, which tries to better understand how microbes interact with one another, and promises to revolutionize our understanding of how the smallest living creatures on Earth carry out their lives.

This dissertation presents a comprehensive review of the current knowledge of the development of biofilms by the Gram-positive model bacterium *Bacillus subtilis*, with a focus on the mechanisms and signals that mediate the interactions that this bacterium can establish, both among its own cells and with those of other species. Further, it includes original research on the interactions that *B. subtilis* can develop with other soil bacteria, both as active members of a predator-prey relationship, and as providers of environmental cues that change the structure of *B. subtilis* biofilms. Additionally, the present work includes investigations on the genetic differences between *B. subtilis* strains and strain variants that impact phenotypic social behavior and biofilm formation. This dissertation also includes the first comprehensive inquiry about the total effect that a family of regulatory phosphatases has upon the population heterogeneity of *B. subtilis* and its adaptability to diverse environments and growth conditions.

ZUSAMMENFASSUNG

Mikrobiologie, die Wissenschaft der kleinsten Lebewesen, hat einen tiefgreifenden Einfluss auf die menschliche Gesellschaft und Belange. Sie hat Lösungen für Krankheiten bereitgestellt, die die Menschheit bis dahin über Jahrtausende hinweg heimsuchten und hat ganze Industriezweige durch verständlichere und einfacher zu kontrollierende Prozesse verändert. Diese wichtigen Vorteile sind traditionell durch die Nutzung von geschüttelten Flüssigkulturen erreicht worden, welche Zellen einer einzigen mikrobiellen Spezies enthalten. Dieses wissenschaftliche Modell reflektiert jedoch nicht den üblichen mikrobiellen Lebensstil.

Mikroorganismen leben in der Natur häufig in sessilen Gemeinschaften, sogenannten Biofilmen. Diese Gemeinschaften besitzen hohe Zelldichten, welche die Entwicklung von auf Signalmolekülen basierten Kommunikationsnetzwerken unterstützen. Dadurch wird ebenfalls die Ausbildung von komplexen Interaktionen zwischen Zellen im Biofilm ermöglicht. Weiterhin kommen Zellen, die in einer natürlichen Umgebung leben, oft in Kontakt mit Mitgliedern anderer Arten. Diese können bei der Bildung des Biofilms zusammenarbeiten, oder um vorhandene Resourcen konkurrieren. Die jüngste Entwicklung von besseren molekularbiologischen Methoden und fortgeschritteneren Mikroskopieverfahren hat es für Wissenschaftler möglich gemacht, diese komplexen mikrobiologischen Interaktionen zu untersuchen. Die Anwendung von Theorien der Sozialwissenschaft und informatische Lösungen zur Analyse großer Datensätze von großen mikrobiellen Populationen führte zur Entwicklung des neuen Bereichs der Soziomikrobiologie. Hier wird versucht, die Interaktionen von Mikroben besser zu verstehen, was eine Revolution unseres Verständnisses der Existenz der kleinsten lebenden Organismen der Erde verspricht.

Diese Dissertation stellt eine umfassende Übersicht des aktuellen Wissensstandes der Entwicklung von Biofilmen des grampositiven Modellbakteriums *Bacillus subtilis* dar. Der Fokus der Arbeit liegt auf den Mechanismen und Signalen, welche die Interaktionen bestimmen, die dieses Bakterium sowohl mit eigenen Zellen und als auch mit anderen Arten ausbilden kann. Darüber hinaus enthält sie eigenständige wissenschaftliche Untersuchungen zu Interaktionen, die *B. subtilis* mit anderen Bodenbakterien ausbilden kann, sowohl als aktives Mitglied einer Räuber-Beute-Beziehung wie auch zum Bereitstellen von Umweltsignalen, welche die Struktur von *B. subtilis* Biofilmen verändern kann. Zusätzlich beinhaltet diese Arbeit Forschung zu genetischen Unterschieden zwischen *B. subtilis* Stämmen und Stamm Varianten, die das phänotypische soziale Verhalten und die Biofilm Bildung beeinflussen. Dies schließt eine erste umfassende Nachforschung über die Effekte einer Familie von regulatorischen Phosphatasen auf die Populationsheterogenität von *B. subtilis* und seine Anpassungsfähigkeit gegenüber diversen Umwelt- und Wachtumsbedingungen mit ein.

OUTLINE AND AIM OF THE DISSERTATION

This is a cumulative dissertation composed of 8 main sections. It aims to provide the reader with a comprehensive review of the different facets of the sociomicrobiology of *Bacillus subtilis* and its biofilms. It presents original single- and multi-species research into this topic that use different approaches; from classic molecular biology and microbiology techniques, to advanced fluorescence microscopy and whole-population tracking in multi-strain experimental competition.

The content and function of the main sections of this dissertation is:

Introduction.
This section presents the historical background of biofilm research and their ecological role, followed by a detailed review of the genetic regulatory mechanisms that govern biofilm formation by *B. subtilis* and the social interactions that may take place within them. Its function is to provide the reader with a solid grasp of these topics, in order to better understand the following sections of the dissertation, either independently or as a complete analysis of the sociomicrobiology of *B. subtilis* biofilms.

Chapter 1 – From environmental signals to regulators: modulation of biofilm development in Gram-positive bacteria.
A comprehensive review of the diverse known signals, both environmental and microbe-generated, that can trigger and modulate biofilm formation in Gram-positive bacteria, with a focus on *B. subtilis* biofilms. The function of this section is to complement the information provided in the introduction by presenting specific examples of signaling that trigger and modulate the biofilm formation process.

Chapter 2 – Specific *Bacillus subtilis* 168 variants form biofilms on nutrient-rich medium.
This is a detailed comparison of the biofilm forming capabilities of diverse *B. subtilis* strains, and variants of strain 168. It conveys an analysis of the genetic causes that underlay differences in biofilm formation, and the role that environmental conditions may play to complement this behavior. This first experimental section serves to provide a clear view of the difficulties faced by sociomicrobiologists while choosing research approaches. It showcases different biofilm research models (complex colonies, pellicles, and plant root biofilms) and their impact on biofilm formation by the same bacterium.

Chapter 3 – *Lysinibacillus fusiformis* M5 induces increased complexity in *Bacillus subtilis* 168 colony biofilms via hypoxanthine.
This section presents a case of multi-species microbial interaction that may occur among soil bacteria. The interaction between *B. subtilis* 168 and *L. fusiformis* M5 is examined in depth, researching the signaling molecule and mechanism that mediate the induction of increased architectural complexity of *B. subtilis* 168 biofilms. Its function is to provide an example of how new interspecies bacterial interactions can be investigated at the population level, from the initial phenotypic changes to identification of mediating molecules and initial probing of the mechanism involved in the studied interaction.

Chapter 4 – Draft genome sequence of the soil isolate Lysinibacillus fusiformis M5, a potential hypoxanthine producer.
The sequenced genome of the soil bacterium *L. fusiformis* M5 is presented here, with the intention that it will facilitate future research with this organism, either in sociomicrobiology or in regards to its biotechnological potential.

Chapter 5 – Unraveling the predator-prey relationship of *Cupriavidus necator* and *Bacillus subtilis*.
This section presents thorough research into the predator-prey relationship that can be established between *C. necator* and *B. subtilis*. The predatory condition and mechanism of *C. necator* are investigated with various prey organisms, as well as the strategy that *B. subtilis* utilizes to survive this interaction. Its function is to depict a case of direct cell to cell relationship between two different bacterial species, its possible ecological implications, and the mechanisms that mediate it.

Chapter 6 – Variance of cell-cell communication networks governs adaptation to distinct life-styles in *Bacillus subtilis*.
This is the first comprehensive study on the role that the entire family of Rap regulatory phosphatases and their cognate Phr peptides plays to fine-tune population heterogeneity and adaptation of one single strain of *B. subtilis,* both in pellicle forming conditions and as a planktonic population. The function of this chapter is to present an example of how high-throughput sequencing and lineage-tracking can be used as an approach to investigate sociomicrobiological issues involving complex genetic regulatory networks and large bacterial populations.

General discussion.
The final section presents an extended discussion on the results obtained by the studies presented in this dissertation. Its function is to frame the information and results provided in the previous sections within the context of the interactions that can take place in biofilms of *B. subtilis*. It also provides general reflections about sociomicrobiology and its relevance to the study of microbial life.

INTRODUCTION

Microbiology is the study of microscopic organisms. The existence of small "unseen" organisms was postulated since ancient times by different authors. In the 6th century BC, the Indian philosopher Mahavira proposed the existence of *nigodas*: microscopic creatures living in groups and forming part of any larger organism (1); another example is the Roman scholar and agriculturist Varro, who, during the 1st century BC, wrote recommendations to avoid swamps due to the existence of small invisible creatures able to cause disease (2). However, it was not until the 17th century AD, when Anton van Leeuwenhoek showed the world his observations of *animalcules* (3), that the study of microbial life was cemented as a scientific enterprise. It is easy to understand why microbiology has become a major field of scientific research when one considers the profound impact that microbes have on human life. Ranging from their pernicious activities, such as causing infections and spoiling food; to their beneficial ones, like their production of useful enzymes; microbes are of paramount importance for mankind (4).

Scientists have enthusiastically investigated the lives and characteristics of microorganisms. These efforts have caused several paradigm shifts in microbiology since its conception as a science. The experimental validation of the germ theory of disease, first in silkworms by the Italian entomologist Agostino Bassi (5), and afterwards in humans with the work of Louis Pasteur and Robert Koch (6–8), caused a revolution in antiseptic practices in medicine; which resulted in impressive gains in human health throughout the 20th century (9). Likewise, the importance of microbes for food production, especially related to fermentation processes, was first shown by Pasteur (6, 8), and later on embraced by the food industry to improve food quality and production through the use of biotechnology (10). A similarly important change in the perception of microorganisms has developed in recent decades: the realization that microbes commonly display complex interactions involving large populations or multi-species communities. This has led to the growth of the novel field of sociomicrobiology, which studies the social relations between microorganisms, and promises to profoundly alter our understanding of the smallest living creatures on Earth (11).

Bacteria are a good model to study the social lives of microbes; they have inhabited this planet for billions of years, and thus have had a long evolutionary history to explore and form relationships among themselves (11–13). Perhaps more importantly from a technical point of view, bacteria have been intensively studied by microbiologists, thus generating a large body of knowledge about the molecular processes that control their metabolism. In a similar fashion, multiple scientific tools have been developed by researchers that allow for an efficient use of bacteria in a laboratory environment. Among the plethora of bacterial species, few have enjoyed the level of attention that *Bacillus subtilis* has received. This bacterium has been studied for over a century in a wide range of topics, making it a superb and robust model organism for the study of sociomicrobiology (14–16). In this work, I present a review of the latest research on bacterial communications and their social interactions, with a focus on *B. subtilis* and its biofilms; as well as original research on the population behavior of *B. subtilis* biofilms, in monoculture, and in interaction with other microbial species.

1. Bacterial biofilms.

1.1 Historical perspective.

Traditionally, researchers have studied bacteria as planktonic monocultures. This research model makes the general assumption that a population of clonal cells will consume the resources provided in the medium, and multiply until the resources are exhausted or the accumulation of waste products reaches toxic levels. Importantly, this model views the cellular population as a suspension of individual organisms, all of them behaving in an almost identical manner. This model of bacterial cultures has been very useful for bacteriology; indeed, much of the knowledge in this field has been obtained using planktonic monocultures of bacteria.

Despite the usefulness of the planktonic monoculture model, microbiologists have long recognized that bacteria can have other lifestyles in nature. In particular, the realization that bacteria can live attached to solid surfaces can be glimpsed from Leeuwenhoek´s original studies, when he observed his *animalcules* directly scratched from his own teeth (3). During the 1930´s, various marine microbiologists noted that bacterial populations were larger when they had an available surface on which to grow, and that low availability of nutrients seemed to promote this phenomenon (17, 18). Later, in 1943, Claude Zobell noted that many marine bacteria could be sessile, and that they grow attached to solid surfaces by "exuding a mucilaginous holdfast" (19). Despite these early insights, the lack of methods to study bacterial populations *in situ* hindered further investigations in this topic during most of the 20th century. However, the development of new microscopy and molecular biology techniques, especially regarding the use of fluorescent protein reporters, made it possible for researchers to study the formation and development of surface-attached bacterial populations (20, 21). In 1978, Costerton presented his landmark work regarding a new paradigm for chronic infections, where he described surface-attached bacterial populations as "bacteria covered by a glycocalyx of fibers that adhere to surfaces and other cells". In posterior studies, Costerton coined the term "biofilm" to refer to this bacterial lifestyle (22–24).

1.2 Biofilm development.

Microbiologists now recognize that the majority of microorganisms live preferentially attached to surfaces, and encased by a matrix of polymeric substances; rather than as single planktonic cells (25). Biofilms can be formed in both biotic and abiotic surfaces, and therefore can develop in multiple forms. In laboratory settings, biofilms are most commonly studied as submerged communities formed by bacteria attached to a solid abiotic surface that is covered by liquid media (Fig. 1A); one more model favored by microbiologists is biofilms as colonies on agar plates, where bacteria can form architecturally complex colonies characterized by a rugose or wrinkled surface (Fig. 1B); furthermore, biofilms are also commonly studied as floating pellicles, formed by bacteria attached to each other forming a community in the air-liquid interface of static liquid cultures (Fig. 1C).

Despite their diverse appearance, biofilms share a general developmental path. The onset of biofilm formation depends on surface sensing and attachment. This is an important decision for

cells because motility and biofilm formation are considered mutually exclusive events, and transition from one to the other implies major metabolic changes (26, 27).

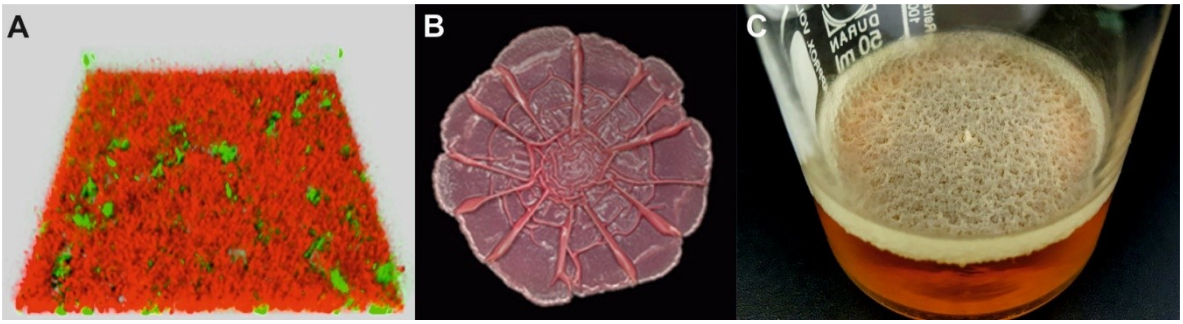

Figure 1: Types of bacterial biofilms commonly studied in the laboratory. **A)** Confocal laser scanning microscopy image of a mixed-species submerged biofilm of *B. subtilis* (green) and *Staphylococcus aureus* (red) (28). **B)** Colony biofilm of *Pseudomonas aeruginosa* (29). **C)** Pellicle biofilm of *B. subtilis* (Image obtained by Marivic Martin).

The flagellum is the main organelle required for bacterial motility; bacteria can quickly rotate these flexible structures to swim in liquid media, or to swarm on semi-solid surfaces. The flagellum and its associated proteins can also function as a mechanosensor, and transduce surface signals to the cell (26). In *Vibrio cholerae*, interactions with surfaces cause mechanical obstructions to flagellar motility and changes in the ion flow through the flagellar motor, which in turn promotes permanent surface attachment (30). Another example occurs in *B. subtilis*, where mechanical inhibition of flagellar rotation induces an increased expression of transcriptional regulators that promote biofilms formation (31). Additionally, membrane-bound signal transduction systems can also sense mechanical changes and stress in cell walls, triggering biofilm formation. For example, in *Escherichia coli* the CpxRA two-component system can sense changes in the cell wall derived from interactions with hydrophobic surfaces, and promote the expression of surface-attachment factors (32). In the case of pellicles, it has been shown that aerotaxis and motility are important for cells of *B. subtilis* and *Pseudomonas aeruginosa* to efficiently initiate pellicle formation. The proposed mechanism is that, as oxygen concentrations decrease in the culture medium, cells need to locate and migrate to the oxygen-rich air-liquid interface (33). Similar mechanisms have been proposed in other bacterial species such as *E. coli* and *Shewanella oneidensis*, where anoxic conditions impair pellicle formation (34, 35).

Once attached to a surface, bacteria multiply and form microcolonies. Interestingly, these microcolonies do not grow uniformly; rather, they are interspersed with open channels that allow an efficient diffusion of nutrients to all the cells and elimination of waste products (36–38). Thus, a hint of the complex architecture and organization of biofilms is revealed early in their development (Fig. 2).

As the microcolonies grow, the cells in these aggregates produce various extracellular polymeric substances (EPS) that form the matrix that surrounds the biofilm. These polymers further mediate cellular attachment to surfaces and to each other. The EPS matrix is mainly comprised of polysaccharides, proteins, nucleic acids and lipids. The production of EPS is a complicated process that has being intensively investigated by researchers. Thus, we now know that the nature of the biofilm matrix shows variation among bacterial species, and even among different strains of the same species, or the same bacterium but grown in different environmental conditions (39, 40). For example, *B. subtilis* NCIB 3610 produces poly-N-acetylglucosamine as its

main matrix component, while *B. subtilis* RO-FF-1 mainly produces γ-poly-DL-glutamic acid (γ-PGA) to form its biofilm matrix (41, 42).

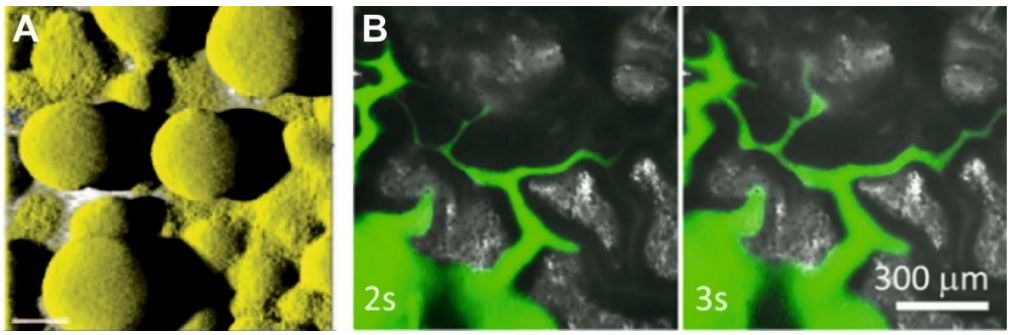

Figure 2: Complex architecture of bacterial biofilms. **A)** Confocal laser scanning image of a submerged biofilm of *P. aeruginosa*. Scale bar represents 40 µm (43). **B)** Series of microscopy images of a region near the center of a *B. subtilis* biofilm. Injection of an aqueous dye (green) reveals a network of channels beneath the wrinkles (44).

Regardless of its composition, the biofilm matrix is the most representative part of any bacterial biofilm. Its production involves a major metabolic investment for cells; and once produced, the biofilm matrix fulfills important roles for the biofilm community. It mediates the long-term adhesion of cells to surfaces and to each other, maintains a hydrated environment for the cells, hinders the diffusion of toxic substances from the environment, and provides an environment ideal for the digestion of macromolecules by limiting the diffusion and loss of nutrients. Furthermore, it enables complex social interactions among the cells by creating microenvironments within the biofilms, where cells can better adapt to specific conditions (45–48). Truly, if biofilms are seen as bacterial cities, then the matrix would be the buildings, roads, postal service and sewer system of those communities.

Finally, bacteria are also able to escape their own biofilms. This may be desirable for cells once the nutrients in the biofilm have been depleted, or the accumulation of waste products reaches toxic levels (49). Cells mediate biofilm dispersal by producing small molecules and enzymes that degrade the biofilm matrix. As is the case with matrix component production, the strategies for biofilm disassembly vary among bacterial species (50). In *Staphylococcus aureus*, the *agr* quorum sensing (QS) system induces the expression of proteinase genes that release cells from the protein fibers that keep them fixed to the biofilm (51). A more complex strategy is found in *P. aeruginosa*, where the surfactant rhamnolipid is beneficial for biofilm formation when produced in low amounts; however, when its concentration increases it promotes biofilm disassembly (43, 52). Another example is the case of "seeding dispersal" used by pathogenic bacteria such as *Serratia marcescens*; in these cases, hollow cavities filled with motile cells are formed within the biofilm, and a breach of the cavity releases all these cells into the surrounding medium (53). Interestingly, some well-characterized bacterial biofilm formers do not seem to require an escape mechanism; *B. subtilis*, for example, produces resistance structures known as spores as part of its biofilm-forming development. Spores are able to survive harsh environmental conditions, and can lay dormant for decades. When the environmental conditions are once again optimal (perhaps after desiccation and mechanical dispersal of the biofilm), spores germinate and produce cells, which can form new biofilms of their own (54, 55).

To summarize, biofilm development can be said to occur in phases; i) motile cells sense and attach to a surface, ii) attached cells form microcolonies and start producing the biofilm matrix,

iii) the community grows and expands the biofilm, iv) as the resources dwindle and local toxicity increases, some cells form spores or escape the biofilm (Fig. 3).

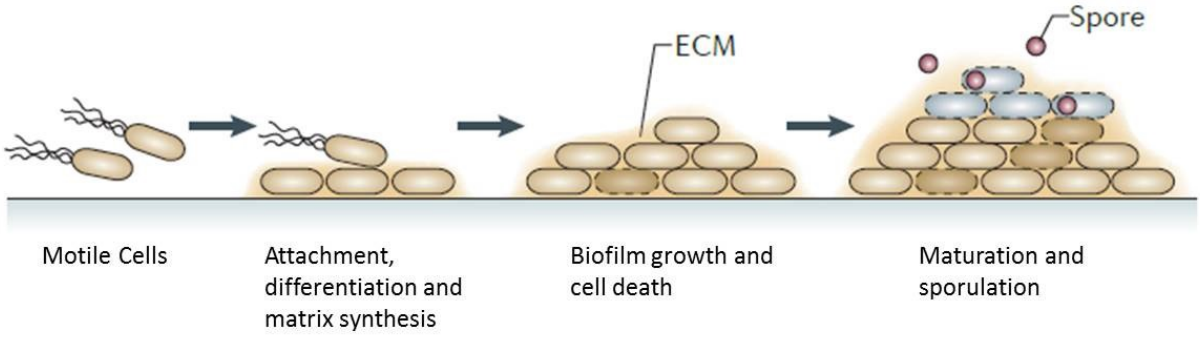

Figure 3: General developmental process of a bacterial biofilm. Solid-line cells represent metabolically active cells, segmented-line cells represent dead cells, ECM = extracellular matrix (56).

1.3 Biofilms in nature.

Bacteria inhabit all environments that are suitable for more complex organisms, and many more that most complex organisms would find intolerable (57). It is therefore not surprising that biofilms can be found in a wide variety of environments. One of the most relevant characteristics of biofilms is their high tolerance to difficult environmental conditions, allowing bacterial survival under conditions where planktonic cells would quickly die (20, 25).

In aquatic environments, biofilms can be found from the middle of the ocean to the bottom of mountain rivers (25). Since nutrients are quickly dispersed in an aqueous medium, biofilm formation and aggregation around a food source is a priority for bacteria living in these environments (58). In the sea, one of the best known examples of biofilm formation is the case of marine "snow". This phenomenon happens when organic matter sinks through the water and is quickly colonized by bacteria to consume its resources. Marine snow can form around any type of organic material usually found in the sea: phytoplankton detritus, discarded animal shells, fecal pellets and dead animals. All this material represents a rich and concentrated source of energy, carbon, macronutrients, and minerals in an otherwise nutrient-poor environment. Bacteria present in the proximity of these particles quickly detect and colonize them, forming a biofilm to minimize the loss of resources by diffusion and to protect themselves against environmental insults and predation (58). In the rivers and lakes of the world, biofilms can be found colonizing the sediment and solid surfaces in their bottom, as well as the roots of plants floating in their surface (59). There, these biofilms play an important ecological role in the remineralization of organic matter, as well as in the course of geological cycles (60, 61).

Biofilms are also ubiquitous and important in soil environments. This is not surprising when one considers that soil is the environment that contains both the highest diversity of bacterial species and the largest average number of cells per volume in the planet (62, 63). One of the most relevant types of soil biofilms are those formed in the rhizosphere; the area immediately adjacent to the roots of plants. There, biofilm formation is related to both symbiotic and pathogenic processes, depending on the colonizing bacteria and general physiological conditions of the

plant (64). As an example, *Agrobacterium tumefaciens* is a pathogenic bacterium able to cause crown gall tumors in several plant species. Recently, it has become increasingly recognized that biofilm formation is important for this bacterium not only for plant colonization, but also as a reservoir mechanism in soil particles when the bacterium is not associated to a plant (65, 66). On the other hand, *B. subtilis* is known as a biocontrol agent able to colonize and form biofilms on the roots of plants, and protect them from invasion by pathogenic agents (67). In this case, the bacterium receives the benefit of nutrients, as the plant secretes polysaccharides to sustain the biofilm (68). The impact of biofilms in the soil does not stop at the biosphere level; nowadays it is known that they affect the cycles of diverse minerals (69, 70) and general geology of the Earth (71, 72), and that this impact may be deep enough to use them as indicators of ancient life in other planets (73).

Biofilms are relevant for human activities as well, both in positive and negative ways. As a beneficial example, multi-species biofilms of archaea and bacteria are routinely used in waste water treatment plants to degrade complex organic contaminant molecules (74). In these cases, the regular metabolic activity of the biofilm microorganisms makes it easier and cheaper to dispose of these contaminants than using chemically synthetized catalysts. On the other hand, biofilms can be highly damaging for human interests, as in the case of biofilms of *Listeria monocytogenes* in food processing plants, where they become a persistent source of contamination that can lead to disease outbreaks (75).

Bacterial biofilms are particularly important for human health. Multiple bacterial species can colonize different cavities and tissues of the human body and form biofilms, which become a persistent infection resistant to antibiotic treatment (76). The issue of infectious biofilms is highly relevant for medicine, and indeed a large portion of the knowledge about biofilms and biofilm control has been obtained using human pathogenic bacteria as research models. The most salient point for treating biofilm-related infections is their high resistance to antibiotics (conferred by the biofilm matrix), which can be 1000-fold higher as compared to planktonic cells (20, 76, 77). There are abundant examples about the severity and consequences of biofilm-related infections: common oral problems, such as tooth decay, are caused by the long-term effects of biofilm-forming bacteria such as multiple *Streptococcus* species (48, 78); another example are the biofilms associated to cystic fibrosis, where the opportunistic pathogen *P. aeruginosa* colonizes the lungs of patients and becomes a major cause of host mortality (79). Finally, biofilms can also cause infections indirectly, by serving as pathogen reservoirs. Besides the above-mentioned example of *L. monocytogenes* in food processing plants, bacteria commonly form biofilms in the water systems of human dwellings, which may cause recurrent infections by contaminating the drinking water (80, 81).

To conclude, bacterial biofilms can be found in a wide variety of environments, and play an important ecological role in all of them (Fig. 4). The study of bacterial biofilms is thus a major point of interest for scientists in order to better understand the microbial world, and to utilize them for biotechnological purposes, or to remove them when they are pernicious for human life.

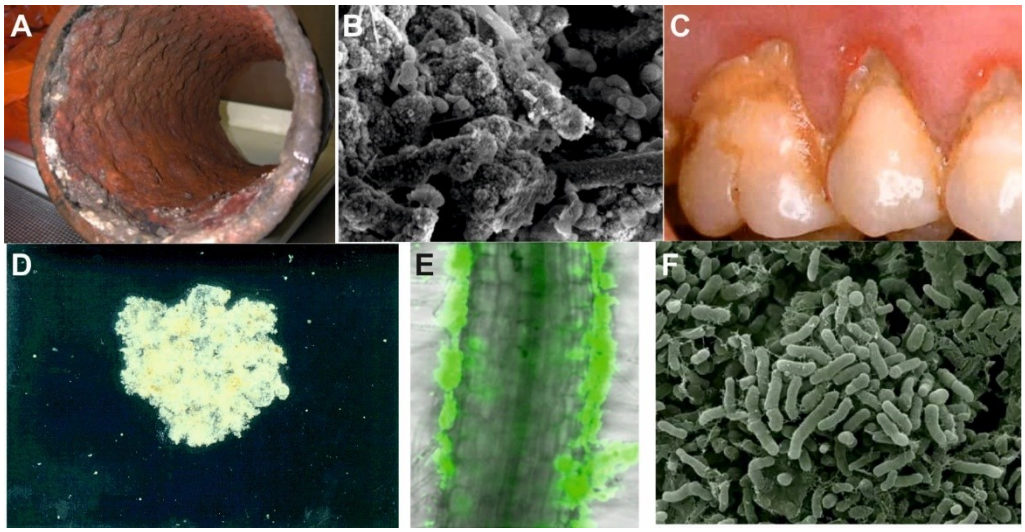

Figure 4: Bacterial biofilms in nature. **A)** Biofilm of an infectious *E. coli* O157:H7 strain in a water pipe, and **B)** Scanning electron microscopy image of that same biofilm (see esemag.com/archive/0105/0105ed.html). **C)** Multi-species dental plaque biofilm in a human patient (see brightonimplantclinic.co.uk/understanding-tartar-part-ii/). **D)** Multi-species biofilm formed on organic matter particles in marine waters (see ocean.si.edu/ocean-news/marine-snow-staple-deep). **E)** Confocal laser scanning microscopy image of a *B. subtilis* biofilm (green) colonizing the roots of a tomato plant (67). **F)** Scanning electron microscopy image of an *E. coli* biofilm growing on lettuce leaf (82).

2. Biofilm formation by *Bacillus subtilis*.

2.1 *B. subtilis* as a model bacterium for scientific research.

B. subtilis is a rod-shaped non-pathogenic Gram-positive bacterium that was originally named *Vibrio subtilis* by Christian Ehrenberg in 1835, and later renamed as *B. subtilis* by Ferdinand Cohn in 1872 (83, 84). *B. subtilis* can be isolated from multiple soil and aquatic environments, and therefore it has been often dubbed as an ubiquitous microorganism of the biosphere (85).

B. subtilis possesses several characteristics that have made it an attractive research organism: like all members of the *Bacillus* genus, it can produce endospores as a survival mechanism against adverse environmental conditions (86); it commonly produces metabolites with antibiotic activity, with up to 5 % of its genome devoted to antibiotic production (87); its robust metabolism makes it ideal for biotechnological applications, such as enzyme production (15); and its widespread distribution make it a good model for studying microbial ecology (85, 88). All these studies have provided microbiologists with extensive knowledge about the general metabolism and genetic regulation of *B. subtilis*, which in turn as made it into a superb research model from Gram-positive bacteria.

In more recent decades, *B. subtilis* has also been studied for its capacity to form biofilms. Numerous studies have thus revealed that biofilm formation in *B. subtilis* proceeds through a complex and tightly regulated genetic network (89), that it produces multiple cells types to divide labor within the biofilm (54), and that this population heterogeneity exhibits a spatiotemporal regulation (90). In laboratory settings, *B. subtilis* biofilms have been most commonly studied as architecturally complex colonies growing on solid agar (Fig. 5A) or as floating pellicles in the air-liquid interface of static liquid cultures (Fig. 5B), both of these types of biofilm develop robust communities with an opaque, dry, and wrinkled surface (89, 91, 92). More

recently, *B. subtilis* biofilms have also been studied as plant root colonizers able to compete against plant pathogens (Fig. 5C) (93); as submerged biofilms attached to the bottom of polystyrene microtiter plates (Fig. 5D) (94); and encased in emulsion drops to simulate microenvironments (Fig. 5E) (95).

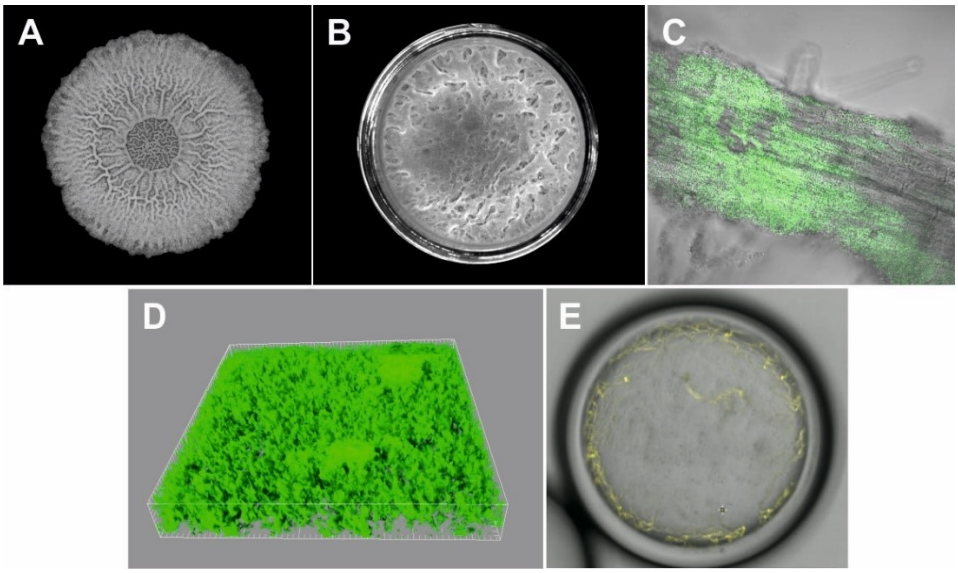

Figure 5: *B. subtilis* biofilms used in research. **A)** Colony biofilm grown on MSgg medium (96). **B)** Pellicle biofilm grown on MSgg broth (96). **C)** Root-colonizing biofilm (green) grown on *Arabidopsis thaliana* (96). **D)** Submerged biofilm grown with LB broth (97). **E)** Biofilms (yellow) contained in LB-emulsion microdroplets (95).

The next sections focus on the process and regulation of biofilm formation by *B. subtilis*, which was used as the model organism for the work presented in this dissertation.

2.2 Regulation of motility and biofilm initiation.

B. subtilis is a multi-flagellated bacterium that possesses at least two types of active motility: swimming and swarming. Swimming is shown by single planktonic cells moving freely in a liquid medium; while swarming occurs when groups of cells move together on a wet semi-solid surface. Both of these types of motility require the production and proper function of rotating flagella (98). Furthermore, *B. subtilis* can also display a type of passive motility known as sliding, which does not require flagella. Sliding occurs via the production of surfactant molecules, which reduce surface tension and allow the cells to spread as they multiply (99).

As is common among bacteria, the decision-making process between motility and biofilm initiation is tightly regulated in *B. subtilis* cells. Besides the regular complexities of producing a functional flagellum (100), *B. subtilis* has the added complication of exhibiting wide population heterogeneity, i.e., even when growing under apparently equal conditions, cells will show stochastic differences in gene expression (101). As an example, during exponential growth *B. subtilis* can multiply forming single independent cells, or chains of cells joined together by their poles (Fig. 6A).

In *B. subtilis*, the long *fla/che* operon (ca. 27 Kbp) is highly important for motility, since its genes codify many components of the flagellar motor. The alternate sigma factor σ^D, which regulates population heterogeneity and expression of flagellar genes, is located almost at the end of this

operon (98, 102, 103) (Fig. 6B). It has been shown that transcript abundance decreases along the *fla/che* operon, and that gene position in this operon can drastically change their corresponding expression (104). Since σ^D is the penultimate gene in its operon, the expression levels of this gene vary among cells. σ^D protein levels are low in chained cells, and remain high in single motile cells. This is an important difference because chained cells can attach to surfaces more efficiently and initiate biofilm formation (98). Thus, stochastic expression of the *fla/che* operon genes can account for differences in regulation of motility and surface attachment of individual cells.

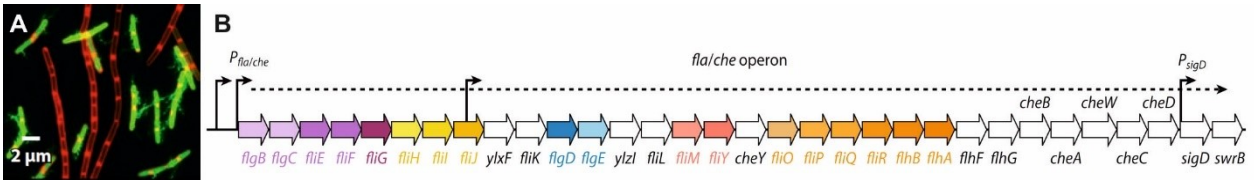

Figure 6: Motile population heterogeneity in *B. subtilis* (98). **A)** Fluorescence microscopy image of a *B. subtilis* liquid culture showing cells expressing a P_{hag}-YFP reporter fusion (main flagellum filament protein, green) and membranes stained with FM 4–64 dye (red). **B)** Schematic representation of the *fla/che* operon of *B. subtilis*: open arrows represent genes (⇨), bent arrows represent promoters (↱). Gene arrows are color coded as follows: flagellum basal body – violet, flagellum protein secretion – yellow/orange, flagellum hook – blue.

A determined regulator of the switch between planktonic and sessile lifestyle in *B. subtilis* is the canonical two-component system DegS-DegU. DegS is a cytoplasmic sensor histidine kinase able to phosphorylate its cognate response regulator, DegU (105). The regulon of DegU is extensive, and includes genes associated to both motility (e.g. flagellum production), and biofilm formation (e.g. exoenzymes needed for substrate degradation). It has been shown that the phosphorylation state of DegU is the factor that determines which genes are transcribed (106). When DegU is not phosphorylated, it preferentially promotes the expression of genes needed for flagellum biosynthesis, such as the *fla/che* operon. On the other hand, when DegU has been phosphorylated (DegU~P) it promotes the transcription of genes whose products are needed for sessile lifestyle, such as amylase, glucanase, and production of γ-PGA. Furthermore, DegU~P promotes the transcription of its own biosynthetic gene, *degU*, and inhibits the expression of some of the flagellar genes that it activates in its non-phosphorylated form (106) (Fig. 7). Thus, the cellular ratio of DegU/DegU~P is an important mechanism that regulates population heterogeneity in *B. subtilis*; as the concentration of DegU~P increases, the cell will transition from being motile to a state that favors biofilm formation (27, 106, 107).

The signals that promote phosphorylation of DegU by DegS are still not completely known. However, it was recently shown that the flagellum can act as a mechanosensor, and cause DegS to phosphorylate DegU when flagellar activity is impaired. Cairns *et al.* studied mutant strains of *B. subtilis* unable to form functional flagella. They observed that a *motB* mutant, unable to produce a functional flagellar motor, showed increased DegU~P levels and overproduction of γ-PGA. More tellingly, when they impeded flagellar rotation using antibodies, they also observed and increase in DegU~P activity (31). Evidence found in other bacteria also supports the general idea that inhibition of flagellar rotation, possibly by contact with surfaces, is a signal that promotes biofilm formation (26, 27).

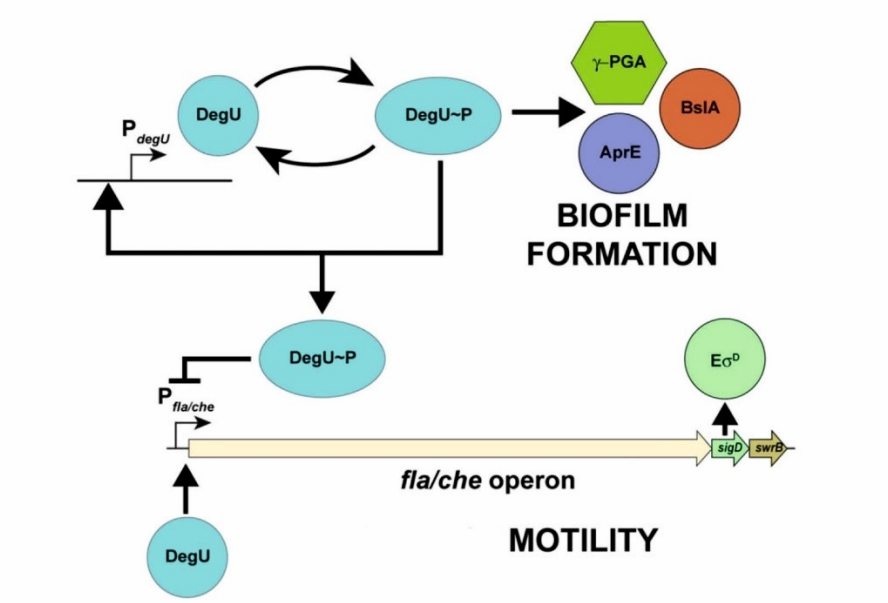

Figure 7: Control of a motility/biofilm switch by DegU (27). Open arrows represent genes (⇨), bent arrows represent promoters (↱), arrows indicate activation (→), T bars indicate inhibition (⊢). γ-PGA, AprE, and BslA are examples of biofilm-related proteins or polymers.

Another important regulatory mechanism for the motile-sessile lifestyle switch is given by the regulator SlrR and the master transcriptional repressor of biofilm formation, SinR. *B. subtilis* cells generally express SinR, this regulator inhibits biofilm-matrix genes and indirectly promotes the expression of motility-related genes (108–110). SinR can also inhibit the expression of *slrR*. SlrR is a repressor of SinR that can titrate it and form a SinR-SlrR complex unable to repress the normal SinR target promoters, but now able to inhibit expression of motility-related genes (111). Thus SinR and SlrR form a double negative feedback loop where each protein can block the activity of the other. This balance can be disturbed when SinR suffers additional repression, for example by its cognate inhibitor SinI. Expression of *sinI* is positively controlled by Spo0A, a master regulator for biofilm gene expression (89)(Fig. 8).

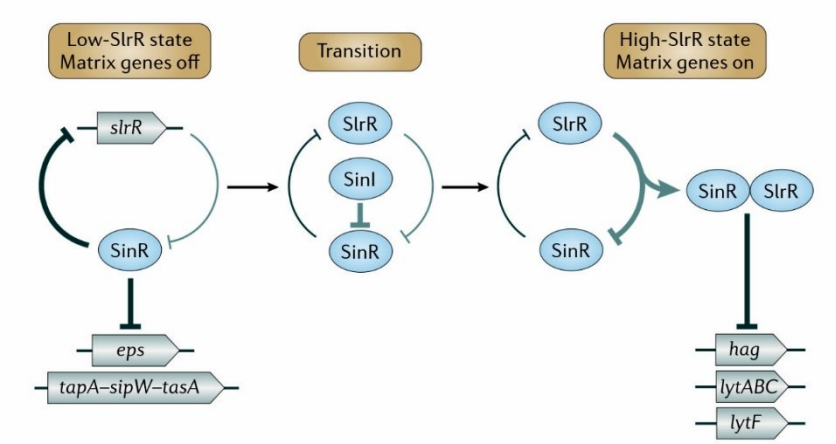

Figure 8: Epigenetic switch of the SinR-SlrR pair (89). A double-negative feedback loop between SinR and SlrR controls the expression of motility- and matrix-related genes. Open arrows represent genes (⇨), arrows indicate activation (→), T bars indicate inhibition (⊢).

As can be appreciated by examining all these mechanisms, the transition from planktonic to sessile lifestyle is a finely tuned process in *B. subtilis*, involving multiple regulatory steps from stochastic gene expression to environmental signal detection.

2.3 Production of the biofilm matrix.

Once biofilm formation has initiated, cells must produce the extracellular matrix that will support all the cells living in the biofilm and provide protection from environmental stresses.

The main components of the biofilm matrix of *B. subtilis* are produced by the biosynthetic operons *epsABCDEFGHIJKLMNO* (*epsA-O*) and *tapA-sipW-tasA*. The *epsA-O* operon is responsible for producing the exopolysaccharide component of the biofilm matrix (89, 112). Although the exact composition of this polysaccharide seems to vary among *B. subtilis* strains (41, 42), the importance of the *epsA-O* operon has been clearly established for various strains and biofilm models of *B. subtilis*. Strains with mutations in the epsA-O genes display defective biofilm formation, developing flat mucoid colonies in agar plates, or thin and weak pellicles in liquid static cultures that tend to collapse and sink (Fig. 9) (113, 114). The differences in biofilm formation between recent soil isolates of *B. subtilis* (wild-type) and strains that have been cultured in laboratory conditions for extended periods of time (domesticated) have also been studied. The domesticated strains usually show defective biofilm formation due to single-point mutations in key genes, including *epsH* , and some of these deficiencies can be complemented using media with specific carbon sources (96, 115).

The proteins encoded by the *tapA-sipW-tasA* operon are the second main component of the biofilm matrix (116). It has been proposed that these genes are responsible for producing amyloid-like proteinaceous fibers among the cells, thus holding them together and providing structural integrity to the biofilm (89, 112). In this regard, TasA is the main component of said amyloid fibers, arranging itself into long polymeric structures; TapA promotes the assembly of TasA fibers and functions as an anchor, attaching the fibers to the cell wall; SipW is a peptidase, responsible for processing and exporting TapA and TasA to the extracellular space (117–120). As is the case for *epsA-O* mutants, strains unable to produce the proteins encoded by the *tapA-sipW-tasA* operon show striking deficiencies in biofilm formation. *tasA* deficient mutants form flat colonies without wrinkles, and thin pellicles that do not develop a rugose surface (Fig. 9) (116). Likewise, mutants in *tapA* and *sipW* show deficient colony biofilm formation (121).

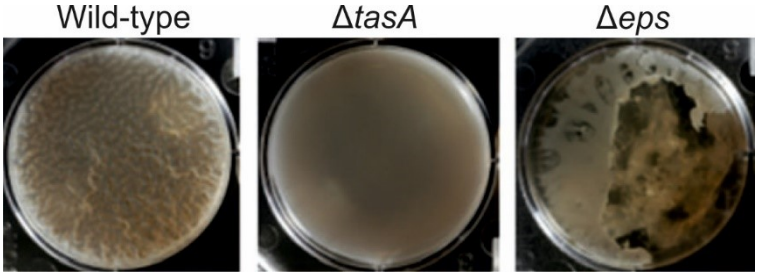

Figure 9: Pellicle biofilms of *B. subtilis* wild-type and mutant strains lacking the main exopolysaccharide (Δ*eps*) or protein (Δ*tasA*) biofilm matrix components (116).

The expression of the *epsA-O* and *tapA-sipW-tasA* operons is controlled by the transcriptional regulator Spo0A, whose activity depends on its phosphorylation state. Phosphorylation of Spo0A

occurs through a phosphorylation cascade that can be initiated by 5 different kinases (KinA, KinB, KinC, KinD, and KinE). Although all the Kin kinases share a high homology, they vary in their ability to activate Spo0A, and in the signals that they can recognize (reviewed in chapter 2 of this dissertation) (89, 122, 123). The Kin kinases, once activated by their cognate signals, phosphorylate the response regulator Spo0F, which in turn transfers the phosphoryl group to Spo0B, which finally phosphorylates Spo0A (124). Phosphorylated Spo0A (Spo0A~P) can then promote the expression of *epsA-O* and *tapA-sipW-tasA*, although this is not done directly. The expression of the two biofilm-matrix component biosynthetic operons is normally repressed by SinR (see previous section) and AbrB (121, 125). AbrB is a repressor of multiple biofilm-related genes, acting directly at the transcriptional level in the case of the *epsA-O* and *tapA-sipW-tasA* operons (110, 125). Spo0A~P relieves AbrB repression by directly blocking *abrB* transcription (126, 127). Spo0A~P can also release the biofilm matrix biosynthetic operons from SinR inhibition by directly promoting the expression of *sinI*. SinI is the cognate repressor of SinR; together they form a SinI-SinR complex that is unable to bind DNA (128). Thus, as the concentration of Spo0A~P increases in the cells, the expression of *epsA-O* and *tapA-sipW-tasA* leads to the formation of the biofilm matrix (Fig. 10).

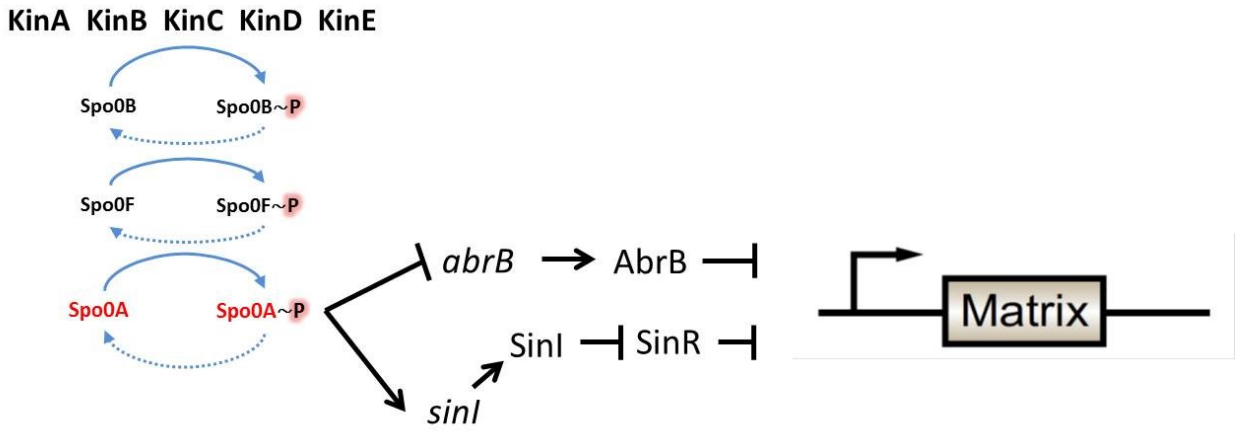

Figure 10: Phosphorelay controlling the activation of the Spo0A transcriptional regulator. The phosphorylation state is represented with (**P**), black arrows indicate activation (→), black T bars indicate inhibition (⊢), solid blue arrows indicate phosphorylation (→), segmented blue arrows indicate dephosphorylation (⇢), the solid box represents diverse genes related to biofilm matrix production.

B. subtilis produces other abundant biofilm-matrix components; although they are not as essential for the development of biofilms as the products of *epsA-O* and *tapA-sipW-tasA* are.

The BslA protein is one such additional matrix component, which provides an hydrophobic characteristic to *B. subtilis* biofilms (129). BslA is a relatively small protein (~19 kDa) that can self-assemble as a stable film in a liquid-oil interface, in the case of biofilms this is reflected as a hydrophobic layer present in the surface of the biofilm (Fig. 11) (130, 131). As can be expected for a protein related to biofilm formation, expression of its biosynthetic gene, *bslA*, is positively controlled both by Spo0A~P and DegU~P (132). *bslA* expression is also controlled by the transcription factor Rok (Regulator of ComK, the competence master regulator), although it is unknown how this regulation integrates into the overall regulatory network of biofilm formation (133). The BslA hydrophobic layer has been proposed to function as a "raincoat", protecting the biofilm from diffusion of unwanted polar compounds (129).

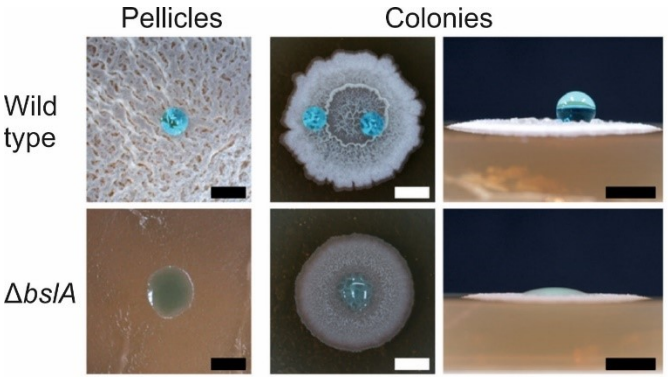

Figure 11: Hydrophobic surface of a *B. subtilis* biofilm (131). Pellicle and colony biofilms of *B. subtilis* wild-type and a mutant strain lacking *bslA*. Blue-colored water drops were placed on top of the biofilms. The scale bars represent 2 mm.

An additional matrix component is γ-PGA. This polymer is produced in large amounts by specific strains of *B. subtilis*; and its production, partially regulated by DegU, has been associated with enhanced cell–surface interactions and improved biofilm formation (42, 134). However, later studies have shown that γ-PGA production is not essential to develop wrinkled colonies or pellicles, and that its production may be medium-dependent or have an altered regulation in certain strains (116).

2.4 Population heterogeneity in *B. subtilis* biofilms.

Regulatory pathways of *B. subtilis* have been reviewed independently in the previous sections, and thus the reader may think that cells in a biofilm transition uniformly in their genetic expression, i.e., that all cells lose their motility behavior and start producing biofilm matrix components at roughly the same time. However, this is not the case; rather, cells monitor the particular environmental conditions that persist in the section of the biofilm where they are located, and express genes accordingly. As the biofilm grows, this results in the division of cells into heterogeneous subpopulations within the biofilm, each with a different phenotype, although genetically similar (54, 102, 135). In this section, cell types beyond motile cells or matrix-producers are described, along with the general mechanisms that mediate the development of population heterogeneity in *B. subtilis* biofilms.

As explained previously, Spo0A~P activates the expression of biofilm-matrix related operons. However, the regulon of Spo0A comprises many more genes (~120) that need to be expressed under different conditions or at different time points. This differential expression of genes that depend on the same transcription factor is regulated by the different affinity of the genes´ promoter regions for Spo0A~P, and by the Spo0A/Spo0A~P ratio in the cells (89, 108, 136). Low and medium levels of Spo0A~P lead to production of biofilm matrix via expression of *sinI*. This gene possesses a promoter with high affinity for Spo0A~P, and its transcription is efficiently induced by it. However, the *sinI* promoter region also possesses low-affinity Spo0A~P operators; as Spo0A~P increases in the cell, these operators are occupied by it and repress *sinI* expression (108).

As Spo0A~P accumulates in the cell, it promotes the expression of two operons: *skf* (sporulation killing factor) and *sdp* (sporulation delaying protein). These operons produce bacteriocins and their corresponding immunity mechanisms. Once produced, these bacteriocins kill neighboring

cells that do not produce the immunity factors (cells with no Spo0A~P), therefore releasing their nutrients to the environment. It has been suggested that this is a strategy used by cells detecting starvation conditions to delay the initiation of the resource-intensive sporulation process. Thus, cells expressing *skf* and *sdp* operons become "cannibals", killing and consuming their siblings for their own benefit (86, 137, 138). The cannibalism and matrix-producing operons are activated by similar levels of Spo0A~P, this overlap eventually results in a population with a majority of matrix-producing cells (139).

One of the most interesting characteristics of *B. subtilis* is its ability to form endospores. These metabolically inactive structures are formed inside *B. subtilis* cells, and show a remarkable resistance to adverse environmental conditions. Spores can survive desiccation conditions for decades, as well as exposure to UV radiation, high temperatures, and toxic chemicals (140, 141); furthermore, spores allow *B. subtilis* to survive predation by ecologically relevant organisms, such as amoeba and nematodes (142, 143). Once the spores find favorable conditions, such as the presence of specific nutrients and sufficient water, they can quickly germinate and produce metabolically active cells (55). The decision to form spores is mainly controlled by Spo0A; once Spo0A~P reaches high levels in the cell, it promotes the expression of several sporulation-related genes which have promoters with low-affinity for Spo0A~P (86, 136). Thus, Spo0A functions as a master regulator of population heterogeneity, where the Spo0A/Spo0A~P ratio determines which particular genes will be expressed in order to fulfill the best possible role for a cell within the biofilm (Fig. 12).

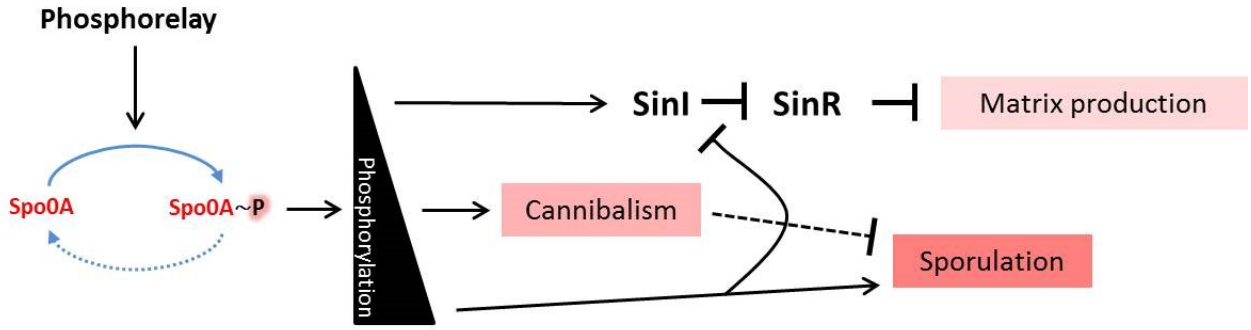

Figure 12: Population heterogeneity regulation by Spo0A. The phosphorylation state is represented with (**P**), black arrows indicate activation (→), black T bars indicate inhibition (⊢), the segmented black T bar indicates indirect inhibition (--⊢), the solid blue arrow indicates phosphorylation (→), the segmented blue arrow indicates dephosphorylation (⇢). The black triangle represents increasing concentrations of Spo0A~P. The solid boxes indicate general cell phenotypes.

DegU is another main regulator of population heterogeneity in *B. subtilis*. As explained in previous sections, DegU plays an important role in the regulation of the planktonic/sessile lifestyle switch; however, this transcriptional factor controls other developmental pathways besides motility. This regulation depends on the phosphorylation level of DegU, and thus can be carefully modulated in response to specific environmental conditions and cell state, much like Spo0A (89, 107, 132).

Besides promoting the expression of motility-related genes, non-phosphorylated DegU positively controls the development of natural competence in *B. subtilis* (144, 145). DegU accomplishes this by directly binding to the promoter region of *comK*, a master regulator of competence development. Once bound, DegU functions as a "primer" for ComK to promote the expression of its own biosynthetic gene at the onset of competence development. Later on, as ComK levels

rise, DegU is no longer necessary to promote competence development (146, 147). This regulation of competence development is highly sensitive to DegU phosphorylation: mutant versions of the DegU protein that cannot be phosphorylated are still able to induce competence, while mutant strains lacking *degU*, or with a modified DegU that remains phosphorylated, show decreased competence development (148).

Once DegU is phosphorylated by DegS, it promotes the expression of genes that favor biofilm formation. Such is the case of *bslA* (previously *yuaB*) and *yvcA*, two genes whose products are associated to increased architectural complexity of colony biofilms and are co-controlled by Spo0A~P and DegU~P (132). In specific *B. subtilis* strains, DegU~P also promotes the production of γ-PGA (42). Additionally, high levels of DegU~P are responsible for producing various exoenzymes such as proteases, α-amylase and levansucrase (149). These enzymes degrade substrate polymers and produce diffusible nutrients, and therefore the cells producing these enzymes are sometimes known as "miners"(150). As is the case for Spo0A, different affinities of the promoter regions of DegU-regulated genes, and the DegU/DegU~P ratio determine which genes are expressed in a given cell (106, 145, 148). Non-phosphorylated DegU induces flagellum synthesis and motility, low and medium levels of DegU~P promote biofilm formation, and high levels promote exoenzyme production (Fig. 13) (106, 107, 145).

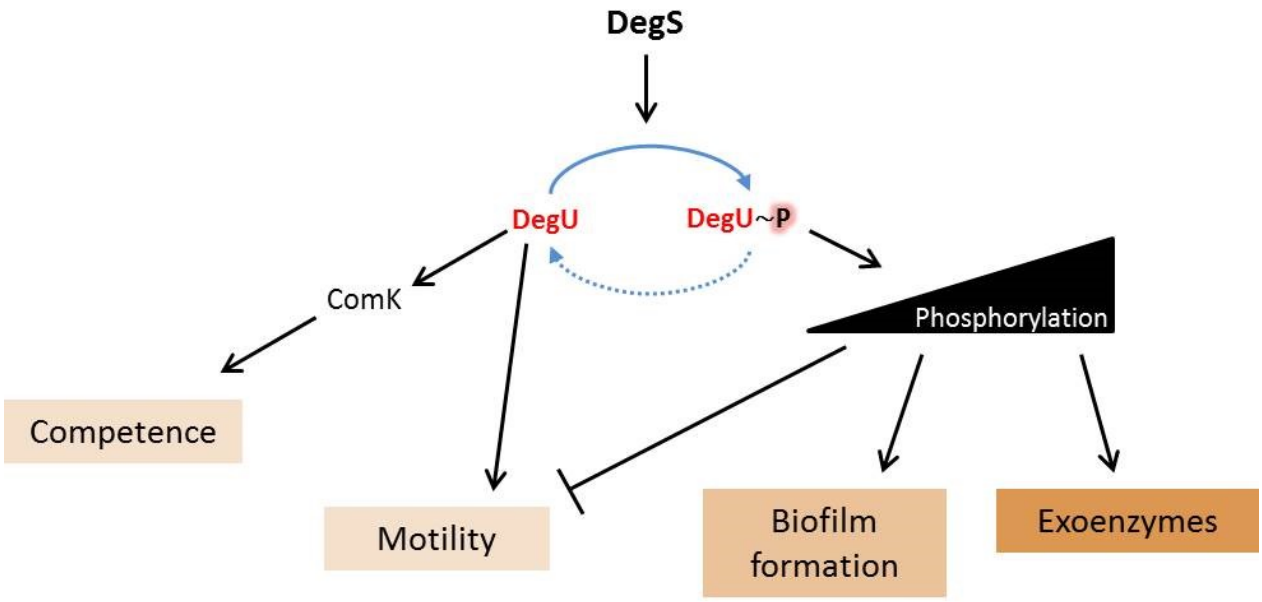

Figure 13: Population heterogeneity regulation by DegU. The phosphorylation state is represented with (**P**), black arrows indicate activation (→), the black T bar indicates inhibition (⊢), the solid blue arrow indicates phosphorylation (→), the segmented blue arrow indicates dephosphorylation (⇢). The black triangle represents increasing concentrations of DegU~P. The solid boxes indicate general cell phenotypes.

The third master regulator of population heterogeneity in *B. subtilis* biofilms is ComA. As Spo0A and DegU, ComA is a transcriptional regulator activated by phosphorylation (150). The activity of ComA is directly regulated by a QS system that uses the 11-amino acid peptide ComX as signal. The signaling pathway is initiated by the expression of *comX*, the translation of this gene produces a 55-amino acid peptide that is then processed and secreted to the extracellular space. The protein ComQ facilitates this process, although the exact mechanism is still unknown.

The processed ComX signal can then be recognized by the ComP sensor kinase, which finally phosphorylates its response regulator, ComA (151–154).

Phosphorylated ComA (ComA~P) directly promotes the expression of the large *srfA* operon (ca. 30 Kbp) (144, 147, 155, 156). The genes of this operon codify an enzymatic complex that is able to produce the non-ribosomal biosurfactant lipopeptide surfactin (157). Surfactin is an important metabolic product of *B. subtilis*, and it serves multiple functions: it is able to disrupt bacterial membranes, and thus possesses antimicrobial activity which allows *B. subtilis* to compete with other organisms (158); due to its surfactant properties, it influences *B. subtilis* motility on diverse conditions (99); and finally, it can cause potassium leakage in *B. subtilis*, triggering KinC to phosphorylate Spo0A and thus influencing matrix production and sporulation (159–161).

The *srfA* operon also contains the gene *comS*, which encodes a small 46-amino acid peptide essential for the development of competence in *B. subtilis* (144, 162, 163). ComS protects the master competence regulator ComK from degradation by the ClpCP protease system; thus allowing ComK to reach levels sufficient to promote the transcription of its own biosynthetic gene (164, 165). By regulating the expression of the srfA operon, the ComA/ComA~P ratio is then able to differentiate cells into a population that produces surfactin and may develop competence (Fig. 14).

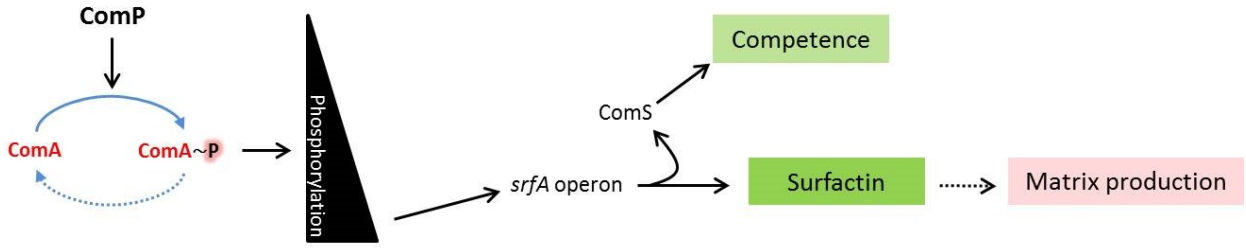

Figure 14: Population heterogeneity regulation by ComA. The phosphorylation state is represented with (**P**), black arrows indicate activation (→), the segmented black arrow indicates indirect activation (⇢), the solid blue arrow indicates phosphorylation (→), the segmented blue arrow indicates dephosphorylation (⇢). The black triangle represents increasing concentrations of ComA~P. The solid boxes indicate general cell phenotypes

To conclude, *B. subtilis* possesses a complex regulatory network that allows its biofilms to generate a heterogeneous population, where each cell can sense particular environmental conditions and adapt to them. This network is mainly controlled by the transcriptional regulators Spo0A, DegU, and ComA. The activity of these regulators depends on their phosphorylation level, and the network includes multiple cross-talk and feedback loops to fine-tune the developmental destiny of each cell (Fig. 15).

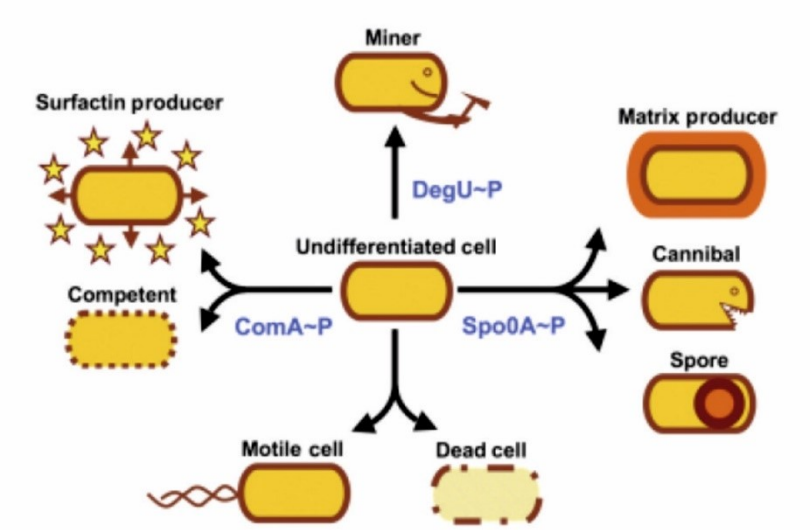

Figure 15: Schematic representation of the distinct cell phenotypes that differentiate in the biofilms of *B. subtilis* (150). Each cell type has been caricaturized, considering its most representative attribute. Arrows indicate the process of differentiation. The master regulator mainly involved is marked in blue.

3. The sociomicrobiology of biofilms.

3.1 The complex social life of bacteria.

Biofilms are complex microbial populations where cells are exposed to a wide variety of signals that inform them about their environmental conditions. Besides signals and chemical cues about the presence of nutrients or accumulation of toxic products; cells can also detect the presence of other organisms in their proximity, and react to them. Although single-species cultures have traditionally been used in microbiological research due to practical reasons, in natural settings microbes are constantly exposed to other microorganisms. Thanks to the development of better microscopy and DNA sequencing techniques, microbiologists are now able to investigate the details of these microbial interactions (11, 12).

Social theory is the collection of frameworks and paradigms used to study how societies change and behave (166). A rapidly growing body of ecological and molecular evidence has shown that microbes engage in complex social interactions, worthy of the same level of interest as the one traditionally given to birds or insects by biologists interested in social theory (13). The best known example may be the case of QS, where cells produce and secrete signaling molecules to the extracellular milieu that can activate sensors in the same secreting cells once the concentration of the signaling molecule reaches certain threshold levels. QS regulates social behavior because the signaling molecules are mainly sensed by cells of the same species, and the more cells that produce the signal, the faster it reaches effective concentrations. More tellingly, QS systems are used to coordinate behavior that is most effective if a large number of cells participate; such as expression of virulence, or production of a biofilm matrix (167, 168).

Microbes not only communicate with members of their own species, they can also sense and engage in cooperative and competitive interactions, such as communal foraging or chemical "warfare", with other species (169, 170). The nature of these behaviors and how they are

established is an increasingly important research area known as sociomicrobiology. It has been shown that microbial communities (i.e. multispecies groups of bacteria) can show an increased virulence in certain infections models (48, 171); at the same time, researchers have studied how the naturally-occurring competitive relationships of certain microbes could be exploited for biocontrol purposes of pathogenic bacteria (172, 173).

A major point of interest for sociomicrobiologists is whether cooperative or competitive interactions are more commonly established and maintained in nature. Many examples in the literature suggest that cooperation easily develops, since it can provide benefits for the whole population; however, social evolutionary theorists maintain that such relationships are fragile or short lived, since they promote the rise of individuals who do not cooperate but receive the benefits of the cooperating partners; if left unchecked, these individuals threaten the whole population. Although sociomicrobiologists intensely discuss these topics, more research needs to be done in order to clarify which kind of relationship is more common, and which environmental conditions will promote the development of competition or cooperation (11, 12, 169, 174, 175).

Biofilms are especially attractive to sociomicrobiologists because they provide a structured environment for the cells living in them. This is to say, cells in a biofilm are relatively static within the biofilm, as opposed to the situation in a liquid agitated culture, where cells mix constantly. This is important because structured environments are known to foster the development of complex social interactions due to the exposure of individual cells to a relatively constant and distinct environment to which they have to adapt. In contrast, a constantly mixed environment means that signals and nascent interactions are constantly dispersed and homogenized throughout the whole population. Furthermore, biofilms possess a high cell density, which also promotes social interactions; and create distinct microenvironments that influence the diffusion of nutrients and other shared goods (Fig. 16) (21, 170, 176–178).

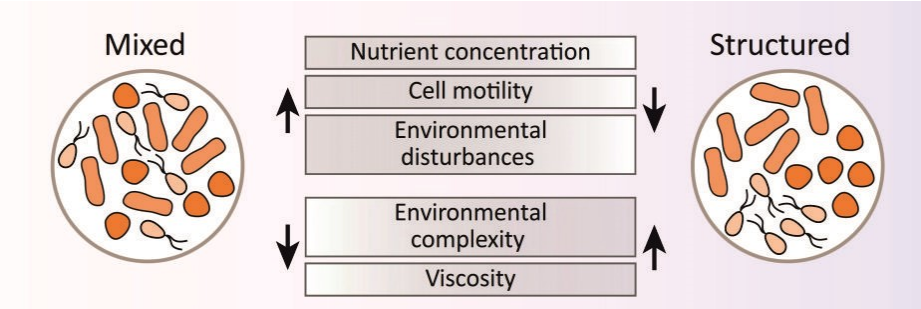

Figure 16: Schematic comparison of structured and mixed environments, and their influence in the ecological conditions where bacterial interactions take place (179).

Interactions in multispecies biofilms are increasingly being directly investigated due to their importance to human health and various ecological settings; and because the findings obtained with single-species studies may not accurately reflect the behavior of microbial communities in nature (180, 181). For example, multispecies oral biofilms have been studied for their relevance to dental health, it has been shown that multispecies biofilms can be more resistant to traditional treatment strategies, and that changes in the ratio of the community members can impact the virulence of the biofilms (78, 182, 183). Multispecies biofilms can also have an indirect impact on human health by functioning as a reservoir of infectious bacteria

(184); or promoting genetic exchange in closely related bacterial species, which may boost virulence by propagating antibiotic-resistance mechanisms (180, 185).

3.2 The role of a family of regulatory phosphatases in *B. subtilis* sociomicrobiology.

Besides the control exerted by the master regulators Spo0A, DegU, and ComA; the population heterogeneity and sociomicrobiology of *B. subtilis* can be fine-tuned by a family of response regulator aspartyl-phosphate (Rap) phosphatases and their associated phosphatase regulator (Phr) peptides (186).

The Rap phosphatases are conserved proteins (>25% of sequence identity) of ca. 380 amino acids that are able to specifically block the phosphorylation of Spo0A, DegU, or ComA; thus preventing the expression of genes that require high levels of the phosphorylated versions of these master regulators (186, 187). Early studies showed that these proteins play their regulatory role by directly dephosphorylating their target regulators. The exception is the regulation of Spo0A, where the cognate Rap phosphatases act on upstream members of the phosphorelay, such as Spo0F (112, 188–190). Later studies have shown that some Rap phosphatases can bind to their target transcriptional regulators, forming a complex that can no longer adhere to DNA. These proteins are still considered part of the Rap family, even when they do not affect the phosphorylation state of their target regulators (191, 192).

The genes that encode for the Rap phosphatases are distributed through the genome of *B. subtilis*, and most of them are followed (and slightly overlapped) by genes that code for small proteins of ca. 40 amino acids that produce the Phr peptides. The expression of the *rap* and *phr* genes is translationally coupled, and thus the *rap-phr* gene pairs are often known as cassettes (193, 194). Once produced, the Rap phosphatases can immediately exert their regulatory function, either by dephosphorylating or preventing the DNA-binding of their target transcriptional regulator. The small proteins encoded by the *phr* genes (known as pre-Phr) follow a more complicated path to become active. Pre-Phr proteins contain export signal sequences in their N-terminal portion, followed by cleavage peptidase signal domains and hydrophilic C-terminal domains. Thus, Pre-Phr proteins seem to be mobilized to the cell membrane, where they are processed by peptidases that produce 5-6 amino acid Phr peptides in the extracellular space. The mature Phr peptides, upon reaching threshold concentrations, can be imported back into the cell by the Opp oligopeptide permease. Once inside the cell, Phr peptides can regulate their cognate Rap phosphatase (produced by the same *rap-phr* cassette) by directly binding to it and inducing conformational changes that block Rap activity (Fig. 17) (186, 187, 193, 195).

In *B. subtilis*, 11 chromosomal and 5 plasmid-encoded *rap-phr* cassettes have been reported and independently investigated (115, 188, 193, 196–198). However, it is important to notice that not all *B. subtilis* strains have the same *rap-phr* cassettes, or even the same amount of them. The *rap-phr* gene pairs are highly prevalent and distributed in the *Bacillus* genus, and it was recently reported that up to 80 different *rap* alleles may be found in the *B. subtilis* group, with an average of 11 ± 2 (mean ± standard deviation) *rap-phr* cassettes per strain among 127 studied strains (199). This variation may be facilitated by the development of natural competence in *B. subtilis*, and social and evolutionary forces shaping the regulatory networks of specific *B. subtilis* strains (199, 200). For example, *B. subtilis* strains isolated from the digestive tract of diverse animals show differences in

the time at which they initiate sporulation, and in the number of *rap-phr* cassettes that they possess. It has been suggested that these differences are adaptations that allow bacteria to sporulate at optimum times according to the ecological niche in which they live (201).

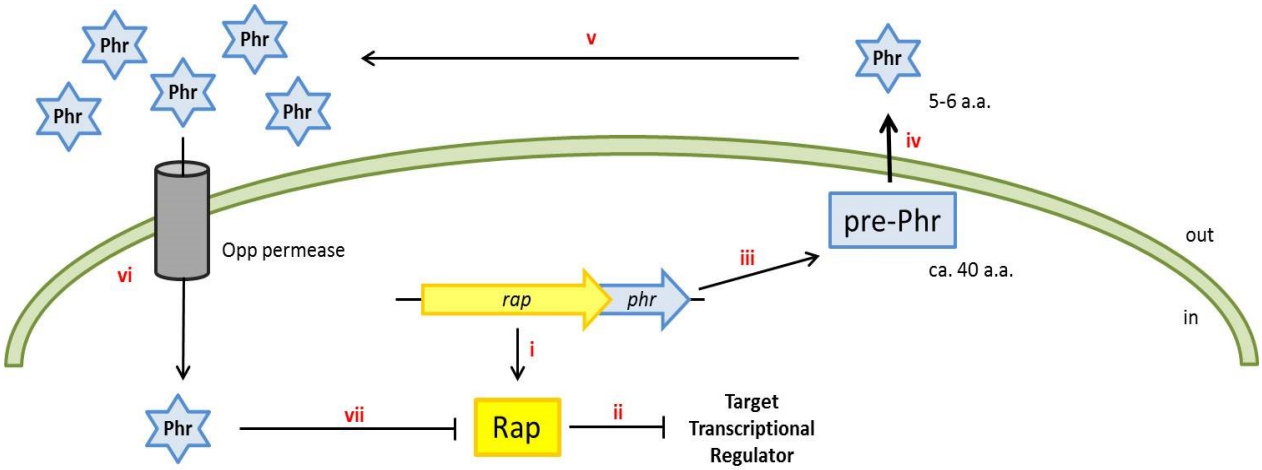

Figure 17: General regulatory mechanism of Rap-Phr pairs. **i)** the Rap protein is produced and, **ii)** carries its regulatory role intracellularly; meanwhile, **iii)** pre-Phr proteins are produced and, **iv)** processed and exported out of the cell as Phr peptides; **v)** upon reaching threshold concentrations, **vi)** mature Phr peptides can be imported into the cell via the Opp permease and, **vii)** inhibit its cognate Rap protein.

The regulatory function and mechanisms of the Rap phosphatases and Phr peptides have been intensively studied during the last decades. Thanks to these efforts, we now know the regulatory targets of many Rap phosphatases, as well as their individual action mechanism. It has also been found that a few *rap* genes do not have a cognate *phr* gene, and that some Rap phosphatases act on multiple targets. Table 1 presents a summary of the latest investigations done with Rap-Phr pairs.

Table 1: Regulatory role of Rap-Phr pairs.

Rap protein	**Phr peptide**	**Target regulator and mechanism of action**	**References**
RapA (chromosomal)	PhrA	Dephosphorylation of Spo0F~P	(202, 203)
RapB (chromosomal)	PhrC	Dephosphorylation of Spo0F~P	(186, 204, 205)
RapC (chromosomal)	PhrC	Blocks binding of ComA to DNA	(191, 206)
RapD (chromosomal)	Unknown	Inhibition of ComA activity	(207)
RapE (chromosomal)	PhrE	Dephosphorylation of Spo0F~P	(208, 209)
RapF (chromosomal)	PhrF	Blocks binding of ComA to DNA	(192, 210)
RapG (chromosomal)	PhrG	Blocks binding of DegU to DNA. Possible inhibition of ComA activity	(211, 212)
RapH (chromosomal)	PhrH	Dephosphorylation of Spo0F~P. Blocks binding of ComA to DNA.	(190, 195, 196, 213)
RapI (chromosomal)	PhrI	Dephosphorylation of Spo0F~P. Involved in the regulation of mobile genetic elements.	(195, 214, 215)

RapJ (chromosomal)	PhrC	Possible dephosphorylation of Spo0F~P	(190, 215)
RapK (chromosomal)	PhrK	Inhibition of ComA activity	(195, 210)
RapP (plasmidic)	PhrP (RapP is resistant to PhrP)	Dephosphorylation of Spo0F~P. Inhibition of ComA activity	(198, 216, 217)
RapQ (plasmidic)	PhrQ	Blocks binding of ComA to DNA. Possible inhibition of Spo0A activity	(218)
Rap40 (plasmidic)	Phr40	Unknown	(197)
Rap50 (plasmidic)	Phr50	Unknown	(197)
Rap60 (plasmidic)	Phr60	Dephosphorylation of Spo0F~P. Inhibition of ComA activity	(197, 219, 220)

The Rap phosphatases fine-tune the sociomicrobiology of *B. subtilis* by modulating the activity of the master regulators of biofilm development and population heterogeneity Spo0A, ComA and DegU. This fine-tuning happens at two levels; first, the Rap phosphatases themselves appear at first sight to have redundant roles (see Table 1), however, this is not entirely the case because the *rap-phr* cassettes are expressed under different conditions. As an example, both RapA and RapB dephosphorylate Spo0F~P; however, *rapA* is promoted by QS-dependent ComA, while *rapB* seems to be promoted only by the "house-keeping" sigma factor σ^A (221–223). This difference means that RapB will be produced earlier and more consistently that RapA, leading to differences in the Spo0A/Spo0A~P ratio in the cell population. The second level of fine-tuning is given by the Phr peptides; their regulatory action is normally delayed by their process-export-import pathway, this gives *B. subtilis* cells the opportunity to detect and integrate further environmental signals into their complex gene regulatory network. Furthermore, the mature Phr peptides function as a QS signal while they remain in the extracellular milieu, the more cells that produce the signals the faster it will reach active concentrations. Even more, their capacity to serve as cell-cell communication signals has been shown by the ability of non-producing cells to detect the Phr signals produced by other cells (112, 186, 188, 189, 205, 224).

Despite recent gains in understanding the social lives of bacteria, much more research is still needed in the novel field of sociomicrobiology. The importance of the relations among microbes, and between microbiomes and their host organism (e.g. humans and their gut microbiota) is increasingly recognized, and promises to revolutionize our understating of microbiology.

CHAPTER 1

From environmental signals to regulators: modulation of biofilm development in Gram-positive bacteria.

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* Contributed equally to this work.

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Bacterial lifestyle is influenced by environmental signals, and many differentiation processes in bacteria are governed by the threshold concentrations of molecules present in their niche. Biofilm is one such example where bacteria in their sessile state adapt to a lifestyle that causes several adaptive alterations in the population. Here, a brief overview is given on a variety of environmental signals that bias biofilm development in Gram-positive bacteria, including nutrient conditions, self- and heterologously produced substances, like quorum sensing and host produced molecules. The Gram-positive model organism, *Bacillus subtilis* is a superb example to illustrate how distinct signals activate sensor proteins that integrate the environmental signals towards global regulators related to biofilm formation. The role of reduced oxygen level, polyketides, antimicrobials, plant secreted carbohydrates, plant cell derived polymers, glycerol, and osmotic conditions are discussed during the transcriptional activation of biofilm related genes in *B. subtilis*.

Introduction.

Bacterial associations and multicellular behaviors in the environment have been a constant debatable issue leading to the realization that microbes in nature mostly exist in biofilm. We now understand that multicellularity in bacteria is very common and includes chains of photosynthetic cyanobacteria, aggregation and formation of *Myxococcus* fruiting bodies, clustering and cyst dormancy, filamentation, and most importantly, biofilm formation (56, 225–227).

Biofilm formation, which was first thought to be the mere orchestration of virulence behavior, has many more aspects like division of labor leading to an improved fitness of the population, co-operating traits, survival to the attack of antimicrobials, and undeniably, evolution of bacteria in their niche (11, 169, 228). What drives bacterial cells to associate in a biofilm? To answer this fundamental question we must understand not only what constitutes the biofilms, but also how do they develop. Biofilms are multicellular associations where the cells encompass themselves in

self-secreted exopolymers, which allow surface attachment and overall protection from severe environmental stress conditions, including antimicrobials. A bacterium often senses and assesses the environment it thrives in and most molecular, as well as metabolic processes are involved in the course of making a settlement in the respective environment. Surface components could also play an important role in coordinating these processes by integrating environmental and regulatory signals (91, 229). Under favorable conditions planktonic cells settle in the form of micro colonies, which then mature to fully developed biofilms. During these conditions, bacterial cells are known to undergo many developmental processes that are not only restricted to differentiation into matrix-producing cells, but also include sporulation, persister phenotype, cannibalism, competence, secretion of various virulence factors, production of antimicrobials, and different metabolic strategies (39). Dispersion is also part of the biofilm life cycle, where some cells in mature biofilm regain motility and disassemble to disperse to other environments.

In recent years several studies have been published that aim to understand and describe the various signals and developmental pathways that drive the formation of bacterial biofilms. Mostly, different environmental signals and their threshold concentrations regulate and trigger biofilm formation leading to the activation of specific signaling cascades (230). This review presents an overview of the various signals that initiate or alter biofilm formation of Gram-positive bacteria. Finally, focus is given on the signal transduction pathways that trigger biofilm development in the Gram-positive model organism, *Bacillus subtilis*.

Carbon and nitrogen source.

Bacteria in nature are exposed to a constantly changing environment, thus, their ability to sense and react to these changes is paramount for their survival. In the following chapters, we review what is known about the signals that are commonly found in the natural habitats of Gram-positive bacteria and their influence on biofilm formation (Table 1).

Table 1: Signal molecules that influence biofilm development in Gram-positive bacteria.

Signal	Affected process	Reference
Glucan and Fructan	Increased bacterial adhesion of *S. mutans*	(78)
Glucose	Biofilm formation and multiple protein expression patterns of *L. monocytogenes*	(231)
	Higher production of PIA in *S. epidermidis* Increased	(232)
	biofilm formation of *S. aureus*	(233)
Carbon source	Biofilm formation through carbon catabolite repression in *L. plantarum*	(234)
	Extracellular polysaccharide production of *S. gordonii*	(235)
Nitrogen availability	Increased biofilm production of *B. cereus* codY mutant	(236)
Starvation of leucine and valine	CodY activation and lower biofilm formation of *S. mutans*	(237)
NaCl	Increased biofilm formation of *L. monocytogenes*	(238)
	Increased production of the extracellular matrix binding protein in *S. epidermidis*	(239)
Temperature	Bacterial adhesion and biofilm formation of *L. monocytogenes*	(240)
Freezing temperatures	Long-term adaptation with increased bacterial adhesion and biofilm formation of *L. monocytogenes*	(241)

Anaerobiosis	Inhibited biofilm formation of *S. aureus*	(242)
Aeration	Reduced biofilm formation of *S. mutans*	(243)
Autoinducer-2	Inhibited biofilm formation of *B. cereus* and dispersion of preformed biofilms	(244)
	Different effects over biofilm formation of *S. suis* depending on its concentration	(245)
S-ribosylhomocysteine (Precursor of AI-2)	Increased biofilm development of *L. monocytogenes*	(246)
4, 5-dihydroxy-2, 3-pentanedione (Precursor of AI-2)	Repression of the ica operon of *S. aureus*	(247)
Cholate	Increased biofilm formation of *L. lactis*	(248)
eATP	Increased bacterial adhesion and biofilm formation of *S. aureus*	(249)
Sialic acid	Increased biofilm formation of *S. pneumonia*	(250)
Spermidine and Norspermidine	Increased biofilm formation of *B. subtilis*	(251, 252)
Indole-triazole and benzothiophene–triazole conjugates	Increased biofilm formation of *S. aureus*	(253)
Volatiles: indole, ammonia, 1-butanol, trimethylamine	Increased biofilm formation of *S. aureus*	(254)
Furanone	Increased PIA production in *S. epidermidis* and *S. aureus*	(255)

Carbon metabolism plays a central role in the metabolic pathways and regulation of gene expression in bacteria, consequently, the influence of carbon sources over biofilm formation has been investigated in several Gram-positive bacteria.

For instance, *Streptococcus mutans*, an important etiological agent of common dental diseases, when grown in the presence of sucrose, produces glucans and fructans that contribute to the formation of a dense and adherent biofilm (78). This mechanism depends on various glucosyltransferases (GtfB, -C, and -D) and glucan-binding proteins, where the synthesized glucans provide a chance for bacterial adhesion to the surfaces and to each other (256). These glucosyltransferases allow *S. mutans* to bind to other bacterial cells, even if these cells lack glucosyltransferases (48). The genes encoding the glucosyltransferases are expressed in response to acidification or to the excess of glucose or sucrose in the environment (78), which suggests that the carbon source has a pivotal role in modifying the biofilm development of *S. mutans*.

Similarly, due to the relevance of *Listeria monocytogenes* as a food borne pathogen, the influence of different nutrients over biofilm formation has also been investigated. Interestingly, various genes related to carbon metabolism show different expression patterns between sessile and planktonic cells in diverse media, implicating the importance of carbon catabolism control for *L. monocytogenes* biofilm formation in response to nutrient availability (231).

Biofilm formation of the opportunistic pathogen *Staphylococcus epidermidis* is promoted by glucose through activation of the ica operon (232), which encodes three membrane proteins with enzymatic activity (IcaA, IcaD, and IcaC) and an additional extracellular protein (IcaB) necessary for synthesis of the intercellular polysaccharide adhesin (PIA) (257), the major protein

component of *S. epidermidis* biofilms (258). The catabolite control protein A (CcpA) is involved in modulating biofilm formation depending on the nutrient sources. CcpA is a member of the GalR–LacI repressor family that mediates carbon catabolite repression and was suggested to promote biofilm formation through suppression of the tricarboxylic-acid cycle (259).

A similar signaling mechanism has been reported for *Staphylococcus aureus*. Addition of glucose to complex growth medium triggers biofilm formation in this bacterium, however, a *ccpA* mutant of *S. aureus* shows an abolished biofilm formation capacity under similar conditions (233). The regulatory role of CcpA in biofilm formation has also been recently reported for *Lactobacillus plantarum* and *Streptococcus gordonii*. In *L. plantarum*, CcpA transcriptionally regulates the expression of *flmA*, *flmB*, and *flmC* genes, which code for homologues of the *S. mutans* BrpA (biofilm regulatory protein A)(234).The *ccpA* mutant strain of *S. gordonii* is severely impaired in biofilm forming ability with a defect in extracellular polysaccharide production (235).

These reports have shown that there is an important association between the particular carbon source present in the environment, the carbon catabolite repression system and the general metabolic state of the cells, to the ability of bacteria to form biofilms.

Nitrogen is another essential factor in bacterial metabolism and therefore several metabolic pathways and gene regulatory networks exist to ensure a proper access to nitrogen sources and to adapt to nitrogen deficiency. As is the case with carbon, the role of nitrogen over biofilm formation has been studied in various Gram-positive organisms.

CodY is a global transcriptional regulator of genes involved in nitrogen metabolism and identified so far in low G+C Gram-positive bacteria including the food poisoning agent, *Bacillus cereus* (236, 260). Recent transcriptome analysis of the *B. cereus codY* mutant confirms the role of CodY in motility and community behavior. A strain with deleted *codY* gene of *B. cereus* ATCC 14579 shows diminished growth during nitrogen starvation and shows increased biofilm formation (236). In contrast, a transposon inactivated *codY* mutant strain of *B. cereus* UW101C develops reduced biofilm (261). It is uncertain whether CodY plays distinct roles in the given strains. However, both studies highlight the connection of nitrogen metabolism and biofilm formation.

Similarly, opposite regulatory roles have been reported for CodY in *S. aureus* in relation to biofilm formation. A *codY* mutant obtained through bacteriophage Mu transposition in *S. aureus* shows reduced biofilm formation (262), while introduction of a *codY*-null mutation into two *S. aureus* clinical isolates results in an overexpression of several virulence genes and more robust biofilm formation associated with an enhanced PIA production (263).

The effect of nitrogen availability on biofilm formation of *S. mutans* has been reported through CodY and the alarmone, guanosine 5'-diphosphate (or 5'-triphosphate) 3´-diphosphate, generally referred to as (p)ppGpp (237). (p)ppGpp is the signal mediator of the "stringent response", a widely distributed bacterial adaptation system that allows global adjustments in gene expression in response to nutrient limitation and certain environmental stresses (264, 265). Correlation between growth in the absence of leucine and valine with (p)ppGpp pools and the activation of CodY was investigated in *S. mutans*, finding a link between (p)ppGpp and CodY. Further, the *codY* mutant strain has a reduced capacity to form biofilms (237).

Role of temperature, oxygen, and osmotic conditions in biofilm formation.

Naturally, carbon and nitrogen availabilities are not the only fluctuating conditions that bacteria encounter in their ecological niches and the importance of other environmental factors over biofilm formation has also been studied.

Sodium chloride (NaCl) has been investigated for its role as biofilm formation promoting signal, especially in pathogens. Both *L. monocytogenes* and *S. aureus* were shown to have lower growth rates, but increased biofilm formation as the NaCl concentration in the media increases (238). The synergistic effect of temperature and NaCl concentration over *L. monocytogenes* biofilm formation has been studied, corroborating that high NaCl concentrations promote biofilm formation at various temperatures (266). Employing osmotic stress via NaCl in *S. epidermidis* causes a slight increase in the production of extracellular matrix binding protein (EmbP) in planktonic cultures, however, a 100-fold increase in *embP* expression can be observed in adherent cultures challenged with NaCl coinciding with altered biofilm morphology (239). EmbP protein is required for host colonization and subsequent biofilm formation of *S. epidermidis* (267). Although these studies suggest that osmotic stress is an important signal for biofilm development, its signaling mechanism remains to be elucidated.

Next to NaCl stress, the effect of temperature on *L. monocytogenes* biofilm formation has been thoroughly investigated. Attachment and biofilm formation on different surfaces highly correlate with increased temperature from 4 to 37 °C (240). Further, the influence of freezing temperatures (-20 °C) over biofilm formation of *L. monocytogenes* was examined and it was found that after 10 months, stressed strains became more adherent and better producers of extracellular slime than the control groups (241). The individual and synergistic effects of glucose, ethanol, NaCl, and temperature over *L. monocytogenes* biofilm formation have also been investigated. Under different temperature conditions, varying concentrations of these substances seem to differentially affect biofilm formation via promotion of the extracellular polymeric substance production (268).

Oxygen is another important environmental factor that affects bacterial metabolism. Anaerobiosis inhibits biofilm development of *S. aureus* quantified by viable bacterial numbers and biomass. In addition, notable differences can be observed in the metabolic profiles of biofilms that are cultivated under normoxia versus anoxia (242). In *S. epidermidis*, the oxidation-sensing regulator, AbfR (aggregation and biofilm formation regulator) is responsible for the oxidative stress response and its mutant showed enhanced bacterial aggregation, but reduced biofilm formation (269). Culturing *S. mutans* under aerated conditions reduces biofilm formation (243) and oxygen availability results in variations in bacterial cell surface composition and production of autolysins (270).

Self-acting molecules that regulate biofilm formation.

Bacteria possess the ability to produce and sense specific signaling molecules that allow them to regulate population behavior, this phenomenon is known as quorum sensing (QS) and it has been the subject of intense study due to its relevance to bacterial communities (271, 272). The QS systems in Gram-positive bacteria typically use secreted peptides as signal molecules and two-component regulatory systems to detect the peptide and trigger changes in gene

expression (273). Several papers have reviewed QS systems in bacteria and their role in biofilm development (167, 230, 272, 274–278). Here, we briefly focus on selected examples and the relevance in coordinating biofilm development in Gram-positive bacteria.

Autoinducer 2 (AI-2) is a QS signaling molecule that is found in both Gram-positive and Gram-negative bacteria, and has been suggested as a universal signal involved in interspecies communications (279). The *luxS* gene encodes S-ribosylhomocysteinase, an enzyme that catalyzes the hydrolysis of S-ribosylhomocysteine to homo-cysteine and 4,5-dihydroxy-2,3-pentadione (DPD), which serves as a precursor of AI-2. The exact role of AI-2 and its synthetic pathway in biofilm formation is still not clear in distinct bacteria, having both positive and negative influence in case of specific biofilm models. In *Streptococcus pneumonia*, the expression of *luxS* peaks at early mid-log phase of growth and *luxS* mutants produce up to 80% less biofilm biomass than the wild-type strain (280). Also, the *luxS* gene in *B. subtilis* is important for robust and morphologically differentiated biofilm formation, as well as for the appearance of surface spreading phenotype (281). In contrast, exogenously added AI-2 inhibits biofilm formation of *B. cereus* and disperses preformed biofilms (244). Similarly, in other Gram-positive bacteria, AI-2 production seems to have a negative effect on biofilm formation.

A *luxS* mutation in *S. mutans* results in biofilms with altered structure and increased resistance against detergents (282). *L. monocytogenes* is able to produce AI-2-like molecules, however, the *luxS* mutant cells attach better to surfaces and the addition of AI-2 has no effect on biofilm formation, but rather, the AI-2 precursor S-ribosylhomocysteine stimulates the increase in biofilm development, explaining the dense biofilm formation when the *luxS* gene is mutated (246). Similarly, *luxS* mutation in *S. epidermidis* enhances biofilm formation via augmented production of PIA (283) and similar mutation in *S. aureus* also positively affects biofilm production under several growth conditions, while exogenous addition of a chemically synthesized precursor of AI-2 activates the transcription of *icaR*, a repressor of the *ica* operon (247). A more complex scenario is described for *Streptococcus suis*, where low concentrations of AI-2 promote biofilm formation and host–cell adherence, but high concentrations of this compound, as well as a mutation in the *luxS* gene, inhibit both phenotypes (245).

It is important to note that the multiple effects of a *luxS* mutation in these above mentioned organisms may not be related to changes in QS signaling but rather to metabolic effects as the products of LuxS (homo-cysteine and DPD) are part of the central activated methyl cycle in the cells, providing methyl groups for RNA, DNA, certain metabolites, and proteins (284). Transcriptomic and metabolomic analyses of *S. mutans* and *Lactobacillus reuteri* have shown that *luxS* mutation influences the expression of more genes than those exclusively affected by changes in AI-2 availability (285, 286).

Thus, LuxS and AI-2 QS system influence biofilm formation in several organisms and affect the development, dispersal, or formation of structurally complex colonies. However, the various *luxS* phenotypes are explained not only by altered QS system but also through modified metabolic functions of the Gram-positive bacterial cells.

Another common and important biofilm related QS system in Gram-positive bacteria is encoded by the *agr* (accessory gene regulator) locus that was first described in *Staphylococcus* species (287). The Agr system contains four genes (*agrBDCA*) which code for the sensing and signal

transducing proteins of a signaling molecule, the autoinducing peptide (AIP) (288). When secreted, AIP is detected by the Agr system, which in turn regulates the transcription of genes involved in different phenotypes, including biofilm formation (289).

Vuong *et al.* (290) reported for the first time that in *S. aureus*, a transposon-mediated mutagenesis of *agrC* leads to a strong enhancement of biofilm formation. A subsequent screen of more than 100 isolates showed an inverse correlation between an active AIP QS system and biofilm formation, suggesting that AIP plays a major role in the dispersal of biofilms and not during the initial maturation stage. While the repression of the AIP QS system is necessary for proper biofilm development of *S. aureus*, activation of this system is necessary for biofilm dispersal (51). Similarly, the isogenic *agr* mutant strain of *S. epidermidis* shows increased biofilm development and colonization in a rabbit model (227). Interestingly, the AIP QS system is required for the full expression of the *codY* transcriptional regulator in *S. epidermidis*. The *agr* mutant strain shows decreased level of CodY protein and increased expression of CodY regulated genes (291). Thus, in the *Staphylococcus* genus, the AIP QS system not only regulates biofilm dispersal but also influences the nitrogen-metabolism related regulator CodY, linking the availability of nutrients in the environment with commitment towards biofilm dispersal.

The Agr QS system, as described for *S. aureus*, also appears to be involved in the modulation of adhesion and early stages of biofilm formation in *L. monocytogenes*. However, the *Listeria* Agr system has an important role in the initial biofilm development, since deletion of *agrA* or *agrD* diminishes biofilm formation during the first 24h (292). The role of the Agr system in *L. monocytogenes* biofilm development has been investigated in more detail using different flow-culture conditions and media, and the QS system was shown to regulate heterogeneity and biofilm development (293). Transcriptome studies on the Agr system of *L. monocytogenes* at saprophytic (25 °C) and in vivo (37 °C) temperatures showed that the AIP QS system regulated genes overlap with various other regulons, including the CodY regulated genes, and therefore it may integrate information on its biotic environment (293).

In Streptococci, the competence signaling peptide (CSP) mediated QS system has an important role in biofilm development. CSP activates the *com* regulon related to natural competence development (294). The early-competence genes encode proteins that include the cell–cell signaling apparatus: the CSP precursor (encoded by *comC*), the histidine kinase receptor (*comD*), the cognate response regulator (*comE*), and the alternative sigma factor (*comX*) (295). Among the genes influenced by the CSP QS system, particular ones are also involved in biofilm formation, dispersal, and architecture. The CSP QS system was first suggested by Loo *et al.* to be involved in biofilm formation. The transposon insertion in the *comD* gene of *S. gordonii* shows an impaired biofilm formation (296). Similarly, *S. mutans* with single or multiple mutations in the *comCDE* or *comX* genes shows abnormal biofilm development (297). Moreover, in *S. mutans* and *Streptococcus intermedius*, CSP appears to specifically regulate the initial stages of biofilm formation. *S. mutans comD* or *comE* mutants show impaired adhesion to surfaces and CSP promotes the early accumulation of *S. intermedius* biofilm cells (297, 298). Interestingly, among the genes that are regulated by the CSP QS system are those that code for autolysins involved in cell lysis and DNA release (298), which is an important biofilm component for several bacteria.

Extracellular DNA (eDNA), identified as part of the biofilm matrix, facilitates the initial stage of adhesion to biomaterials, forms a structural backbone, and acts as a glue that promotes biofilm

aggregation (45). eDNA is formed by the release of bacterial genomic DNA mostly by cell lysis or, less commonly, by active excretion (299). The importance of eDNA in bacterial biofilm formation has been demonstrated in several studies that present evidence that addition of DNase I to the culture medium strongly inhibits biofilm formation and that DNase I can also dissolve established biofilms of different bacterial species (300–302).

In *S. aureus*, eDNA concentration is increased due to the major autolysin Atl and the bacteriophage-like holin–antiholin system, Cid-Lrg (303, 304). The GdpS protein in *S. aureus* acts as a negative regulator of eDNA release by influencing expression of *lrgAB* and *cidABC* genes, thus contributing to cell-to-cell interactions during early biofilm formation (305).

Various reports have established the importance of eDNA for biofilm formation, however, it is still unclear if eDNA functions as a signal that promotes biofilm formation, or if it acts solely as an early component of biofilms mediating cell-adhesion to surfaces and other cells.

Heterologous (signaling) molecules that alter biofilm formation of Gram-positive bacteria.

Several signaling molecules or chemical substances have been identified that can promote biofilm formation of diverse Gram-positive bacteria. In particular cases, molecules that promote biofilm formation in a given species, actually attenuate biofilm development in others, suggesting that signal molecules can have a specialized role in the diverse biofilm systems.

Biofilm formation by pathogenic bacteria receives a unique attention due to chronic infections that are highly resistant to normal antibiotic therapeutics (78, 306, 307). As biofilm formation depends on successful attachment of bacteria to a surface, several studies have investigated the role of various molecules that are normally found in the host and how they are involved in initial bacterial attachment.

EmbP is a giant fibronectin-binding protein that mediates binding of *S. epidermidis* to solid- phase attached fibronectin, constituting the first step of biofilm formation. Interestingly, it has been reported that in *S. epidermidis*, EmbP is expressed only in the presence of serum (267). Bacteria possess different mechanisms to recognize fibronectin and other proteins that are present in host-cell membranes (308–310). However, it is still unclear if these molecules are signals that trigger biofilm formation or if they only play a physical role in cell-surface interactions.

Nevertheless, there are other host-generated molecules that seem to have a clear role as signaling molecules for biofilm formation. Cholate, a general component of human bile salts, was recently reported to stimulate biofilm formation of *Lactococcus lactis* when used at sub-inhibitory concentrations (248). Extracellular adenosine 5´-triphosphate (eATP) plays an important role in diverse patho-physiological processes and is used ubiquitously amongst eukaryotes as an intercellular "danger" signal (311). eATP was reported to stimulate bacterial adhesion and biofilm formation of *S. aureus* and other Gram-negative pathogens (249). Another example of biofilm promoting signals produced by the host is the monosaccharide sialic acid (N-acetylneuraminic acid), which can be found in normal human saliva as a terminal carbohydrate of the O-glycan chains of mucins (312). The effect of various sugars was tested over biofilm formation of *S. pneumonia* using a murine model and it was found that among the tested carbohydrates, only sialic acid significantly increases the pneumococcal invasion and biofilm formation (250)(Fig. 1).

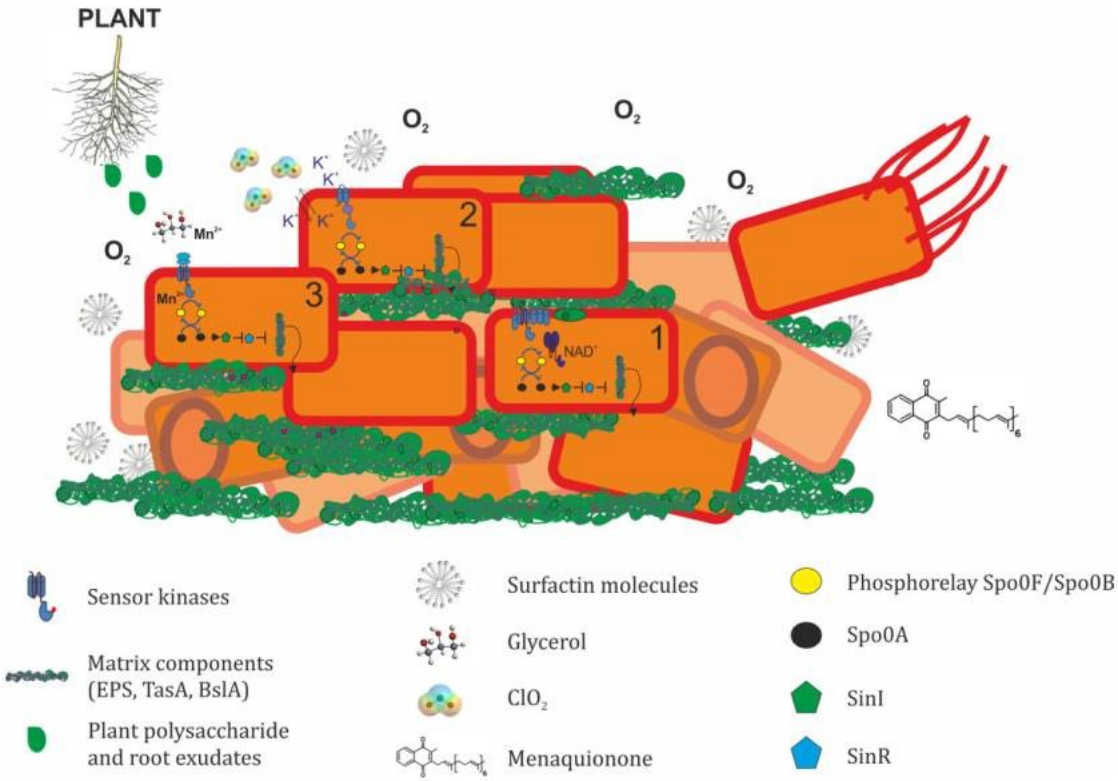

Figure 1. Signaling molecules are highlighted that trigger biofilm formation of *B. subtilis*. Bacterial cells are depicted at the onset of biofilm formation. Molecules and signals are indicated below the figure. Arrows represent activation, while T shapes indicate repression. Activation of KinA/KinB (1), KinC (2), or KinD (3) kinases by their respective signaling molecules at the cellular level is indicated by numbers.

There is a myriad of molecules that are produced by diverse organisms and that can promote biofilm formation of diverse bacteria. As is the case with host-provided molecules, it is still not clear if many of these molecules are real specific signals for biofilm formation or if they are generally sensed by bacteria as environmental stresses or threats and thus the bacterial population forms biofilms as a defensive response. The fact that some of these molecules promote biofilm formation only when present in small sub-inhibitory concentrations, at which point the biofilm-forming population becomes highly resistant to the molecule's antimicrobial activity, seems to favor the later hypothesis.

Polyamines are linear, organic polycations found as secondary metabolites in most bacteria (313). These molecules play an important role in biofilm formation of diverse Gram-negative and Gram-positive bacteria. Biosynthetic pathways and transport systems for diverse polyamines have been identified as key components of bacterial biofilm formation (313). In various Gram-negative bacteria, the biosynthesis of norspermidine has been reported to promote biofilm development. In *Vibrio cholerae*, extracellular norspermidine enhances biofilm formation, and disruption of norspermidine biosynthetic enzymes leads to a reduced biofilm formation. Similarly, *Yersinia pestis*, the causative agent of plague, requires polyamine biosynthesis and the *speA* and *speC* genes for biofilm formation (314). In *B. subtilis*, both the endogenously produced spermidine and the exogenously added norspermidine enhance biofilm formation (251, 252). At high concentration, norspermidine inhibits growth and biofilm development of *B. subtilis* (252) suggesting a particular role on biofilm promotion only at lower concentrations.

Indole is a ubiquitous small molecule signal that controls a variety of phenotypes in both Gram-positive and Gram-negative bacteria. Indole has been shown to play a role in acid tolerance, antibiotic resistance, and biofilm formation (315). In a recent study, indole–triazole and benzothiophene–triazole conjugates have been reported to promote biofilm formation of *S. aureus*, however, the signaling pathway for this mechanism remains to be elucidated (253). In addition to indole, ammonia, 1-butanol, and trimethylamine promote biofilm formation of *S. aureus* through aerial signaling pathway. These volatile compounds are produced by *Escherichia coli* as secondary metabolites (254).

Interestingly, some antimicrobial molecules are able to trigger biofilm formation via inhibition of the AI-2 QS system. Sub-inhibitory concentrations of furanone, which is a bactericidal compound produced by marine algae, can promote PIA production in *S. epidermidis* and *S. aureus* via interference with *luxS* expression (255).

***B. subtilis*, the Gram-positive model bacterium for environmental biofilm formation.**

Among the Gram-positive bacteria, *B. subtilis* is one of the most studied bacteria for its phenotypic heterogeneity leading to the various differentiated cell types and sub-population level of gene expressions (316). In addition to this, biofilm formation of Bacilli is well studied due to its connection to the onset of sporulation.

B. subtilis, in the stationary phase, changes from peripatetic lifestyle to a sessile mode of growth. Like in other biotic systems, biofilm forming bacteria sense the environmental stimuli and modify its lifestyle to survive in a dynamic niche (317). During development of B. *subtilis* biofilms, a subpopulation of isogenic community expresses certain genes related to biofilm formation. The *epsA–O*, 15 gene-containing operon is important for the production of exopolysaccharide, while the *tasA* and *bslA* genes code for the protein components of biofilm: the amyloid fibers and the surface hydrophobic protein component, respectively, that all together protect the population from environmental stresses (113, 116, 117, 130, 131, 318). These matrix components help cells to adhere to numerous surfaces and the neighboring cells, thus forming a microcommunity, where numerous cell-to-cell interactions exist in order to cooperate as well as to differentiate into various cell types.

Under laboratory conditions, the biofilm models in *B. subtilis* include pellicle formation on the air–liquid surface, architecturally complex colonies on the agar surface, as well as submerged surface attached biofilms. Robust biofilm formation of *B. subtilis* is promoted on various defined minimum or broth containing complex media that contain components involved in triggering the transcription of genes involved in matrix production and development. Common constituents include K^+, Mg^{2+}, Fe^{2+}, Mn^{2+} ions, carbon source in form of glucose and/or glycerol (91, 106). Interestingly, various components of MSgg medium also induce sliding motility of the flagellum-less *B. subtilis* strains resulting in tendril-like cluster formation, called sliding (319). Though biofilm formation of *B. subtilis* is a natural process (320), numerous environmental factors, and extracellular signals are known to affect these complex changes in *B. subtilis*. Most of these environmental triggers allow the expression of genes important for matrix production through complex networking and signaling pathways. Several global transcription regulators have been described to be involved in the processes that lead to biofilm formation, including among others Spo0A, SinR, AbrB, SlrR, DegU, ComA, and Rok (89, 106, 107, 109, 125, 133, 150, 321–323).

Global regulators affecting biofilm development of *B. subtilis*.

The main biofilm repressor, SinR, directly represses the biofilm genes *epsA–O* and *tapA–sipW–tasA*. When the phosphorylated levels of Spo0A (Spo0A–P) reach a given threshold level, it activates the transcription of *sinI* gene that codes an anti-repressor (108). SinI then forms a complex with SinR and thus permits the expression of matrix forming genes (128, 321). SlrR, a homologue of SinR plays another important role on the SinI–SinR switch during biofilm formation and motility (108, 109). SlrR forms a complex with SinR and therefore it titrates away SinR that would otherwise repress biofilm genes, while SlrR–SinR complex also represses the genes related to motility and cell lysis (111, 324). The SlrR–SinR interaction has an additional de-repressive effect on the *slrR* gene resulting in a positive feedback loop, observed as hysteresis or epigenetic switch that keeps the cells in the "high-SlrR" state for several cell divisions (324). Transcriptional activation of the biofilm genes and repressed expression of motility and lysis genes result in chaining of *B. subtilis* cells (321, 324). The switch to the "high-SlrR" state and chaining occurs independently from the environmental signals, it is memoryless (i.e., switching is stochastic), and exists for around 7.6 generations under laboratory conditions in a microfluidic device (325). In the presence of environmental signals, the "high-SlrR" state and therefore the de-repression of biofilm gene transcription by SinR is maintained. This results in the production of biofilm matrix for extended periods of time (324).

The threshold level of Spo0A–P required for biofilm formation is lower than for sporulation, and high level of Spo0A–P has rather inhibitory effects on the expression of biofilm genes. However, like for sporulation, the membrane-bound kinases-facilitated phosphorylation of Spo0A is required for biofilm formation. The diverse roles of the different *B. subtilis* kinases and the biofilm related triggering molecules are discussed below in detail.

The activity of another global transcriptional regulator in *B. subtilis*, DegU is controlled by the sensor kinase DegS through phosphorylation (145). The exact signal for DegS is unknown. In its unphosphorylated form, DegU activates the expression of *comK*, the main regulator of competence (146). When the level of DegU–P slightly increases, the expression of motility genes is induced, followed by activation of biofilm formation at an intermediate DegU–P level and exoprotease production at a high level (106, 107). DegU–P also has an impact on the transcription of its own gene, which is additionally inhibited by SinR during exponential growth phase. During late log phase, as levels of SlrR begin to rise, it sequesters SinR from the promoters of biofilm related genes and *degU* by forming a complex. Low phosphorylated levels of DegU–P alters the SinR–SlrR switch thus affecting its auto regulation (326). In laboratory strains, the role of DegU–P in the production of γ-poly-DL-glutamic acid is related to the mucoid colony phenotype giving rise to the biofilm formation (42).

Next to the global regulators described above, the carbon source has important impact on biofilm formation of *B. subtilis* as it is also observed for other Gram-positive bacteria described above. Previous transcriptomic study reported that over 500 genes were differentially expressed at one or more time points as cells transitioned to a biofilm state (327). The signal that appeared to affect gene expression during submerged biofilm formation is glucose depletion in the laboratory strains. Low concentration of glucose is required to promote submerged biofilm formation, however high amount of glucose inhibits biofilm formation via CcpA, the common catabolite repressor (327).

The role of Kin kinases in *B. subtilis* biofilm development.

Phosphorylated Spo0A controls the transcription of many genes next to biofilm related genes, including sporulation, competence, and cannibalism toxin and immunity genes. The level of phosphorylated Spo0A is controlled by the action of membrane bound histidine sensor kinases (KinA–E). KinA has been the most actively studied kinase so far due to its major role in sporulation. While the active kinase phosphorylates Spo0F response regulator, Spo0B phosphotransferase transfers the phosphoryl group from Spo0F to Spo0A. In absence of KinA and KinB kinases, KinC and KinD also phosphorylate Spo0A via Spo0F to levels sufficient to activate biofilm genes but not sporulation (122). In this part of the review, the extracellular signals that mediate the activation of membrane kinases are discussed related to biofilm development. Each kinase needs different and specific concentration of the environmental cues to phosphorylate Spo0A and hence induce matrix-forming genes (Table 2). However, not a single kinase is alone responsible for activation of the signaling cascade, but rather the interplay between the different kinases determines the optimal expression induction of biofilm related genes.

Table 2: Overview of the molecules and the signaling cascade in *B. subtilis* biofilms.

Molecules/ processes triggering biofilm formation	**Effect at the cellular level**	**Sensor kinase**	**References**
Impaired oxidative phosphorylation	Decrease in NAD^+ levels	KinA	(328)
	Reduced electron transfer through cytochromes	KinB	(328)
Surfactin, nystatin, gramicidin	K^+ leakage	KinC	(159, 329)
ClO_2	Altered membrane potential	KinC	(330)
Nisin	Increased population of matrix producers	Unknown	(139)
Plant polysaccharides (*Arabidopsis thaliana*)	Carbon sources via incorporating the galactose residues in the matrix	KinC and KinD	(68)
Root exudates (tomato)	Maltose and other metabolites	KinD	(331)
Glycerol	Glycerol uptake and metabolism	KinD	(332)
Mn^{2+}	Cofactor in Spo0A phosphorylation via Spo0F	KinD	(332)
Menaquinones	Respiration and growth	Unknown	(333)
Osmosensitivity	Physiological forces governed by the biofilm matrix	KinD	(334)

KinA and KinB kinases regulate biofilm formation in response to anaerobiosis.

The role of KinA and KinB in sporulation is known since many decades. However, the exact nature of the signal to which these two kinases respond is still unknown. While KinA autophosphorylates itself with the aid of Spo0F, there are reports that it may respond to ATP (335, 336). Recent studies showed that impaired respiration or oxidative phosphorylation stimulate wrinkling and matrix gene expression via KinA and KinB (328). The membrane bound KinB interacts with the aerobic respiratory apparatus and is activated by the decrease in electron transport through the cytochrome chain. This interaction varies with the concentration of iron (Fe^{3+}), which is required for the function of heme-containing cytochromes. KinA also contributes to anaerobic condition sensing by directly responding to the decrease in NAD^+ levels in the cytoplasm.

The paracrine signaling network.

While studying the association between *Streptomyces coelicolor* and *B. subtilis*, it was discovered that the production of surfactin by *B. subtilis* itself is required for the development of the aerial structures in the colony and that the mutant in surfactin production (*srfA*) is unable to form such aerial structures and forms thin, fragile pellicles (91, 337). Later studies on various compounds showed that surfactin, similar to many polyketides like nystatin, does not only reverse the phenotype in *srfA* mutant but also induces biofilm formation when supplemented in LB medium, which is not optimal for biofilm formation (159, 338). Nystatin and other structurally and functionally similar polyketides cause cation leakage by producing pores in the cell membrane of *B. subtilis*. This leakage triggers biofilm formation signaling through the KinC kinase. The role of KinC in this process was identified in a screen of various kinase mutants and it was observed that kinC mutant failed to respond to the presence of surfactin or nystatin in LB. By constructing altered kinC alleles lacking various domains in KinC, a PAS–PAC sensor domain was found to be important for KinC-mediated induction of biofilm genes (159). The importance of the PAC–PAS domain was also proved by replacing the phosphorylating domain of KinC with the phosphorylating domain DegS from the above-described DegU–DegS two component system. Having the chimera kinase, *B. subtilis* cells respond to the presence of nystatin by increasing the transcription of the *aprE* gene, which is a member of the DegU–DegS regulon. Along with nystatin, many other compounds like amphotericin, gramicidin, valinomycin, and others found in soil and mainly produced by soil dwelling bacteria are known to trigger biofilm formation through a similar mechanism (159, 338, 339). Since K^+ is the most abundant cation present in the cytoplasmic membrane, it is expected that its leakage is sensed leading to the activation of biofilm genes and hence pellicle formation. Surfactin fails to induce pellicle formation, when 150mM KCl is present in the medium (159). KinC thus responds to the internal K^+ concentration or its leakage and not just the presence of surfactin. The effect of nystatin on fungal population is mediated by its binding to a membrane sterol (ergosterol) and therefore inhibiting growth (340). Importantly, the effect of surfactin and nystatin on the bacterial cell is mediated indirectly through altered ion concentration.

As ergosterol is derived from squalene, genes related to squalene production were screened and it was observed that the mutations in a non-essential gene *yisP* lead to the complete loss of pellicle formation (329). The *yisP* gene is involved in the synthesis of squalene that is required for the assembly of membrane microdomains analogous to lipid rafts of eukaryotic cells. KinC localize to these punctate microdomains together with the flotillin like proteins, FloT and FloA. These results were further corroborated by the observation that the inhibitor of squalene biosynthesis, zaragozic acid reduces both biofilm formation and the punctate localization of KinC and flotillin proteins. Interestingly, zaragozic acid is a potent inhibitor of biofilm formation and of the presence of lipid rafts in *S. aureus* (329). Future experiments are necessary to reveal the role of these lipid rafts in *S. aureus* biofilm formation. It is also intriguing what the exact role of lipid rafts in Gram-positive bacteria is and how these membrane structures play a role in various developmental pathways, including motility, genetic competence, and cell morphology (341, 342). Biofilm formation is reduced in the *floA* and *floT* mutants and this is also related to the delocalization of the FtsH protease (343). As presented above, induction of biofilm formation and sporulation requires given levels of Spo0A–P. FtsH influences the levels of Spo0A–P by degrading the proteins RapA, RapB, RapE, and Spo0E that modulate the Spo0A phosphorelay (343–345). The subpopulation of cells producing the matrix components in the *B. subtilis* biofilm also exhibits

antimicrobial activity towards the non-producers in an attempt to lessen the competition as well as to thrive on molecules released by lysed cells in nutrient limiting, biofilm conditions (139, 346). By producing toxins, these producers are also resistant to the antimicrobial peptides as they themselves express immunity machinery required to repel the effects of these toxins (347). The production of toxins, especially Skf, also leads to an increased production of extracellular matrix observed through increased colony wrinkling. Surfactin triggers the production of these cannibalism factors by activating the expression of *skfA–H* and *sdpABC* genes through KinC mediated activation of Spo0A–P (139). The presence of the Skf and Sdp peptides and analogous molecules like nisin are also known to delay sporulation and thus increase the ratio of matrix-producing cells in the biofilm population.

More evidence of how biofilm provides protection against toxic compounds is provided by a recent study where chlorine dioxide was found to trigger biofilm pellicle formation. ClO_2 is regarded as effective biocide agent but at sub-lethal concentrations activates KinC by acting on membrane, resulting in membrane potential changes (330).

Integrating environmental signals: from glycerol to plant polysaccharides.

In natural environments, bacterial species are constantly evolving different mechanisms due to the interactions between different species and genera. For *B. subtilis*, molecules produced by neighboring cells of same and related species are known to trigger biofilm formation and cannibalism through membrane kinase KinC as discussed above. Recent report also highlights how distant species when associated with *B. subtilis* stimulate biofilm formation (348). The given Kin sensors seem to play different roles in integrating the various environmental signals toward the activation of biofilm formation. During association of *B. subtilis* with *Arabidopsis thaliana* roots, bacteria require production of EPS and other matrix components for the colonization on the roots. The expression of biofilm genes under these conditions is triggered through contact with the roots. To investigate further what initiates biofilm formation during *A. thaliana* and *B. subtilis* interactions, plant extracts were purified. Plant cell wall polysaccharides such as xylan, pectin, and arabinogalactan trigger biofilm formation via KinC and KinD in cells grown on otherwise non-biofilm inducing medium. While kinC and kinD mutants show reduced and fragile pellicle formation induced by the presence of xylan, pectin, and arabinogalactan in the medium, *kinCkinD* double mutant showed complete loss of pellicle formation indicating their involvement in plant polysaccharide mediated signaling (68). Extracts from other plants harboring different cell wall polysaccharides fail to induce pellicle formation under the same conditions. Also, as galactose constitutes an essential part of EPS, many plant polysaccharides are used as carbon source and its galactose residues are incorporated into matrix via GalE by converting UDP-glucose to UDP-galactose (349). This study brings KinD into spotlight as one of the kinases involved in mediating environmental signals for biofilm establishment, but the molecules and factors that directly activate transcription of biofilm genes are still not clearly understood.

Structurally, KinD consists of two transmembrane helices, two cytoplasmic catalytic domains, and a periplasmic sensor domain. It differs from other Kin proteins of *B. subtilis* due to the presence of a CACHE domain, known as a calcium channel and chemotaxis receptor. This CACHE domain is responsible for rhizosphere associated biofilm induction on tomato plants (331, 350). High amount of L-malic acid found in tomato root exudates activates the biofilm colonization on the root surfaces through KinD. However, ligand- binding studies using malic acid failed to show specific

binding to the sensor CACHE domain of KinD. L-malate was therefore suggested to act as a carbon source and to indirectly activate KinD through CACHE or other unknown domains (331). Electron density studies showed that this sensor domain preferably binds pyruvate and other carboxylic acids like propionate and butyrate. Residue R131 within the sensor domain is required for pyruvate binding, while the membrane distal domain showed preference to acetate binding (350). Thus, R131 residue was suggested to be required for binding pyruvate and carboxylic acids through which KinD might be activated. Possibly, L-malate is converted to pyruvate and this molecule binds to the KinD sensor domain. Thus, KinD mediated activation of biofilm formation requires a complex response involving the CACHE domain that may recognize the small molecules secreted by plants or *B. subtilis* itself. KinD mediated activation process may require a partner membrane molecule similar to the lipoprotein KapB protein that is necessary for KinB activity related to sporulation (351). The membrane anchored lipoprotein, Med, is required for KinD dependent phosphorylation of Spo0A (352). Such an association with KinD sensor is functionally similar to the LuxP–LuxQ interaction during quorum sensing in *Vibrio harveyi*, where LuxQ is structurally similar to KinD (331, 353). However, the study performed with the Med-KinD interaction suggests an indirect or transient partnership as chemical crosslinking and pull-down experiments failed to show interactions between the two proteins, Med and KinD. Spo0A–P has a negative effect on the expression of *med* gene and the elevated levels of Spo0A–P reduce the sensor activity of KinD through a negative feedback loop (136).

Similar to root exudates and plant polysaccharides, another carbon source, glycerol, and its derivatives were also reported to activate biofilm genes of *B. subtilis* via KinD. The effect of glycerol on robust biofilm formation under otherwise non-inducing medium (i.e. Luria broth medium) depends on the addition of manganese (Mn^{2+}). Similar to the above-described examples in *B. subtilis*, glycerol and Mn^{2+} induce biofilm gene expression via KinD mediated phosphorylation of Spo0A–P (332). Mn^{2+} seems to act as a cofactor creating a complex with Spo0F and possibly promoting the efficiency of phosphotransfer towards Spo0A. Importantly, Mn^{2+} is an essential component of various biofilm media used for *B. subtilis*. Mn^{2+} has also other important effects in the bacterial cells, e.g. it is required for sporulation (354, 355). Mn^{2+} seems to play a complex role in *B. subtilis* and its presence modulates the various differentiation processes during biofilm development (E. Mhatre & Á. T. Kovács, unpublished results).

Recently, osmotic pressure increase due to certain dextran polymers and PEG supplementation in the medium was reported to reduce the expression of matrix-related genes and pellicle robustness. The effects on biofilm development during osmotic level shift is connected to the KinD sensor kinase, adding a physical clue in addition to the chemical signals (334).

The examples listed above show the diverse signals that determine the kinase activity of KinD resulting in increased level of Spo0A in the cell. In addition to this, KinD also acts as a checkpoint for delaying sporulation under biofilm conditions. KinD operates as a kinase keeping Spo0A–P level high enough to activate matrix production, but on the other hand its phosphatase activity keeps the Spo0A–P level at an intermediate level to prevent the initiation of sporulation (356). Interestingly, sporulation is not reduced in cells grown in liquid cultures, thus the Spo0A–P level is kept at an intermediate level only in matrix-deficient cells of colony biofilms. The presence of extracellular matrix components of the mature biofilm is required for KinD to switch the phosphatase mode to the kinase activity. Hence, matrix-embedded cells possess high concentration of Spo0A–P, and thus are the ones that enter the sporulation first. This is the reason

for late onset of sporulation in the *eps* and *tasA* mutants of *B. subtilis*. In accordance with this, a kinD mutant sporulates early in biofilm (356).

Recent studies with menaquinones (Vitamin K2, PubChem Compound-ID 15956540) have highlighted how these compounds aid in complex colony formation in *B. subtilis* (333). The wild-type strain when grown on agar plates supplemented with diphenylamine (inhibitor of menaquinone biosynthesis), shows small colonies with absence of irregular dendrite-like structures, which are otherwise the character of complex colony biofilms. A similar defect is observed in mutants with impaired menaquinone biosynthesis, which is reverted with externally supplemented menaquinone (333). Previously, menaquinone was also shown to be important for sporulation (357). However, the direct sensing and triggering process of menaquinone on biofilm formation is unknown and it is undetermined whether one of the kinases plays a role in the activation pathway.

Conclusions.

Biofilm formation of Gram-positive bacteria is regulated by various environmental signals. On one hand, determination of signaling processes and their specific inhibitions may provide novel anti-biofilm strategies to reduce chronic infections, contamination of medical devices and food poisoning, while on the other hand, isolation of small molecules that improve biofilm development can serve as biofilm-promoting compounds in the biocontrol area and in advantageous biotechnological processes.

Studying model organisms, such as *B. subtilis* allows us to determine the action of diverse environmental factors that manifest their effects on the same pathway, resulting in central signal perception. While dozens of signaling molecules are described and their putative sensor is identified, the exact molecular details on how the signaling molecules are sensed still remain unknown and it encourages conveying of detailed analysis of the identified systems. Crystallization studies of the various signaling molecules with their putative sensors could enlighten us whether the signal is directly perceived and may also highlight other mediating molecules involved.

Finally, scrutinizing mixed biofilms could also expose novel compounds that shape the community structure and biofilm properties. Thus, more details on the induction of biofilm formation should also answer the challenging questions about how organisms shift from one mode of lifestyle to another.

Acknowledgments.

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CHAPTER 2

Specific *Bacillus subtilis* 168 variants form biofilms on nutrient-rich medium.

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Bacillus subtilis is an intensively studied Gram-positive bacterium that has become one of the models for biofilm development. *B. subtilis* 168 is a well-known domesticated strain that has been suggested to be deficient in robust biofilm formation. Moreover, the diversity of available *B. subtilis* laboratory strains and their derivatives have made it difficult to compare independent studies related to biofilm formation. Here, we analyzed numerous 168 stocks from multiple laboratories for their ability to develop biofilms in different set-ups and media. We report a wide variation among the biofilm-forming capabilities of diverse stocks of *B. subtilis* 168, both in architecturally complex colonies and liquid–air interface pellicles, as well as during plant root colonization. Some 168 variants are indeed unable to develop robust biofilm structures, while others do so as efficiently as the non-domesticated NCIB 3610 strain. In all cases studied, the addition of glucose to the medium dramatically improved biofilm development of the laboratory strains. Furthermore, the expression of biofilm matrix component operons, *epsA-O* and *tapA-sipW-tasA*, was monitored during colony biofilm formation. We found a lack of direct correlation between the expression of these genes and the complexity of wrinkles in colony biofilms. However, the presence of a single mutation in the exopolysaccharide-related gene *epsC* correlates with the ability of the stocks tested to form architecturally complex colonies and pellicles, and to colonize plant roots.

Introduction.

Bacillus subtilis is a non-pathogenic model Gram-positive bacterium that has been extensively studied for over a century. Microbiologists have used *B. subtilis* to investigate a broad variety of biological questions, ranging from the intricacies of cell metabolism to community behaviour and evolution (85, 135). As a consequence of the extended use of *B. subtilis*, multiple strains exist in laboratories and strain collections all over the globe. Some of these strains have been isolated from distinct environments and are used as wild-type reference strains, e.g. NCIB 3610 (from here onwards, 3610) and PS216. Other commonly used strains have been described as 'domesticated' due to their prolonged use under laboratory conditions, which have conferred

them with characteristics that make them ideal research models, i.e. ease of genetic manipulation and efficient growth on commercially available media.

One of the key features of *B. subtilis* is its ability to form biofilms. Biofilms are complex multicellular communities that can develop in diverse environments and potentially have a major impact on multiple human activities, among others including an ominous progression of common infections or hampering of biotechnological and industrial applications (75, 306). Formation of biofilms can be desirable under certain circumstances; *B. subtilis* biofilms, for example, have been implicated in crop protection by prevention of colonization of plant roots by pathogenic organisms (93).

B. subtilis has become one of the model organisms used for biofilm research. Studies performed over the years have provided many insights regarding the processes involved in the development of these bacterial populations (112, 358). Strain 168 is the most well-known and widely used laboratory strain; it is an easily transformable tryptophan auxotroph that was obtained by X-ray mutagenesis (359) and has been used in a multitude of academic and industrial studies. The intensive use of strain 168 has generated various derivative strains, several of which have been sequenced by a joint European–Japanese consortium and later re-sequenced using single strains (360–362).

The biofilms formed by *B. subtilis* have traditionally been studied as complex structured colonies on agar plates or as pellicles formed at the liquid–air interface of static liquid cultures (89, 363). Branda *et al.* (91) were the first to report the various biofilms developed by certain *B. subtilis* strains, noticing that domesticated laboratory strains derived from strain PY79 formed deficient biofilms. Since then, laboratory strains, including 168, have largely been considered as poor or non-biofilm formers at best (364). Different studies have investigated the genetic differences between this strain and wild-type 3610, reporting that these disparities are responsible for strain 168's small, unstructured colonies and flat, featureless pellicles (115, 217). In particular, a deficiency in the production of exopolysaccharide (EPS) has been highlighted as an important flaw of strain 168 related to biofilm formation (115, 321).

In *B. subtilis*, EPS is produced by the proteins encoded in the *epsA-O* operon (113) and is a major component of the biofilm matrix (45). Due to its relevance to biofilm formation, the chemical nature of this polymer has been investigated by various groups. However, these studies have normally used different non-domesticated strains and media, obtaining disparate results (41, 349, 365). This phenomenon is a testament to the robustness of *B. subtilis*, a soil bacterium that has evolved the ability to survive on different nutrient sources and therefore can use diverse compounds to produce the polymers that form the backbone of the biofilm matrix (366, 367).

A problem that permeates *B. subtilis* research is the plethora of available strains and methods, making it difficult to compare experimental results. Here, we compared various biofilms developed by divergent laboratory stocks of strain 168 originating from various research groups around the globe. We also analyzed the expression of the *eps* and *tapA-sipW-tasA* operons using fluorescent reporter fusion. We report that the formation of complex colonies varies greatly among the various 168 strains, some of them being able to form architecturally complex colonies similar to those developed by 3610 when grown on complex or supplemented media. In addition, we show that the expressions of P_{eps}-GFP and P_{tapA}-GFP fusion does not necessarily correlate with the formation of architecturally complex structures in these biofilms.

Material and Methods.

Strains and media.

All strains used in this study are listed in Table 1. These strains were pre-grown overnight in LB medium (lysogeny broth, Carl Roth; 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract and 5 g l^{-1} NaCl) and later grown on LB supplemented with 0.1 mM $MnCl_2$ and either 0.1% glucose (hereafter, LB-Glu) or 1% glycerol (hereafter, LB-Gly), 2×SG medium [16 g l^{-1} nutrient broth (Difco), 2 g l^{-1} KCl, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 1 mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2 \cdot 4H_2O$, 1 µM $FeSO_4$ and 0.1% glucose] (92) or defined MSgg medium [5 mM potassium phosphate buffer (pH 7), 100 mM MOPS, 2 mM $MgCl_2$, 700 µM $CaCl_2$, 100 µM $MnCl_2$, 50 µM $FeCl_3$, 1 µM $ZnCl_2$, 2 µM thiamine, 0.5% glycerol, 0.5% glutamate, 50 µM L-tryptophan and 50 µM L-phenylalanine] adapted from Branda *et al.* (91). Murashige and Skoog (MS) medium was used for *Arabidopsis thaliana* germination [MS basal salt mixture, Sigma-Aldrich; 2.2 g l^{-1} MS medium (pH 5.6–5.8)], while MSNg medium was used for root surface colonization assays [5 mM potassium phosphate buffer (pH 7), 100 mM MOPS, 2 mM $MgCl_2$, 50 µM $MnCl_2$, 1 µM $ZnCl_2$, 2 µM thiamine, 0.2% NH_4Cl, 0.05% glycerol and 700 µM $CaCl_2$]. Media were supplemented with Bacto agar 1.5 or 1% when solid plates were needed for bacterial colonies or plant seed germination, respectively. Unless otherwise stated, all liquid cultures were grown at 37°C with shaking at 225 r.p.m.

Table 1: Strains used in this study.

Strain	**Characteristics**	**Abbreviation used here**	**Reference**
NCIB 3610	Prototroph, wild-type	3610	Bacillus Genetic Stock Center (BGSC)
DK1042	3610 *comI*^{Q12I}		(368)
NRS2243	3610 *sacA*::P_{epsA}-*gfp* (*neo*), *hag*::*cat*		(369)
NRS2394	3610 *sacA*::P_{tapA}-*gfp* (*neo*)		(369)
JH642	Δ*trpC2* Δ*pheA1 citS642*, derived from Marburg strain		Laboratory Stock (Grau, R., originally from Hoch, J.A.)
168 (Boston)	Δ*trpC2*, derived from 168	168 Bo	Laboratory Stock (Romero, D., originally from Kolter, R.)
168 (Braunschweig)	Δ*trpC2*, derived from 168	168 Br	Laboratory Stock (Härtig, E.)
168 (Göttingen)	Δ*trpC2*, derived from 168	168 Gö	Laboratory Stock (Stülke, J.)
168 (Jena)	Δ*trpC2*, derived from 168 1A700	168 Je	Laboratory Stock (Terrestrial Biofilms Group, originally from University of Groningen and BGSC)
168 (Ljubljana)	Δ*trpC2*, derived from 168 1A1	168 Lj	Laboratory Stock (Mandić-Mulec, I., originally from BGSC)
168 (Malaga)	Δ*trpC2*, derived from 168	168 Ma	Laboratory Stock (Romero, D.)
168 (Münich)	Δ*trpC2*, derived from 168	168 Mü	Laboratory Stock (Thorsten, M., originally from Stülke, J.)
168 (New Castle)	Δ*trpC2*, derived from 168 1A1	168 NC	Laboratory Stock (Veening, J.-W. and Errington, J., originally from BGSC)
168 (Paris)	trp+, tryptophan-prototrophic derivative of 168 (with reconstituted *trpC* gene)	168 Pa	Laboratory Stock (Briandet, R., BaSysBio reference strain, BSB1) (370)

168 (Pavia 1)	Δ*trpC2*, derived from 168	168 P1	Laboratory Stock (Calvio, C., originally from Anagnostopoulos)
168 (Pavia 2)	Δ*trpC2*, derived from 168	168 P2	Laboratory Stock (Calvio, C., originally from Burkholder and Giles)
168 (Tokai)	Δ*trpC2*, derived from 168	168 To	Laboratory Stock (Ogura, M., originally from the EU sequencing consortium)
TB356	168 NC *sacA*::P_{epsA}-*gfp* (*neo*)		This study
TB357	168 Gö *sacA*::P_{epsA}-*gfp* (*neo*)		This study
TB358	168 To *sacA*::P_{epsA}-*gfp* (*neo*)		This study
TB359	168 Br *sacA*::P_{epsA}-*gfp* (*neo*)		This study
TB360	168 P1 *sacA*::P_{epsA}-*gfp* (*neo*)		This study
TB361	168 P2 *sacA*::P_{epsA}-*gfp* (*neo*)		This study
TB362	JH642 *sacA*::P_{epsA}-*gfp* (*neo*)		This study
TB363	DK1042 *sacA*::P_{epsA}-*gfp* (*neo*)		This study
TB365	168 Je *sacA*::P_{epsA}-*gfp* (*neo*)		This study
TB392	168 Bo *sacA*::P_{epsA}-*gfp* (*neo*)		This study
TB394	168 Ma *sacA*::P_{epsA}-*gfp* (*neo*)		This study
TB366	168 NC *sacA*::P_{tapA}-*gfp* (*neo*)		This study
TB367	168 Gö *sacA*::P_{tapA}-*gfp* (*neo*)		This study
TB368	168 To *sacA*::P_{tapA}-*gfp* (*neo*)		This study
TB369	168 Br *sacA*::P_{tapA}-*gfp* (*neo*)		This study
TB370	168 P1 *sacA*::P_{tapA}-*gfp* (*neo*)		This study
TB371	168 P2 *sacA*::P_{tapA}-*gfp* (*neo*)		This study
TB372	JH642 *sacA*::P_{tapA}-*gfp* (*neo*)		This study
TB373	DK1042 *sacA*::P_{tapA}-*gfp* (*neo*)		This study
TB375	168 Je *sacA*::P_{tapA}-*gfp* (*neo*)		This study
TB393	168 Bo *sacA*::P_{tapA}-*gfp* (*neo*)		This study
TB395	168 Ma *sacA*::P_{tapA}-*gfp* (*neo*)		This study
TB34	DK1042 *amyE*::$P_{hyperspank}$-*gfp* (*cat*)		(371)
TB49	168 Je *amyE*::$P_{hyperspank}$-*gfp* (*cat*)		(372)
TB707	168 Bo *amyE*::$P_{hyperspank}$-*gfp* (*cat*)		This study
TB709	168 P1 *amyE*::$P_{hyperspank}$-*gfp* (*cat*)		This study
TB722	168 Gö *amyE*::$P_{hyperspank}$-*gfp* (*cat*)		This study
TB723	168 Lj *amyE*::$P_{hyperspank}$-*gfp* (*cat*)		This study

Strain construction.

B. subtilis P_{eps}-GFP and P_{tapA}-GFP strains were obtained via natural competence transformation (373) using genomic DNA from strains NRS2243 and NRS2394, respectively. Briefly, overnight cultures of the receiver strains were diluted to a 1:50 ratio with GCHE medium [1% glucose, 0.2% glutamate, 100 mM potassium phosphate buffer (pH7), 3 mM trisodium citrate, 3 mM $MgSO_4$, 22 mg l^{-1} ferric ammonium citrate, 50 mg l^{-1} L-tryptophan and 0.1% casein hydrolysate], and these cultures were incubated for 4 h, after which 5–10 µg of genomic DNA was mixed with 500 µl of competent cells and further incubated for 2h before plating. $P_{hyperspank}$-GFP labelled strains were obtained by the same method using plasmid phyGFP that integrates into the *amyE* locus of *B. subtilis* (372). Transformants were selected on LB plates with 5 µg ml^{-1} kanamycin or 5 µg ml^{-1} chloramphenicol. In all transformations, several clones were examined for colony morphology and no phenotypical differences regarding wrinkleability were observed between the obtained

transformants and their corresponding parental strains. Successful transformation was validated using the fluorescence reporter activity of the strains or amylase-negative phenotype on 1% starch agar plates (374). DK1042 was used rather than 3610 to obtain fluorescently labelled strains, due to its improved transformability (368).

Biofilm development as pellicles and architecturally complex colonies.

The study strains were pre-grown in 3 ml LB medium over- night, following which 2 ml of 1:50 dilution of these cultures in 2×SG and MSgg media were used to inoculate 24-well plates for pellicle formation. For biofilm colony structures, the four types of media (LB-Glu, LB-Gly, 2×SG and MSgg) were supplemented with 1.5% agar and tempered to 55°C, and 25 ml of the medium was poured into a 90 mm-diameter Petri dish. Once solid, plates were dried completely open in a laminar airflow bench for exactly 15 min. The plates were closed and 2 µl of each strain was inoculated on to the plate. To avoid growth inhibition and constraint resulting from two different *B. subtilis* strains, only three strains were inoculated per plate and each had the reference 168 Je strain for comparison of morphology. The plates for the pellicles and colonies were incubated at 30°C for 72 h.

Biofilm development on root surfaces.

Seeds of *A. thaliana* ecotype Col-0 were surface sterilized by incubation in 1 ml of 2% NaClO solution for 20 min on an orbital mixer. The seeds were washed five times with 1 ml of sterile distilled water and placed on MS medium supplemented with 1% agar (375). The seeds were planted 15 mm apart in order to avoid entanglement of the roots. The MS plates were parafilm sealed and incubated at 4°C for 72 h, following which they were placed at room temperature on a windowsill for 10–12 days to allow the seeds to germinate and develop roots of approximately 1 cm in length. Overnight cultures of the test strains were adjusted to OD_{600} of 0.2 and then further diluted 10-fold using MSNg medium. The seedlings were then placed in 300 µl of these cultures in a 48-well microplate. The roots of the seedlings were completely submerged in the bacterial dilutions. The microplate was incubated at 28°C with 90 r.p.m. shaking for 24 h. After the incubation period, the seedling roots were gently washed three times with sterile MSNg medium, placed on microscopy slides, covered with coverslips and examined without further treatment.

Microscopy and image analysis.

All bright-field and green fluorescence images of colonies and pellicles were obtained with an Axio Zoom V16 stereomicroscope (Carl Zeiss) equipped with a Zeiss CL 9000 LED light source, HE eGFP filter set (excitation at 470/40 nm and emission at 525/50 nm) and an AxioCam MRm monochrome camera (Carl Zeiss). For colony and pellicle morphology comparison, images were obtained after 72h incubation at ×5 and ×20 magnifications. For P_{eps}-GFP and P_{tapA}-GFP reporter fusion expression comparison, images were taken at different time points using ×3.5 magnification and exposure times of 2500 ms for green fluorescence and 10 ms for bright field.

The expression of green fluorescence of colonies was analyzed using ImageJ (National Institutes of Health). Briefly, images were batch processed to subtract a background value, and this value was calculated using a circular region of interest (ROI) of 1 mm radius and measuring the average fluorescence intensity in 36 non-colony areas selected from random images at all time points using only the green channel information. Afterwards, the colony area of each image was selected using the bright- field channel and the tracing tool with legacy mode and a tolerance

of 100. Using the selected ROI, the corresponding colony areas were marked on the green fluorescence images and their average fluorescence intensity was measured.

All bright-field and green fluorescence images of *A. thaliana* roots were obtained with an Axio Observer 780 Laser Scanning Confocal Micro- scope (Carl Zeiss) equipped with a Plan-Apochromat 63×/1.4 Oil DIC M27 objective, an argon laser for stimulation of green fluorescence (excitation at 488 nm and emission at 540/40 nm), a halogen HAL-100 lamp for transmitted light microscopy and an AxioCam MRc colour camera (Carl Zeiss). The images were obtained as a Z-stack of 10 slices covering 3.8 µm on the Z-axis. The images were later merged with ImageJ using an average-intensity Z-projection. The average fluorescence intensity of the roots was measured by selecting the root area as an ROI in the bright-field channel and then measuring the fluorescence intensity in the corresponding area of green channel Z-projections only.

Sequencing of *epsC*, *swrA*, *degQ* and *sfp* alleles.
Fragments of *epsC* (788 bp), *swrA* (935 bp), *degQ* (552 bp) and *sfp* (827 bp) were PCR amplified from genomic DNA of the tested *B. subtilis* stocks using corresponding primer pairs (Table 2). The fragments were sequenced using primer oTB110 (*epsC*), oTB131 (*swrA*), oTB132 (*degQ*) or oTB134 (*sfp*) (GATC Biotech).

Table 2: PCR primers used in this study.

Primer	Sequence (5´ → 3´)	Target Locus
oTB110	CGAACTGCCGGACAAATC	*epsC*
oTB111	ACGGGCTCTCCCATATC	*epsC*
oTB130	GGTTATGGCTTTTCAGGATCAAAAC	*swrA*
oTB131	TCTATCAAATATTAAATGGCTTGGATAT	*swrA*
oTB132	CTGTCGTTTCTTTAATATC	*degQ*
oTB133	ACCAGGGATAACGATATCTC	*degQ*
oTB134	GGTGTCAAGCTGTTGATGAG	*sfp*
oTB135	AAGCATCTCCGCCTGTACAC	*sfp*

Results.

Various stocks of *B. subtilis* 168 show diverse complex colony and pellicle morphologies.
Different nutrients are known to have a profound effect on general metabolism and the production of signaling molecules that regulate biofilm formation in *B. subtilis* (358). Previous publications suggest that the availability of complex nutrients might supplement the metabolic shortcomings of strain 168 and allow it to develop architecturally complex biofilms (133, 376). We were interested in testing various stocks of the 168 strain that are used by different laboratories. Importantly, these stocks might have diverged due to additional genetic differences, possibly resulting in contrasting observations among the work groups investigating biofilm formation.

We grew complex colonies of the 168 variants obtained, the domesticated laboratory strain JH642 and the wild isolate 3610 on defined (i.e. MSgg) and complex (i.e. supplemented LB and 2×SG) media for 72 h. LB, although rich in nutrients and commonly used by microbiologists, is not a biofilm-promoting medium. The colonies developed by *B. subtilis* on LB agar are small, flat and featureless, and no pellicles develop on static liquid LB cultures (data not shown). We investigated the possibility that the addition of both manganese and carbon sources (glycerol or

glucose) similar to those present in other media could be sufficient to promote the development of complex *B. subtilis* biofilms on LB medium (Figs. 1 and S1, all supplementary material for this chapter is available in annexum A).

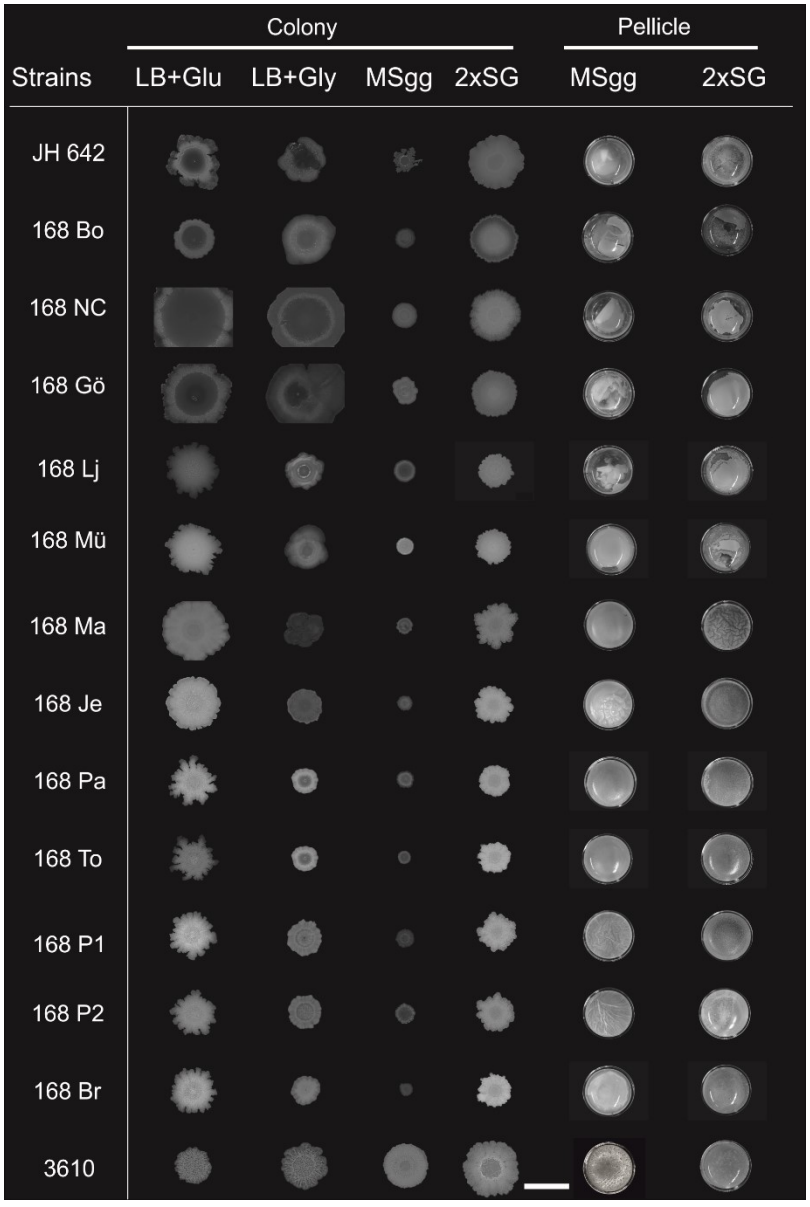

Figure 1: Comparison of complex colonies and pellicles of *B. subtilis* strain 3610 and 168 variants. Strains were grown on different media and imaged after 72 h incubation, arranged by increasing colony wrinkleability on 2×SG medium. The scale bar shown at the bottom represents 20 mm. Strain abbreviations are described in Table 1.

Using the chemically defined MSgg medium, we observed a marked difference between the colonies developed by all 168 variants and the non-domesticated prototroph; namely, 3610 developed large (18–20 mm), opaque colonies with wrinkles, while all 168 variants developed smaller (5–8 mm) colonies with smooth, bright surfaces on the periphery that show small or no wrinkles (Figs. 1 and S1). The opacity in the wrinkles of a complex colony is suggested to be associated with increased sporulation as the biofilm matures (138, 377). These phenotypes are in line with previous reports that define the domesticated 168 strain as a non-biofilm former when grown on MSgg medium (91, 115). However, the appearance of the biofilms changed drastically

when grown on complex medium. On 2×SG, 3610 showed large colonies with increased architectural complexity, i.e. its colonies had larger and seemingly taller wrinkles with opaque white summits, while leaving a clear flat and smooth area in the centre of the colony. On this medium, the 168 variants formed colonies almost as large as those developed by 3610 (15–20 mm), and their topography showed great diversity. 168 P2 and 168 Je, for example, formed wrinkled opaque colonies resembling those formed by 3610 on MSgg, while 168 Gö and 168 NC showed a uniformly rugose, bright surface. On the other hand, JH642 and168 Bo colonies remained flat with a smooth surface, although they were also larger than those developed on MSgg (Figs. 1 and S1). The biofilm colonies grown on LB medium with additional manganese and carbon sources (glycerol and glucose) showed an improvement in the development of wrinkles. 3610 showed an increased formation of wrinkles accompanied by a slightly smaller colony size (15– 17 mm), while most 168 variants produced large colonies that even surpassed those of 3610, especially when the medium was supplemented with glucose. On LB-Glu medium, the 168 variants developed opaque colonies that appeared indistinguishable from those of 3610 on MSgg, which suggests that abundance of glucose might be sufficient to overcome the deficiencies observed while developing on MSgg. Strikingly, this is not the case for glycerol, which is the carbon source normally available in MSgg. The colonies developed on LB-Gly, although larger than those on MSgg, still showed reduced complexity with brighter surfaces, as shown before for stock 168 Je (376).

We tested the development of pellicle biofilms by all 168 variants and 3610 using common biofilm media 2×SG and MSgg after 72 h (Figs. 1 and S1). MSgg promotes the formation of densely wrinkled pellicles by 3610, while most 168 variants form thin and flimsy pellicles (e.g. 168 Ma, Pa and To). The most fragile of these pellicles even collapse and sink to the bottom of the well (e.g. 168 Bo, NC, Lj, Gö and JH642). Notable exceptions are the pellicles developed by 168 P1 and P2 strains, which develop thick, wrinkled pellicles similar to those of 3610. Importantly, 2×SG medium promotes the appearance of dense floating biofilm in most 168 variants, except in those that form fragile or collapsed pellicles in MSgg (i.e. 168 Mü, Lj, Gö, NC, Bo and JH642) (Figs. 1 and S1). In sum, these experiments suggest that certain variants of *B. subtilis* 168 do develop complex structures on biofilm-promoting, nutrient-rich medium.

<u>Lack of wrinkle formation of complex colony biofilms does not depend exclusively on expression of epsA-O and tapA-sipW-tasA operons.</u>

Biofilm formation by *B. subtilis* has been associated with the development of architecturally complex communities. In this regard, the most recognizable visual phenotype is the development of wrinkles or folds, in both colonies and pellicles (91, 92). As the production of EPS is closely associated with biofilm development in diverse species (45), we investigated whether the relative expression of the *epsA-O* operon correlates with the appearance of architecturally complex communities. Specifically, we expected that the expression levels from a P_{eps}-GFP reporter fusion would be higher in those strains and under conditions that efficiently develop opaque wrinkles, as compared to those that remain flat and featureless.

To this end, we monitored the levels of green fluorescence during the development of complex colonies of 168 variants on 2×SG. We selected this medium because the biofilm structures showed a wide range of complexity from the highly wrinkled 3610 reference strain towards the flat and featureless JH642. Surprisingly, we did not observe a correlation between the measured fluorescence levels and the appearance of complex structures in the colonies (Figs. 2 and S2).

The expression levels of the reporter fusion increased in all 168 variants over time, especially after the onset of stationary phase when colony size extension lessened (around 24 h). However, differences in fluorescence expression appeared without regard to colony wrinkleability. 3610, for example, developed typically complex colonies while maintaining low fluorescence throughout colony development. An inverse correlation was not evident either; JH642 displayed flat, featureless colonies but its fluorescence levels were inferior to those shown by 168 Ma and 168 Br. This result suggests that the expression of the *epsA-O* operon is not directly related to the development of wrinkles during complex colony biofilm formation.

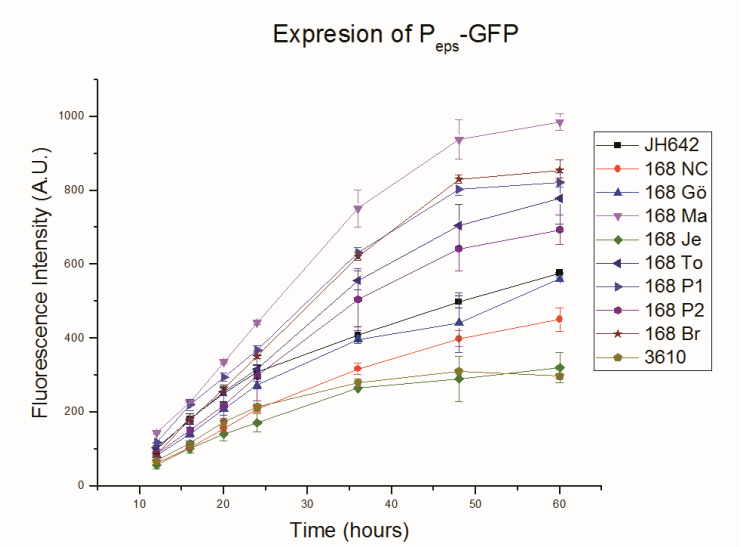

Figure 2: Fluorescence expression profiles of colonies of *B. subtilis* strain 3610 and 168 variants carrying a transcriptional P_{eps}- GFP fusion. Strains were grown on 2×SG medium and fluorescence was determined at regular intervals as described in Methods. Data points represent the average of three independent colonies. Error bars represent SD. Strain abbreviations are described in Table 1.

To further test the involvement of other extracellular matrix components over the formation of wrinkles, we measured the expression levels of a P_{tapA}-GFP reporter fusion in the 168 variants. The *tapA-sipW-tasA* operon encodes the protein component of *B. subtilis* biofilms (116). *B. subtilis* strains that lack both the *epsA-O* and *tapA-sipW-tasA* operons are unable to form biofilms (116, 118, 121). We monitored the expression levels of a P_{tapA}-GFP reporter fusion during the development of complex colonies of 168 variants. As in the case of the *epsA-O* operon, 3610 developed a complex wrinkled colony while showing the lowest level of reporter fusion expression, while all 168 variants showed higher fluorescence levels without a clear correlation to wrinkle formation or colony opacity (Figs. 3 and S3).

Taken together, these results indicate that the relative expression of genes coding for the EPS and TasA protein matrix components does not directly correlate with the formation of wrinkles in complex colony biofilms of *B. subtilis*. Importantly, it is worth noting that, in both reporter strains, the expression of the corresponding reporter fusion was lowest in the undomesticated 3610 strain.

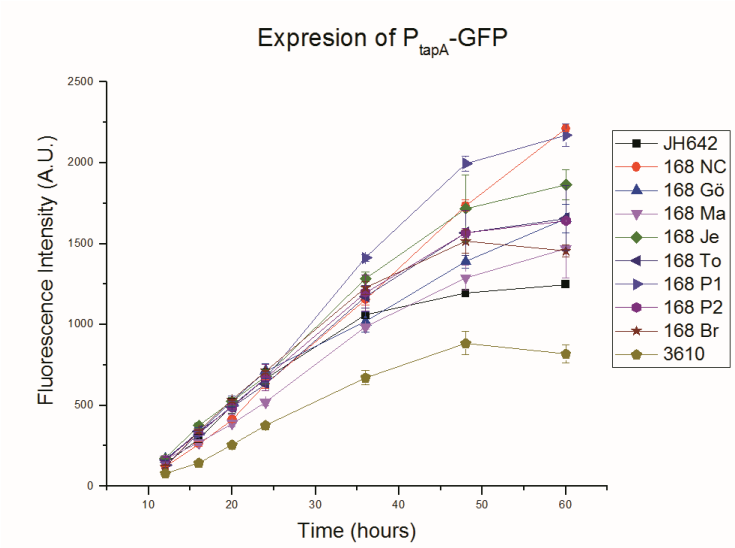

Figure 3: Fluorescence expression profiles of colonies of *B. subtilis* strain 3610 and 168 variants carrying a transcriptional P_{tapA}- GFP. Strains were grown on 2×SG medium and fluorescence was determined at regular intervals as described in Methods. Data points represent the average of three independent colonies. Error bars represent SD. Strain abbreviations are described in Table 1.

Differential biofilm formation on roots of *A. thaliana* by diverse 168 stocks.

B. subtilis is a soil bacterium known for its ability to colonize plant roots via biofilm development (67, 68). We tested whether our previous observations on colony and pellicle robustness would hold true for a root colonization assay, which is a more natural biofilm development model than colonies on agar plates. We examined the colonization of *A. thaliana* roots after 24 h incubation with selected 168 stocks. The stocks used are representative of the diverse colony morphologies previously observed, and were labelled with a $P_{hyperspank}$-GFP reporter fusion in order to detect the bacterial cells using fluorescence microscopy analysis. We observed that the ability to colonize *A. thaliana* roots differs greatly among the 168 stocks tested. Importantly, there is a correlation between a stock's ability to form architecturally complex colonies and root colonization by biofilm development. The non-domesticated isolate 3610 readily colonized the roots over 24 h by developing a large, multi-layered biofilm (Fig. 4a). In contrast, stocks 168Bo, Gö and Lj were barely present on the root surface, forming small, mono-layered attachments (Fig. 4b–d). These stocks are all poor biofilm formers on agar plates. On the other hand, stocks 168 Je and P1 colonized the root surface in a similar fashion as 3610, by forming large biofilms with multiple cell layers (Fig. 4e, f).

To quantitatively strengthen our observations, we estimated the attached bacteria per area of the root surface by measuring the fluorescent intensities originating from the bacterial cells. Using this approach, we corroborated that the average fluorescence intensity per root area was significantly higher in those roots colonized by strain 3610 or stocks 168 Je and P1 as compared to roots colonized by stocks 168 Bo, Gö and Lj (Fig. 4g).

These experiments directly correlate pellicle and colony wrinkleability of *B. subtilis* 168 with the ability to attach and develop biofilm on the plant root surface. Additionally, this also suggests that the biofilm-proficient variants of 168 could be exploited to the study of biofilms in ecological settings.

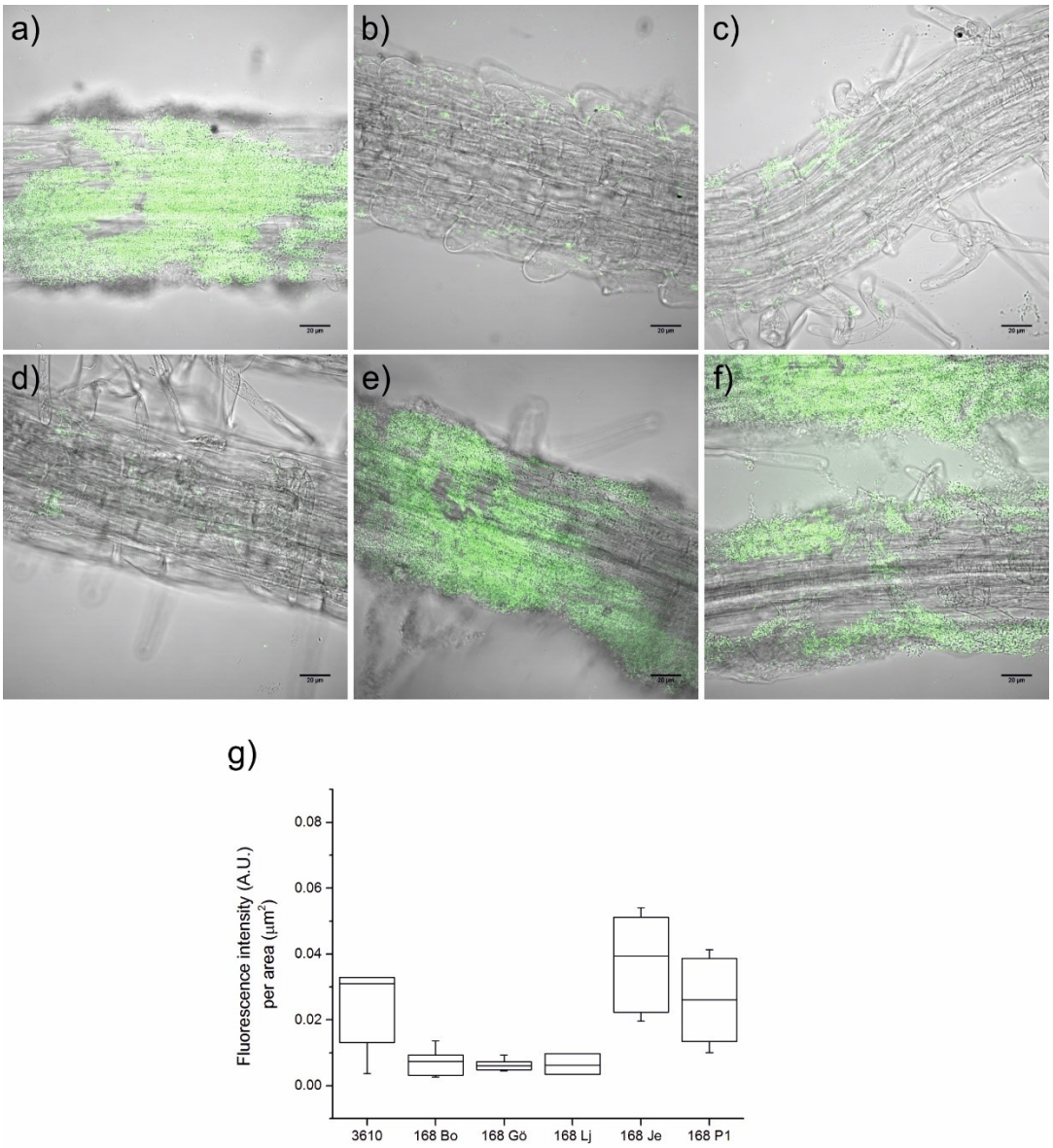

Figure 4: Colonization of *A. thaliana* roots by selected *B. subtilis* strains. Bacterial cells harbouring a $P_{hyperspank}$-GFP fusion were visualized using fluorescence (false-coloured green) attached to 10- to 12-day-old seedlings of *A. thaliana*. Images are representative of at least six independent roots. The colonization of the plant root surface by *B. subtilis* strains 3610 (a), 168 Bo (b), 168 Gö (c), 168 Lj (d), 168 Je (e) and 168 P1 (f) was quantified as described in Methods and the fluorescent area covered is presented using box plots (g) from at least three scanned areas of three independent roots. Strain abbreviations are described in Table 1.

B. subtilis 168 stocks with a point mutation in *epsC* are poor biofilm formers.

Previously, it was reported that a single nucleotide mutation (C to T) at base pair 827 of the *epsC* gene of domesticated *B. subtilis* variants was partially responsible for deficiencies in biofilm development (115). This mutation is responsible for a change from alanine codon 276 (GCG) to a valine codon (GTG), which possibly impairs EPS production. We sequenced a fragment of the *epsC* gene from all the 168 stocks studied in order to investigate whether a consistent relationship exists between the presence of this mutation and deficiencies in biofilm development. We found that all 168 stocks that develop large and flat colonies on 2×SG agar show the C-to-T base substitution in *epsC* (Fig. 5). Among these stocks are 168 Bo, Gö and Lj, which show feeble root colonization. Conversely, stocks that develop architecturally complex colonies on rich media

and show efficient root colonization possess the wild-type allele of *epsC* (Fig. 5). The only exception to this trend is 168 Pa, which has the C-to-T mutation but develops architecturally complex colonies on rich media and robust pellicles. 168 Pa was obtained from a 168 parental strain transformed with genomic DNA to repair the tryptophan auxotrophy (Table 1), and *epsC* may have reverted to its wild-type allele during this process while leaving other loci mutated similar to the 168 strains that develop complex wrinkles.

Figure 5: Allelic variation of the *epsC* gene in *B. subtilis* stocks. Alignment of re-sequenced *epsC* gene between base pairs 817 and 837 in all studied *B. subtilis* strains and variants; coded amino acids are indicated above the triplet sequences. The nucleotide at position 827 denoted by a black box for those sequences that present the mutated C-to-T allele. The sequences are arranged by the corresponding phenotype of increasing colony wrinkleability on 2×SG medium. Strain abbreviations are described in Table 1.

Additionally, we sequenced the relevant fragments of *swrA*, *degQ* and *sfp* to further investigate previously reported gene defects that may be responsible for biofilm formation deficiencies (115). Similar to previous observation (133), all 168 variants studied and the JH642 strain were found to contain the frameshift (*swrA* or *sfp*) or point (*degQ*) mutations as compared to the corresponding wild-type alleles in 3610 (data not shown); it is thus likely that other loci are different among the various 168 stocks. These results confirm that minimal genetic differences in biofilm-related genes exist among diverse 168 stocks and can lead to drastic phenotypical differences.

Discussion.

Despite *B. subtilis* being one of the most thoroughly studied bacteria, the process by which it develops its characteristic biofilms is still not completely understood. This may be due to the fact that previous studies focused mainly on the bio-technological potential and metabolic aspects of this organism (370, 378, 379), while its development as a bacterial population has not enjoyed intense research interest until more recently. Detailed investigations on *B. subtilis* interaction with other bacterial and fungal species have been described only recently (348, 380–384). The availability of diverse strains of *B. subtilis* has also hindered biofilm formation research, leading to contrasting observations about this phenomenon. In addition, other phenotypic features, including flagellum-dependent and -independent surface spreading, called swarming and sliding, respectively, are influenced by the strain and conditions applied (160, 385, 386). Spurred by the reported differences among variants of *B. subtilis* 168 strain used in different research laboratories, we present here a thorough comparison of the biofilms developed by various stocks and compared to those of strain 3610. We note that complex, rich media, especially those

containing abundant glucose as a carbon source (2×SG and LB-Glu), enable most 168 variants to form large structured colonies. This is strikingly different from the results obtained with MSgg, a chemically defined medium commonly used for biofilm development research, where all 168 variants were unable to develop architecturally complex colonies. Interestingly, carbon-enriched LB medium shows similar observations: the addition of glucose improves the biofilm development of most 168 variants, while glycerol is unable to do so. Additionally, we observed that the same 168 variants that form robust pellicles and complex colonies are able to efficiently colonize *A. thaliana* roots by developing biofilms over the root surface. Interestingly, the medium used in this assay (MSNg) utilizes glycerol as carbon source. The presence of plant exudates may be responsible for promoting efficient biofilm formation in this medium despite the lack of glucose (68, 387). Furthermore, all 168 variants that consistently show poor biofilm formation have the same base-pair substitution in the *epsC* gene. This mutation has previously been suggested to be responsible for decreased production of EPS and impaired biofilm formation in 168 variants (115).

The development of wrinkles in *B. subtilis* biofilms is perhaps the most recognizable characteristic of this bacterium. However, the process by which these structures are formed is complex. Localized cell death was shown to determine the location of wrinkle formation (388). The channels formed below the wrinkles facilitate liquid flow toward the middle of *B. subtilis* colony biofilms, possibly facilitating nutrient and oxygen transport (44). Our experiments did not show a direct correlation between the magnitude of *epsA-O* and *tapA-sipW-tasA* expression and the formation of said wrinkles. Moreover, strain 3610 showed the lowest expression of the reporter fusion used, with no sign of expression peaks during early biofilm development, as would have perhaps been expected for an efficient biofilm former strain. It is plausible that the expression levels of *epsA-O* and *tapA-sipW-tasA* operons observed in 3610 are sufficient to allow complex colony development, while other genetic factors account for the observed differences in wrinkle formation. In such case, a feedback mechanism might exist that is responsible for increased expression of these reporter fusions in stocks that have reduced wrinkle development. Previously, it was reported that activation of sporulation in colony biofilms of 3610 is delayed in strains lacking EPS production (356). In such case, the variants with a mutated *epsC* gene would have been expected to have the highest expression levels from P_{eps}-GFP, as they have impaired production of EPS. However, these variants showed only medium expression levels of the reporter fusions, with some of the variants with wild-type alleles of *epsC* showing the highest expression levels of the reporter fusions.

To conclude, the development of biofilms by *B. subtilis* is markedly influenced by available nutrients, especially the carbon source. Due to the variation present in strain 168, we recommend that researchers determine the origin of the particular variant used in their studies. Although more research is necessary to solve the intricacies of these bacterial communities, researchers have to consider the metabolic needs of their laboratory strains before dismissing them as research models.

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CHAPTER 3

Lysinibacillus fusiformis M5 induces increased complexity in *Bacillus subtilis* 168 colony biofilms via hypoxanthine.

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In recent years, biofilms have become a central subject of research in the fields of microbiology, medicine, agriculture, or systems biology amongst others. The sociomicrobiology of multispecies biofilms, however, is still poorly understood. Here, we report a screening system that allowed us to identify soil bacteria, which induce architectural changes in biofilm colonies when cocultured with *Bacillus subtilis*. We identified the soil bacterium *Lysinibacillus fusiformis* M5 as inducer of wrinkle-formation in *B. subtilis* colonies mediated by a diffusible signaling molecule. This compound was isolated by bioassay-guided chromatographic fractionation. The elicitor was identified to be the purine hypoxanthine using mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. We show that the induction of wrinkle formation by hypoxanthine is not dependent on signal recognition by the histidine kinases KinA, KinB, KinC, and KinD, which are generally involved in phosphorylation of the master regulator Spo0A. Likewise, we show that hypoxanthine signaling does not induce the expression of biofilm-matrix related operons *epsA-O* and *tasA-sipW-tapA*. Finally, we demonstrate that the purine permease PbuO, but not PbuG, is necessary for hypoxanthine to induce an increase in wrinkle formation of *B. subtilis* biofilm colonies. Our results suggest that hypoxanthine-stimulated wrinkle development is not due to a direct induction of biofilm-related gene expression, but rather caused by the excess of hypoxanthine within *B. subtilis* cells, which may lead to cell stress and death.

Biofilms are a bacterial lifestyle with high relevance regarding diverse human activities. Biofilms can be favorable, for instance in crop protection. In nature, biofilms are commonly found as multispecies communities displaying complex social behaviors and characteristics. The study of interspecies interactions will thus lead to a better understanding and use of biofilms as they occur outside laboratory conditions. Here, we present a screening method suitable for the identification of multispecies interactions, and showcase *L. fusiformis* as a soil bacterium that is able to live alongside *B. subtilis* and modify the architecture of its biofilms.

Introduction.

Biofilms are microbial populations formed by cells living in high density communities attached to biotic or abiotic surfaces. These cells are often encased in a matrix of polymeric substances that provide the whole population with an increased resistance against environmental stress (20, 46). Furthermore, these communities exhibit highly complex structural organization and social behavior. Thus, biofilms have become an increasingly studied research subject by microbiologists, especially when it became apparent that this lifestyle is widely spread among bacteria and involved in a multitude of biological processes (12, 389). Although much attention has been given to medically relevant biofilms (306, 390), scientists have also studied biofilms in the context of industrial applications (75), bioremediation (391), and crop protection (392).

In nature, biofilms rarely occur as single-species populations, but rather as mixed communities of diverse bacteria and other microorganisms. This leads to complex interactions between the different members of the community, usually involving communication networks based on chemical signals (168). Additionally, microorganisms need to sense and efficiently adapt to a wide array of environmental cues in order to efficiently regulate biofilm formation (358).

Bacillus subtilis is a soil-dwelling Gram-positive bacterium that has become a model for biofilm research. On agar plates, *B. subtilis* can form large colonies with remarkably complex architecture, while in liquid medium it forms robust floating biofilms known as pellicles. Both forms of biofilms are characterized by a wrinkled surface, which has been associated to the production of exopolysaccharides, biofilm maturation, and mechanical forces concomitant with an increased population complexity (44, 388, 393). Moreover, these biofilms display intricate cell heterogeneity, i.e. some cells become matrix producers, while others either produce exoenzymes to harvest nutrients, or form resistant structures known as spores (54, 89). The development of this population heterogeneity is regulated by a complex gene regulatory network involving various sensing kinases i.e. Kin kinases, DegS, and ComP, and their concomitant response regulators: Spo0A, DegU, and ComA respectively; and other downstream regulators, e.g. SinI and SinR (89, 112, 321).

Biofilms produced by *B. subtilis* are not only a good research model, they are also currently applied in crop protection (67, 93), and spores of this organism are readily commercialized as a biocontrol agent for agriculture. *B. subtilis* is a prolific producer of secondary metabolites and many potent antimicrobial compounds inhibiting both bacteria and fungi have been identified (87, 394, 395). In addition, it has also been shown that *B. subtilis* activates biofilm-related gene expression in response to chemicals produced by other bacteria closely related to it, for instance by other members of the Bacillus genus (348). Interestingly, the signaling role of the molecules can be independent from other effects of the compounds, as in the case of antimicrobial thiazolyl peptides, which can induce biofilm-matrix production in *B. subtilis* even when separated from their antibiotic activity (396). Moreover, other organisms such as *Pseudomonas protegens*, are able to inhibit cell differentiation and biofilm gene expression in *B. subtilis*, possibly as a competition strategy during root colonization (382).

B. subtilis successfully inhabits a congested and competitive ecological niche (63, 397, 398), and it is to be expected that this organism has a finely tuned regulatory network governing community behavior. Therefore, further study of the signaling mechanisms that influence *B.*

subtilis biofilm formation may enhance the use of this organism, both in biotechnological applications as well as a research model. However, the identification of signals that induce biofilm formation is a poorly investigated field of study, possibly due to the greater general interest in the removal of biofilms in various medical and industrial settings (390, 399–401). Thus, we have established a co-cultivation-based screening method to identify signaling molecules that promote the development of wrinkles in colony biofilms of *B. subtilis*. Using this system, we identified ecologically relevant soil bacteria that are able to induce the formation of large wrinkles in colony biofilms of *B. subtilis*. The majority of these bacteria are members of the family *Bacillaceae*, to which *B. subtilis* belongs. The strain with the clearest wrinkle-inducing effect was identified as *Lysinibacillus fusiformis* M5. The observed effect on *B. subtilis* is dependent on a diffusible signaling molecule, which was identified as hypoxanthine using bioassay-guided fractionation and subsequent structure elucidation using various spectroscopic and spectrometric methods. The induction of wrinkles by hypoxanthine was not dependent on Kin kinases signal transduction, and the expression levels of operons responsible for the production of biofilm matrix components, *epsA-O* and *tapA-sipW-tasA*, remained unaffected. We show that uptake of hypoxanthine by permease PbuO is necessary for the increased induction of wrinkle formation in *B. subtilis* biofilm colonies. We therefore suggest that hypoxanthine induces the formation of wrinkles by introducing a metabolic change in *B. subtilis* cells, rather than by direct stimulation of biofilm-related gene expression.

Material and Methods.

Strains, media, and general culture conditions.

All strains used in this study are listed in Table 1. When fresh cultures were needed, these strains were pre-grown overnight in Lysogeny broth medium (LB-Lennox, Carl Roth; 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 5 g L^{-1} NaCl) at 37°C and shaken at 225 r.p.m. LB medium was used for all *B. subtilis* and *Escherichia coli* transformations, and to screen soil samples. 2×SG medium (16 g L^{-1} nutrient broth (Difco), 2 g L^{-1} KCl, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2 \cdot 4H2O$, 1 µM $FeSO_4$, and 0.1% glucose) (92) was used to grow cultures intended for supernatant production. This medium was also used for all strain interaction assays and wrinkle-induction assays. Tryptone Soya broth (CASO-Bouillon, AppliChem; 2.5 g L^{-1} glucose, 5 g L^{-1} NaCl, 2.5 g L^{-1} buffers (pH 7.3), 3 g L^{-1} soya peptone, and 17 g L^{-1} tryptone) was used for screening soil samples. GCHE medium (1% glucose, 0.2% glutamate, 100 mM potassium phosphate buffer (pH: 7), 3 mM trisodium citrate, 3 mM $MgSO_4$, 22 mg L^{-1} ferric ammonium citrate, 50 mg L^{-1} L-tryptophan, and 0.1% casein hydrolysate) was used to induce natural competence in *B. subtilis* (373). Our developed Gallegos Rich medium was used to grow *Lactococcus lactis* MG1363, in order to purify pMH66: 21 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 8.3 g L^{-1} NaCl, 3 g L^{-1} soya peptone, 2.6 g L^{-1} glucose, and 2.5 g L^{-1} $MgSO_4 \cdot 7H_2O$. Overnight cultures of *L. lactis* were incubated at 30°C without shaking. Media were supplemented with Bacto agar 1.5 % when solid plates were needed. Antibiotics were used at the following final concentrations: kanamycin, 10 µg mL^{-1}; chloramphenicol, 5 µg mL^{-1}; erythromycin-lincomycin, 0.5 µg mL^{-1} and 12.5 µg mL^{-1} respectively; ampicillin, 100 µg mL^{-1}; spectinomycin, 100 µg mL^{-1}; tetracycline, 10 µg mL^{-1}. Specific growth conditions are described in the corresponding methods section.

Importantly, all 2×SG plates used in this study were prepared with 25 mL of medium, and dried for a minimum of 20 minutes before use. Insufficient drying resulted in excessive colony expansion without development of architecturally complex colonies. To dry the plates, they were first

allowed to solidify at room temperature for 1 hour, afterwards, they were kept completely open in a laminar flow sterile bench for the duration of the drying period. These drying conditions were followed for all assays that examined changes in colony architecture.

Table 1: Strains and plasmids used in this study.

Strain	Characteristics	Reference
B. subtilis		
168	168 1A700 *trpC*. Jena Stock	(96)
TB48	168 *trpC2 amyE*::$P_{hyperspank}$-mKATE (cat)	(372)
JH12638	JH642 *trpC2 phe*-1 *kinA*::*Tn917*(cat)	(402)
JH19980	JH642 *trpC2 phe*-1 *kinB*::*tet*	(403)
RGP0203-4	JH642 *kinC*::Sp^R	(160)
DL153	NCIB 3610 *kinD*::tet	(159)
BKE06370	168 *trpC2 pbuG*::Erm^R	(404)
BKE13530	168 *trpC2 kinE*::Erm^R	(404)
BKE29990	168 *trpC2 pbuO*::Erm^R	(404)
NRS2243	NCIB 3610 *sacA*::$P_{epsA\text{-}gfp}$ (neo), *hag*::*cat*	(369)
NRS2394	NCIB 3610 *sacA*::P_{tapA}-gfp (neo)	(369)
Δeps	168 *trpC2 epsA*-O::*tet*	(405)
ΔtasA	168 *trpC2* tasA::Km^R	(405)
TB150	168 *trpC2* amyE::$P_{hyperspank}$-mKATE (*cat*) epsA-O::*tet*	This study, TB48→Δeps
TB171	168 *trpC2 amyE*::$P_{hyperspank}$-mKATE (*cat*) tasA::Km^R	This study, TB48→ΔtasA
TB812	168 *trpC2 amyE*::$P_{hyperspank}$-mKATE (*cat*) *pbuG*::Erm^R	This study, BKE06370→TB48
TB813	168 *trpC2 amyE*::$P_{hyperspank}$-mKATE (*cat*) *pbuO*::Erm^R	This study, BKE29990→TB48
TB822	168 *trpC2 amyE*::$P_{hyperspank}$-mKATE (*cat*), Δ*pbuO*	This study
TB823	168 *trpC2* amyE::$P_{hyperspank}$-mKATE (*cat*), Δ*pbuO*, pbuG::Erm^R	This study, BKE06370→TB822
TB833	168 *trpC2 amyE*::$P_{hyperspank}$-mKATE (*cat*) *kinA*::Tn917(cat)	This study, JH12638→TB48
TB834	168 *trpC2 amyE*::$P_{hyperspank}$-mKATE (cat) *kinB*::*tet*	This study, JH19980→TB48
TB835	168 *trpC2 amyE*::$P_{hyperspank}$-mKATE (*cat*) *kinC*::Sp^R	This study, RGP0203-4→TB48
TB836	168 *trpC2 amyE*::$P_{hyperspank}$-mKATE (*cat*) *kinD*::*tet*	This study, DL153→TB48
TB869	168 *trpC2 amyE*::$P_{hyperspank}$-mKATE (*cat*) sacA::$P_{epsA\text{-}gfp}$ (neo)	This study, NRS2243→TB48
TB870	168 *trpC2 amyE*::$P_{hyperspank}$-mKATE (*cat*) sacA::P_{tapA}-gfp (neo)	This study, NRS2394→TB48
TB911	168 *trpC2 amyE*::$P_{hyperspank}$-mKATE (*cat*) *kinE*::Erm^R	This study, BKE013530→TB48
E. coli		
MC1061	Cloning host; K-12 F^- λ^- Δ(ara-leu)7697 [araD139]B/r Δ(codB-lacI)3 galK16 galE15 e14$^-$ mcrA0 relA1 rpsL150(Str^R) spoT1 mcrB1 hsdR2(r^- m^+)	(406)

Soil Isolates		
Lysinibacillus sp. M2c		This study
Lysinibacillus fusiformis M5		This study
Bacillus pumilus P22a		This study
Acinetobacter variabilis T7a		This study
Plasmid	**Characteristics**	**Reference**
pMH66	pNZ124-based Cre-encoding plasmid, Tet^R Ts	(407)

Strain construction.

All *B. subtilis* strains generated in this work were obtained via natural competence transformation (373) using genomic or plasmid DNA from donor strains as indicated in Table 1. Briefly, overnight cultures of the receiver strains were diluted to a 1:50 ratio with GCHE medium, these cultures were incubated at 37°C for 4 h with shaking at 225 r.p.m. After this incubation period, 5–10 µg of genomic or plasmid DNA were mixed with 500 µL of competent cells and further incubated for 2 h before plating on LB plates added with selection antibiotics. Strain TB822 was obtained by using the Cre recombinase expressed from plasmid pMH66 to eliminate the Erm^R cassette of TB813, and subsequently curating pMH66 via thermal loss of the plasmid (408). Briefly, TB813 was transformed with 10 µg of pMH66, selecting transformants via incubation at 37°C on LB plates added with tetracycline. Candidates were then screened for their capacity to grow at 37°C on LB plates added with macrolide antibiotics (erythromycin-lincomycin), those that were not able to grow were further incubated on LB plates at 43°C for 18 h to induce the loss of pMH66. Candidates that were then unable to grow at 37°C on LB plates added with tetracycline were considered to have lost pMH66.

Successful construction of all used strains and plasmids was validated via PCR and restriction pattern analysis using standard molecular biology techniques, and by the lack of amylase activity on 1% starch LB plates (374) and emission of red fluorescence. All PCR primers used in this study are listed in Table 2. Primer pairs were used to amplify the indicated loci (see Table 2) in order to confirm the proper mutation of the corresponding gene. To confirm the correct construction of strains TB869 and TB870, primer oGFPrev2 was used in combination with oRGM38 (for *sacA*::P_{tapA}-*gfp*) or oRGM40 (for *sacA*::P_{epsA}-*gfp*).

Table 2: PCR primers used in this study.

Primer	**Sequence (5´ → 3´)**	**Target locus**
oGFPrev2	TTGTGCCCATTAACATCACC	*gfp*
oRGM110	GGAATCCGCGCCGTTACATC	*pbuG*
oRGM111	CAGCCCATATAGCAAAGACC	*pbuG*
oRGM116	GCGGTGCGGAATAAGTAAAG	*pbuO*
oRGM117	TACTGAGCGGCACTTGCTTG	*pbuO*
oRGM130	TATCCACGCCTACGCAGAGC	*kinA*
oRGM131	CTCAATGGACACGCTGAGAG	*kinA*
oRGM132	GAAGACCAGCAAGCAAATCG	*kinD*
oRGM133	GCGGCTGATCGCCTTTATGG	*kinD*
oRGM38	GAGAATTCGTGGTGCCAAAGACGAGAAG	*tapA* promotor
oRGM40	GAGAATTCCCAGCTGATTAATAGAATAG	*epsA* promotor
oTB55	CATGGGATCCTGGCGGAGAAGGATTTATG	*kinB*
oTB56	CACGGAATTCTGTCTCAAACGTGCTCATC	*kinB*

oTB61	CATGGGATCCATTACGCTAAGCCCTGAG	*kinC*
oTB62	CACGGAATTCTTGTGCCAGCAAATGATG	*kinC*
oTB237	TGACGGTAAGGATCGTAG	*kinE*
oTB238	GTTTCGGCTGTCGTATAG	*kinE*
27F	AGAGTTTGATCMTGGCTCAG	16S rRNA
1492R	TACGGYTACCTTGTTACGACTT	16S rRNA

Isolation of bacteria from soil samples.

Two independent Mexican soil samples were screened to isolate bacteria able to grow on LB or tryptone soya broth media. The first sample was collected from Tepoztlán, Morelos (18° 59′ 7″ N, 99° 5′ 59″ W), a humid and verdant region of central Mexico. The second sample was collected from Tehuacán, Puebla (18° 25′ 47.71″ N, 97° 27′ 58.1″ W), a semidesertic dry region in east central Mexico. Both samples were collected with a clean metal spatula 5 cm below surface level, and at 15 to 20 cm of the roots of local trees.

1 g of each soil sample was suspended in 9 mL of a sterile 0.85% NaCl solution, and 50 µL of Tween 80 were added. The resulting suspensions were vortexed for 5 minutes at maximum speed. The bigger soil particles were allowed to sediment by keeping the suspensions still for 10 minutes. The supernatants were then diluted with sterile 0.85% NaCl to 1:1000, 1:10 000 and 1:100 000 ratios. 50 and 100 µL of these dilutions were spread on LB and tryptone soya broth agar plates. These plates were incubated at 30°C for a maximum of 5 days. Bacterial colonies that grew during the incubation period were further isolated by cross-streaking them on LB or tryptone soya broth plates and incubating them at 30°C for 48 hours. Single isolated colonies obtained from this secondary cultivation were used to prepare liquid cultures on 3 mL of LB media. These cultures were incubated at 30°C with shaking for a maximum of 48 hours. Bacteria that grew efficiently during this incubation period were used to prepare glycerol stock solutions (20% v/v) and stored at -80°C for further use. In total, 242 soil strains were obtained and subsequently tested.

Soil strains interaction screening.

Overnight liquid cultures of *B. subtilis* strain TB48 and the obtained soil strains were adjusted to OD_{600} 0.2 using LB medium. These diluted cultures were then mixed in 1:1, 10:1, and 1:10 ratios, and 2 µL of the mixed and pure cultures were inoculated on 2×SG plates. For neighbor colonies interaction assay, 2 µL of the pure cultures were inoculated at a distance of 5 mm from each other. Plates were incubated at 30°C for 72 h. The obtained colonies were used for microscopy analysis without further treatment.

Identification of soil strains.

Genomic DNA of selected soil strains was extracted with the GeneMATRIX Bacterial and Yeast Genomic DNA Purification Kit, according to the manufacturer's instructions (EURx, Poland). This DNA was used to PCR amplify a fragment of the 16S rRNA gene using primers 27F and 1492R (409). Amplicons were purified with the High Pure PCR Product Purification Kit according to the manufacturer's instructions (Roche Diagnostics, Switzerland) and sequenced with primers 27F and 1492R (GATC Biotech, Germany). Sequencing results were then compared with sequences in the National Center for Biotechnology Information (16S ribosomal RNA Bacteria and Archaea database) using the BLASTn algorithm (410). Soil strains' identities were established using minimum query coverage of 98% and minimum identity values of 99%.

Purification and treatment conditions of soil strain supernatant.

To obtain cell-free supernatants of selected soil strains, 10 mL cultures on 2×SG medium of the corresponding strains were incubated at 30°C for 24 hours with shaking at 100 r.p.m. These cultures were then centrifuged at 7000 r.c.f. for 15 min. The supernatants were collected and filter-sterilized using a 0.22 µm pore-size filter (Carl Roth, Germany).

Colony wrinkle formation assay.

We developed the following assay to assess the effect that the supernatants of soil strains, and compound solutions, may have upon the architectural complexity of *B. subtilis* colony biofilms. Sterile 12 mm-diameter cotton discs were placed on 90 mm-diameter 2×SG agar plates, in such a way that there is a maximum amount of available space among the discs themselves and between the discs and the border of the plates. 3 cotton discs were used per plate. 50 µL of the tested supernatant or compound solution were deposited on the center of the cotton discs, and the plates were dried for 3 min by keeping them completely open in a laminar flow sterile bench. This drying period was done in addition to the regular 20 min drying previously described. 2 µL of an overnight culture of the tested strains were then inoculated at 5 mm from the edge of the cotton discs. The plates were incubated at 30°C for a total of 72 hours. Every 24 hours the cotton discs were reimpregnated with 25 µL of the corresponding supernatant or compound solution. After the incubation period, the plates were used for microscopy analysis without further treatment.

Bioassay-guided fractionation.

A 50 mL culture of *L. fusiformis* M5 isolate was grown for 24 h under standard conditions. Bacterial cells were pelleted by centrifugation for 10 min at 6.000 r.c.f. and the supernatant was filtered through a 0.2 µm filter. 25 mL of the supernatant were freeze-dried and the remaining foam was dissolved in 1 mL water. The solution was applied to a Sephadex G20 column (3 cm × 40 cm) and eluted with water collecting 3 mL fractions (50 fractions). Fractions were further analyzed using the colony wrinkle formation assay. The fraction with the largest activity was analyzed using an LCMS (Shimadzu Deutschland, Germany) equipped with a Hypercarb column (100 × 3 mm, 3 µM, Thermo Fisher Scientific, flow rate = 0.6 mL min^{-1}, method: 0-10 min: 100% (v/v) water). The main compound of this fraction was purified using a semi-preparative HPLC (Shimadzu Deutschland, Germany) equipped with Hypercarb column (100 × 10 mm, 5 µM, Thermo Fisher Scientific, flow rate = 5 mL min^{-1}, method = 0-20 min: 100% (v/v) water). The pure compound was analyzed using ^{1}H-NMR spectroscopy, LCMS, and HR-ESIMS. Obtained analytical data were in good agreement with a hypoxanthine standard from Sigma Aldrich.

Comparison of hypoxanthine production.

Supernatant of different isolates were heated to 80°C for 15 min and filtered through a 0.2 µm syringe filter. The samples were analyzed using an LCMS (Shimadzu Deutschland, Germany) equipped with a Hypercarb column (100 × 3 mm, 3 µM, Thermo Fisher Scientific, flow rate = 0.6 mL min^{-1}, method: 0-10 min: 100% (v/v) water).

Cell death assessment.

To visualize cell death in colony biofilms we performed the colony wrinkle formation assay on plates supplemented with 0.25 µM Sytox Green nucleic acid stain (Thermo Fisher Scientific, U.S.A.). After 72 h of incubation, sectors of the colonies that grow around the cotton discs were manually sliced with a scalpel to produce thin cross-sections that include the supporting agar

and a sliver of colony biofilm. The cross-sections were placed on a glass slide and used for microscopy without further treatment. All transmitted light and fluorescence images of colony biofilm cross-sections were obtained with an Axio Observer 780 Laser Scanning Confocal Microscope (Carl Zeiss, Germany) equipped with an EC Plan-Neofluor 10x/0.30 M27 objective, an argon laser for stimulation of fluorescence (excitation at 488 nm for green fluorescence and at 561 nm for red fluorescence, with emissions at 528/26 nm and 630/32 nm respectively), a halogen HAL-100 lamp for transmitted light microscopy and an AxioCam MRc color camera (Carl Zeiss).

Stereomicroscopy and Image Analysis.

All bright-field and fluorescence images of colonies were obtained with an Axio Zoom V16 stereomicroscope (Carl Zeiss, Germany) equipped with a Zeiss CL 9000 LED light source, a PlanApo Z 0.5× objective, HE 38 eGFP filter set (excitation at 470/40 nm and emission at 525/50 nm), HE 63 mRFP filter set (excitation at 572/25 nm and emission at 629/62 nm), and AxioCam MRm monochrome camera (Carl Zeiss, Germany).

Images were obtained with exposure times of 20 ms for bright-field, and 2500 ms for red fluorescence or 3000 ms for green fluorescence when needed. For clarity purposes, the images of colonies are presented here with adjusted contrast and the background has been removed, so that the colony structures can be easily appreciated. The modified pictures were not used for any fluorescence measurements.

To assess the expression levels of P_{epsA}-*gfp*, P_{tapA}-*gfp*, and $P_{hyperspank}$-*mKATE* fluorescent reporter fusions in colonies we used ImageJ (National Institutes of Health, USA). Briefly, the average fluorescence emission intensities were measured in the green fluorescence channel for GFP and red fluorescence channel for mKATE by using a region of interest (ROI) that surrounds the cotton discs in the pictures as a partial ring, taking care to avoid the disc area itself. The ROI was drawn in such a way that it avoids the region of the colony that first makes contact with the cotton discs. This ROI had a width of 0.5 mm for measurements done at 40 and 50 hours, and a width of 1 mm for measurements done at 65 hours. The ROI was positioned in each colony image using the bright-field channel, and the average fluorescence intensity was then measured on the corresponding green and red fluorescence images.

To assess cell death, the average green fluorescence intensity of the cross-sections of colonies treated with Sytox Green was measured. All measurements were done with ImageJ. The colony area was selected on the transmitted light channel of cross-section images using the tracing tool (Legacy mode, tolerance 60). The average fluorescence intensity was then measured in the corresponding areas of the green and red fluorescence channels.

Nucleotide sequence accession numbers.

Sequences used in this study have been deposited in GenBank under accession numbers KY705015 (*Lysinibacillus* sp. M2c), KY698015 (*Bacillus pumilus* P22a), and KY703395 (*Acinetobacter variabilis* T7a). Further, the draft genome sequence of *L. fusiformis* M5 is available in GenBank under accession number MECQ00000000, and the strain was deposited in the Jena Microbial Resource Collection (ST-Number: ST036146, see http://www.leibniz-hki.de/en/jena-microbial-resource-collection.html).

Results.

Screening of soil bacteria that induce structural changes in colonies of *B. subtilis*.

We screened a collection of 242 strains isolated from two distinct soil sampling sites in Mexico, in order to identify bacteria that are able to induce biofilm formation or increased complex colony architecture of *B. subtilis*. Importantly, our assay aimed to discover alterations in biofilm colony architecture that was different from the previously described method that identified soil-derived microbes that induce gene expression related to biofilm formation of *B. subtilis* (348). While some *B. subtilis* strains, such as *B. subtilis* NCIB 3610, easily and spontaneously form biofilms, we used a strain that would form architecturally complex colonies only in the presence of specific inducers or nutrient rich conditions. Thus, even weak biofilm-inducing effects would not be overseen. We used *B. subtilis* 168 (Jena stock), a strain that can only form architecturally complex colonies when grown on glucose-rich medium or exposed to signaling molecules as those present in plant root exudates (96). The strain of *B. subtilis* used for the assay (TB48) carried a $P_{hyperspank}$-*mKATE* reporter fusion in order to facilitate the identification of *B. subtilis* from the soil strains in mixed colonies. The reporter strain was mixed in different ratios with the bacterial isolates and allowed to form colonies on 2×SG medium for 72 hours. Single strain colonies of *B. subtilis* and the soil-derived isolates were also grown as neighboring colonies under the same conditions, inoculated with a spatial distance of 5 mm between each other to examine their interactions. The majority of these interactions resulted in the apparent killing of one strain by the other, producing a colony identical to the pure culture colony of the surviving partner (data not shown). However, 36 soil strains were able to grow alongside *B. subtilis*, mainly by creating a colony where the strains segregate in sectors. Interestingly, the *B. subtilis* sectors of these mixed colonies showed an increased architectural complexity by forming large wrinkles and a rugose colony surface, compared to its pure colonies, which remained flat (Fig. 1).

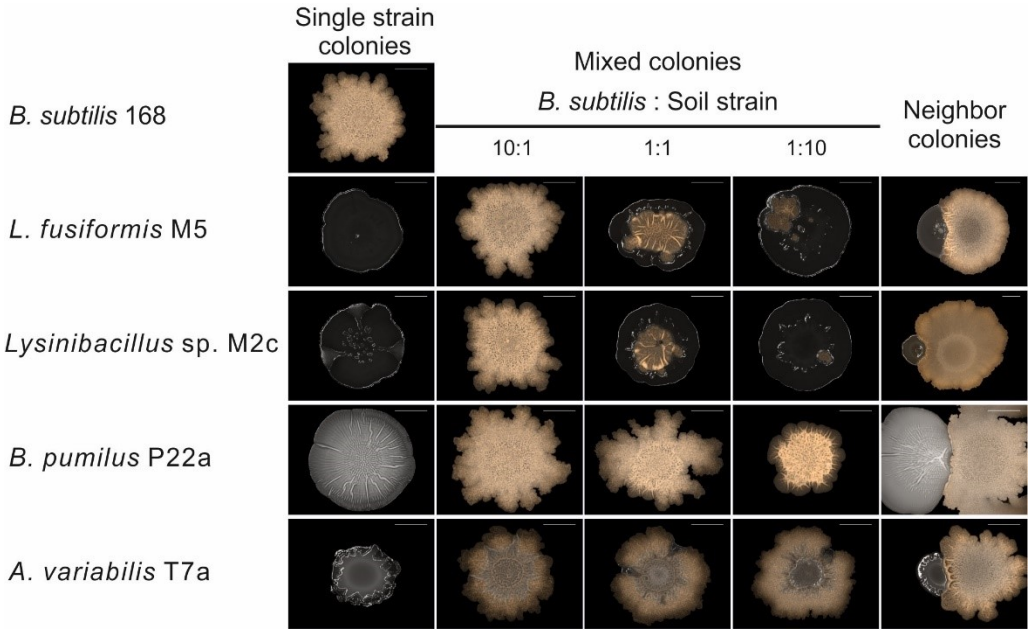

Figure 1: Single strain and mixed colonies of *B. subtilis* and selected soil strains. *B. subtilis* was differentiated from the soil strains using fluorescence emission (false-colored orange) from a reporter expressed by the $P_{hyperspank}$-mKATE construct. Colonies are shown after 72 h of incubation. Neighbor colonies were inoculated at 5 mm from each other. Scale bars represent 5 mm.

Furthermore, when single strain colonies of these soil-derived isolates were grown close to *B. subtilis* colonies, the induction of wrinkle formation could be observed in the areas of the *B. subtilis* colony that are closest to the soil strain, but not in the other regions of the colony (Fig. 1). These results suggest that the aforementioned bacteria produce signals that can induce an increased architectural complexity in *B. subtilis* colony biofilms.

Structural changes in *B. subtilis* colonies are induced by diffusible signals produced by soil bacteria.

In order to elucidate if the observed induction of wrinkle formation is caused by a diffusible signal molecule or due to direct cell-cell interactions, we designed an assay to test the cell-free supernatants of the selected soil strains. In this assay, we used cotton discs infused with cell-free supernatant to simulate colonies of the tested soil strains. *B. subtilis* was inoculated at a distance of 5 mm next to the cotton discs and allowed to grow for 72 hours. Over this period, the growing colony surrounded the cotton discs, coming into contact with the freely diffusing compounds in the supernatants.

Using this assay, we observed that the cell-free supernatants of four bacterial isolates were able to induce efficiently the formation of wrinkles in the adjacent *B. subtilis* colony. Importantly, this phenomenon was observed in the periphery of the cotton discs, but not in the areas of the colony farther away from it (Fig. 2). We note that neither the cell-free supernatant of *B. subtilis* itself, nor the medium used to obtain the supernatants, showed the capacity to induce increased wrinkle formation in *B. subtilis* colonies under our tested conditions (Fig. 2). We concluded that the induction of wrinkle formation was due to a diffusible signaling molecule produced by these soil organisms.

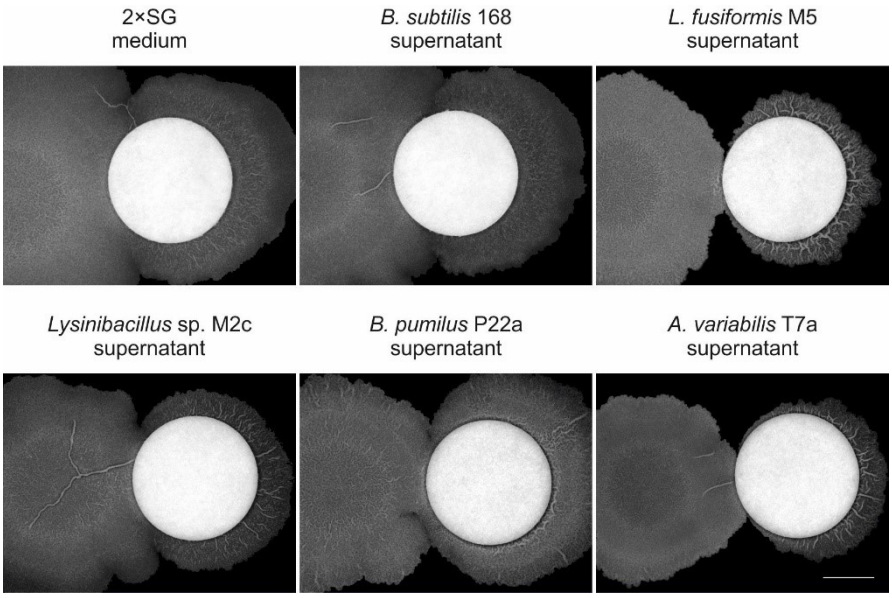

Figure 2: Effect of cell-free supernatants of soil strains on the development of *B. subtilis* 168 biofilm colonies. Colonies were inoculated with 2 µL of culture at 5 mm from white cotton discs impregnated with 50 µL of cell-free supernatant of soil bacteria or 2×SG medium. The discs were reimpregnated with 25 µl of the corresponding medium or supernatant every 24 h. Bright-field images of colonies are shown after 72 h of incubation. The scale bar represents 5 mm.

Using 16S rRNA locus sequencing, we characterized those soil strains whose supernatant could best stimulate wrinkle formation in *B. subtilis*. The majority of the sequenced strains were found to

be members of the same phylogenetic family as *B. subtilis*, such as *Bacillus pumilus* or *L. fusiformis*. The only exception was a strain identified as the γ-proteobacterium *Acinetobacter variabilis*.

Hypoxanthine identified in the supernatant is responsible for wrinkle induction.

The strongest induction of wrinkle formation (defined as the appearance of tall wrinkles and a rugose colony surface) was observed with the supernatant of the soil derived strain identified as *L. fusiformis* M5. For this reason, we decided to further investigate the respective signaling molecule produced by this bacterium using a wrinkle formation assay. Bio-assay-guided fractionation allowed us to identify a compound from *L. fusiformis* M5 that induced a similar phenotype as observed when *B. subtilis* and *L. fusiformis* M5 were co-cultured. To this end, supernatant of *L. fusiformis* M5 was lyophilized and fractionated using Sephadex G20 as stationary phase. Each fraction was applied to cotton discs and placed on an agar plate in the vicinity of *B. subtilis*. The fraction that induced wrinkle formation was sub-fractionated by high-performance liquid chromatography (HPLC) using a hypercarb column as stationary phase. Repeating this procedure led to the isolation of a homogeneous compound whose structure was subsequently elucidated via a combination of nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry (HR-MS). Finally, hypoxanthine was identified as the inducer of wrinkle formation (Fig. 3). We further validated our findings using commercial hypoxanthine, which showed the same retention time as the isolated hypoxanthine (Fig. 3).

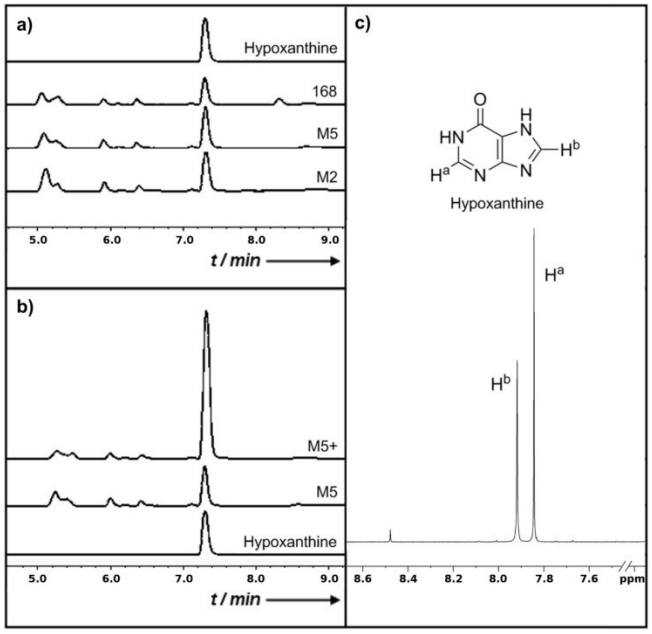

Figure 3: Characterization of hypoxanthine standard and cell-free supernatants of *B. subtilis* 168 (168), and soil isolates *L. fusiformis* M5 (M5) and *Lysinibacillus* sp. M2c (M2). a) HPLC chromatograms of cell-free supernatants compared to a standard solution of hypoxanthine. b) HPLC chromatogram of *L. fusiformis* M5 cell-free supernatant compared with a standard solution of hypoxanthine, and the *L. fusiformis* M5 cell-free supernatant spiked with a standard of hypoxanthine (M5+). c) 1H NMR spectrum of isolated hypoxanthine (DMSO-d6, 600 MHz).

We used the colony wrinkle formation assay to test if hypoxanthine (as a 25 mM solution in 0.05 N NaOH) alone can induce the formation of wrinkles in *B. subtilis* colony biofilms. In addition, guanine and xanthine were also tested using this methodology, since these purines can be found in the same metabolic pathways as hypoxanthine in *B. subtilis*. We found that hypoxanthine and guanine were able to induce the formation of tall wrinkles in *B. subtilis* colonies, while xanthine could not (Fig. 4a). Interestingly, guanine can be deaminated during

purine catabolism to produce hypoxanthine, which can then be oxidized to produce xanthine (Fig. 4b). These results suggest that a metabolite derived from guanine or hypoxanthine, but not xanthine, may be responsible for the observed formation of wrinkles.

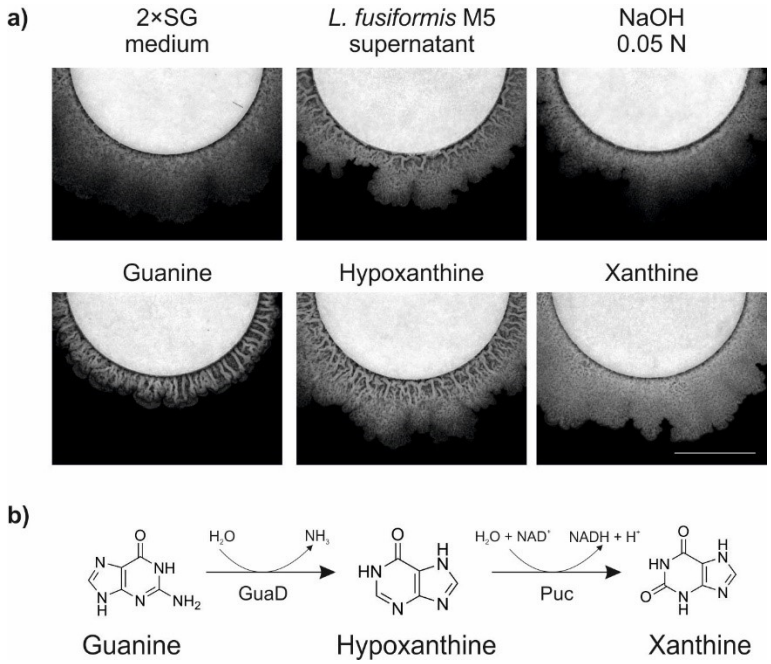

Figure 4: Effect of cell-free supernatant of *L. fusiformis* M5, guanine, hypoxanthine, and xanthine on the development of biofilm colonies of *B. subtilis* 168 (a) and catabolic pathway of guanine, hypoxanthine and xanthine (b). GuaD: guanine deaminase. Puc: hypoxanthine/xanthine dehydrogenases (PucA-E). Bright-field images of colony areas adjacent to the cotton discs are shown after 72 h of incubation. The scale bar represents 5 mm.

Hypoxanthine signaling is not mediated by the activity of individual Kin kinases.

In *B. subtilis*, the transcriptional regulator Spo0A controls the expression of several biofilm-related operons, including those responsible for the production of the exopolysaccharide and protein components of the biofilm matrix (*epsA-O* and *tapA-sipW-tasA*, respectively) (112). Five sensor kinases (KinA, KinB, KinC, KinD, and KinE) have been identified in *B. subtilis*, and four of them (KinA-D) are known to activate Spo0A via a phosphorelay depending on environmental signals (89, 122, 358). We wanted to determine if any of these kinases is involved in hypoxanthine-mediated induction of wrinkles. Therefore, we compared the effect that the supernatant of *L. fusiformis* M5 has on kinase-mutant strains of *B. subtilis* using the colony wrinkle formation assay. We expected that, should one of these kinases be responsible for sensing hypoxanthine, the corresponding mutant strain would no longer show increased induction of wrinkle formation. Interestingly, all the mutant strains were still able to develop highly wrinkled colonies when exposed to the supernatant, when compared to the corresponding colonies exposed to 2×SG medium (Fig. 5). This result suggests that hypoxanthine-mediated induction of increased architectural complexity is not mediated by the activation of Spo0A via a single Kin kinase. We note that more than one Kin kinase could be responsible for detecting hypoxanthine, in which case the deletion of a single kin kinase gene would not prevent *B. subtilis* to form wrinkled colonies when exposed to hypoxanthine.

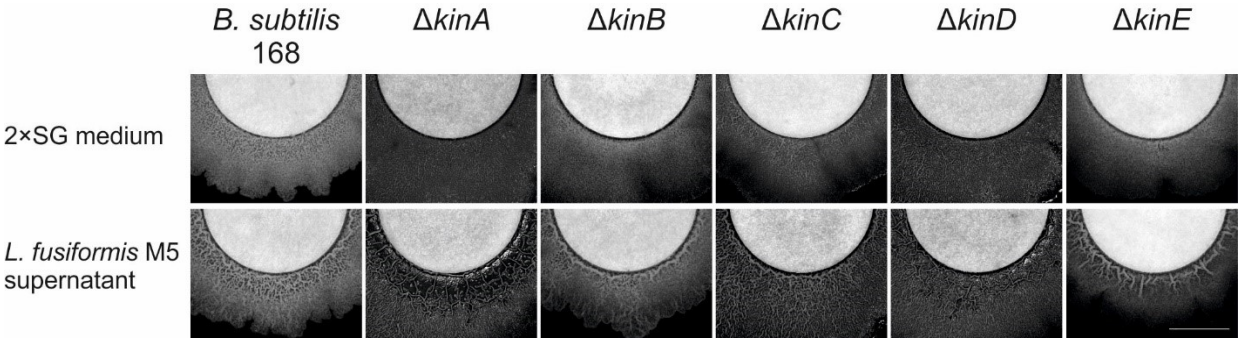

Figure 5: Effect of cell-free supernatant of *L. fusiformis* M5 on the development of biofilm colonies of *B. subtilis* 168 and knock-out mutants of kin-kinase genes. Colonies were inoculated with 2 μl of culture at 5 mm from white cotton discs impregnated with 50 μL of cell-free supernatant of *L. fusiformis* M5 or 2×SG medium. The discs were reimpregnated with 25 μL of the corresponding medium or supernatant every 24 h. Bright-field images of colony areas adjacent to the cotton discs are shown after 72 h of incubation. The scale bar represents 5 mm.

Expression levels of genes responsible for biofilm matrix production are not affected by hypoxanthine signaling.

The *epsA-O* and *tapA-sipW-tasA* operons are related to the production of the exopolysaccharide and protein components of the *B. subtilis* biofilm matrix, respectively. Changes in the expression levels of these operons are associated to a maturating biofilm, and show spatiotemporal variation during its development (54, 90, 150).

To further examine if biofilm matrix-related genes are involved in the induction of wrinkles by hypoxanthine, we monitored the expression of P_{epsA}-*gfp* and P_{tapA}-*gfp* fluorescent reporter fusions in colonies of *B. subtilis* using the colony wrinkle formation assay. Fluorescence emission was examined only in the sections of the colonies directly adjacent to the cotton discs at 3 time points: (i) when *B. subtilis* has encircled the discs (40 hours), (ii) when the colony started to expand from the disc and showed the onset of wrinkle formation (50 hours), and (iii) when the colony has developed wrinkles and expanded (65 hours). The examined strains also carried a $P_{hyperspank}$-*mKATE* reporter fusion to adjust for colony growth. Under these conditions, the expression from P_{epsA} and P_{tapA} in these colonies showed no statistical differences when exposed to cotton discs infused with 2×SG medium or supernatant of *B. subtilis*, as compared to those infused with supernatant of *L. fusiformis* M5 (one-way ANOVA: $P<0.05$, n=4-8 independent colonies) (Fig. 6).

We decided to further test if the products of the *epsA-O* and *tapA-sipW-tasA* operons are necessary for the observed development of wrinkles. We used the wrinkle formation assay to test the effect of the supernatant of *L. fusiformis* M5 on mutant strains of *B. subtilis* that are unable to produce the exopolysaccharide (Δ*epsA-O*) or protein (Δ*tasA*) matrix component. After 72 hours of incubation, the tested *B. subtilis* strains expanded and surrounded the infused cotton discs, but were unable to develop wrinkles and showed a flat and mucoid colony surface (Fig. 7).

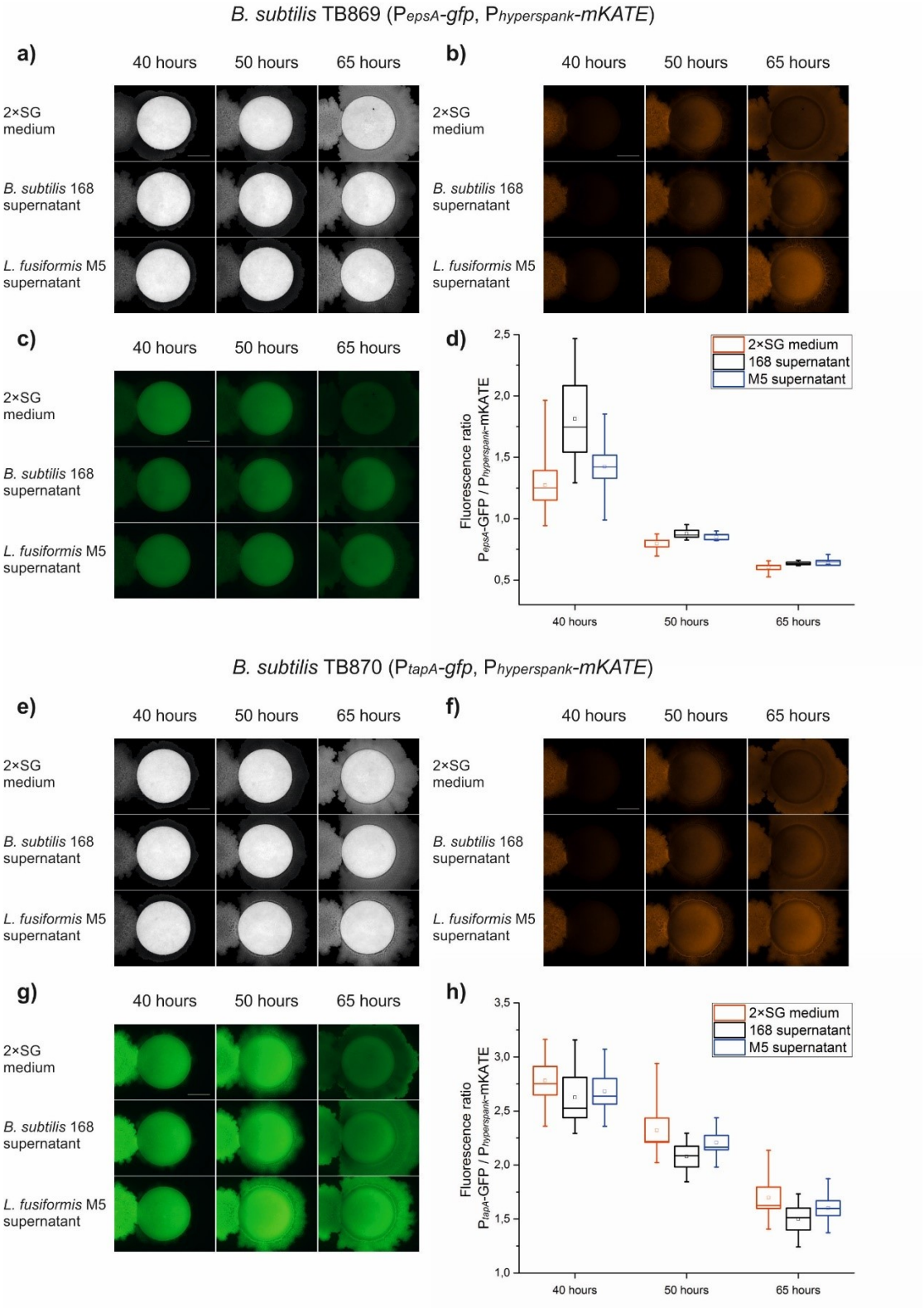

Figure 6: Comparison of fluorescence emission of *B. subtilis* strains carrying the constitutive $P_{hyperspank}$-mKATE, and the P_{epsA}-gfp (a-d) or P_{tapA}-gfp (e-h), reporter fusions. Bright field (a and e), red fluorescence (b and f), and green fluorescence images (c and g) of representative *B. subtilis* biofilm colonies exposed to cell-free supernatants of *B. subtilis* 168, *L. fusiformis* M5 and 2×SG medium. Box plots of the ratio of green and red fluorescence emission of biofilm colonies of *B. subtilis* TB869 (d) and TB870 (h) exposed to cell-free supernatants of *B. subtilis* 168, *L. fusiformis* M5 and 2×SG medium at different time points. Scale bars represent 5 mm. Box plots (d and h) represent fluorescence ratios of at least 4 independent colonies, processed as described in material and methods. (one-way ANOVA: $P<0.05$, n=4-8 independent colonies).

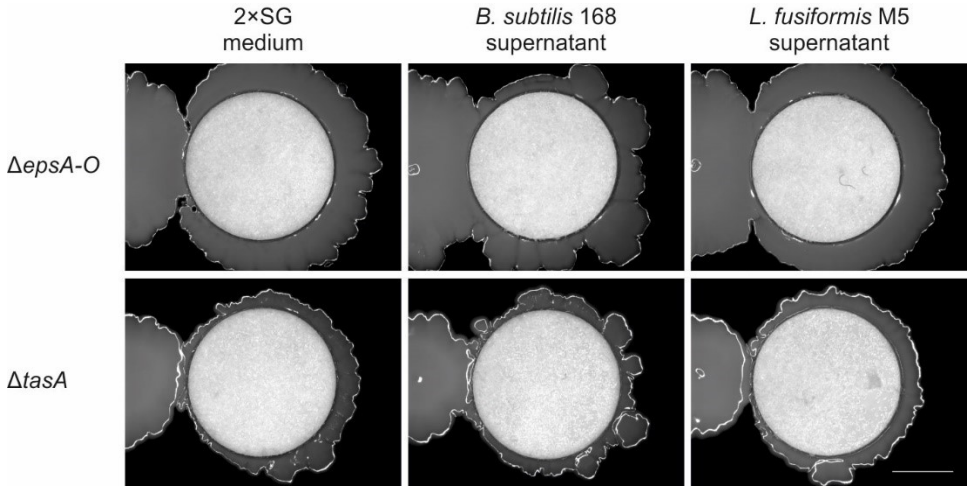

Figure 7: Effect of cell-free supernatant of *L. fusiformis* M5 on the development of colonies of *B. subtilis* 168 knock-out mutants of biofilm matrix biosynthetic operon *epsA-O* and *tasA* gene. Colonies were inoculated with 2 µL of culture at 5 mm from white cotton discs impregnated with 50 µL of cell-free supernatants of bacterial culture or 2×SG medium. The discs were reimpregnated with 25 µL of the corresponding medium or supernatant every 24 h. Bright-field images of colony areas adjacent to the cotton discs are shown after 72 h of incubation. The scale bar represents 5 mm.

Taken together, these results suggest that the increased colony wrinkle formation induced by hypoxanthine is not directly associated with a large increase in expression from the biofilm matrix operons; however, the production of a biofilm matrix is necessary for the development of wrinkles.

Cell death correlates with wrinkle formation.

It has been shown previously that localized cell death can be a trigger for wrinkle formation in biofilm colonies. This happens as a consequence of mechanical forces converging on zones of cell death, which lead to a buckling of the biofilm and the rise of tall wrinkles (388). Based on our previous results, we hypothesized that hypoxanthine, or a metabolite formed during its catabolism, may cause cell death in *B. subtilis*. This would produce mechanical stress in the developing biofilm and lead to buckling and wrinkle formation. Thus, we used Sytox Green to assess the distribution of dead cells in the colony wrinkle formation assay. Sytox Green is a commercially available fluorescent nucleic acid stain that has been established as a reporter of cell death for bacteria (411). For this assay, we used a *B. subtilis* strain that carries a $P_{hyperspank}$-*mKATE* reporter fusion (TB48) in order to facilitate the identification of *B. subtilis* cells that are metabolically active from those that are readily stained by Sytox Green. After 72 hours of incubation, we examined thin cross-sections of colonies that were exposed to 2×SG medium or supernatant from *L. fusiformis* M5. The examined cross-sections corresponded to areas of the colonies adjacent to the cotton discs (Fig. 8a and f). We observed that cell death is localized at the bottom of the biofilm, both on those exposed to 2×SG medium or *L. fusiformis*M5 supernatant (Fig. 8). However, in the cross-sections obtained from the flat colonies of *B. subtilis* exposed to 2×SG medium, the dead cells appear as thin layer of similar width along the length of the cross-section (Fig. 8b-e). In contrast, the cross sections from wrinkled colonies exposed to *L. fusiformis* M5 supernatant revealed aggregates of dead cells that correlate with the wrinkles seen through the colony (Fig. 8g-j). Furthermore, we compared the average green fluorescence of the colony cross-sections (produced by cells stained with Sytox Green) with their own red fluorescence (produced by cells expressing the $P_{hyperspank}$-*mKATE* reporter fusion). We found that the average ratios of green/red fluorescence were significantly higher in the cross-sections of colonies

exposed to *L. fusiformis* M5 supernatant (0.69 AU ± 0.10 (standard deviation)), than those of cross-sections from colonies exposed to 2×SG medium (0.33 AU ± 0.02 (standard deviation) (one-way ANOVA: P>0.05, n=3 cross-sections from independent colonies). These results confirm that the formation of wrinkles is facilitated by cell death, a phenomenon observed in the presence of the *L. fusiformis* M5 supernatant.

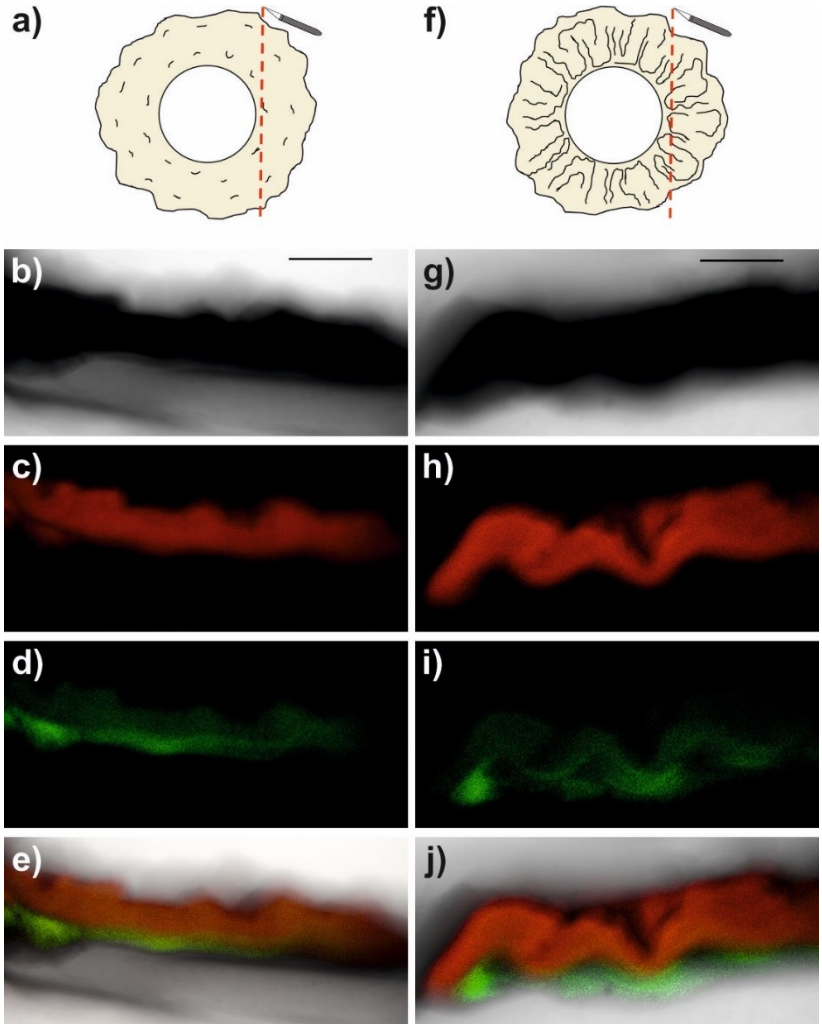

Figure 8: Detection of localized cell death in biofilm colonies of *B. subtilis* exposed to 2×SG medium (b-e) or cell-free supernatant of *L. fusiformis* M5 (g-j). Schematic representations of the cross-sections are shown from areas adjacent to cotton discs (a and b). Transmitted light (b and g), red fluorescence (c and h), green fluorescence (d and i), and composite images (e and j) of colonies of a *B. subtilis* strain carrying the constitutive $P_{hyperspank}$-mKATE reporter fusion are shown after 72 h of growth on plates with 0.25 µM of Sytox Green. The brightness and contrasts of the images have been enhanced to facilitate the appreciation of fluorescence signals and colony wrinkles. The scale bars represent 250 µm.

<u>The permease PbuO is necessary for hypoxanthine-induced development of wrinkles.</u>

We hypothesized that the observed induction of wrinkle formation may be due to metabolic effects on *B. subtilis* cells derived from an excess of available hypoxanthine provided by the culture supernatant of *L. fusiformis* M5, rather than to direct signal-dependent expression of biofilm-related genes. In this case, hypoxanthine alone would be sufficient to induce increased wrinkle formation, and its uptake by *B. subtilis* would be necessary for this phenomenon.

Using the colony wrinkle formation assay, we observed that different concentrations of hypoxanthine (as solution in 0.05 N NaOH) were able to induce the formation of wrinkles in *B. subtilis* colonies as efficiently as the supernatant of *L. fusiformis* M5. To test whether hypoxanthine internalization is required for the observed wrinkle induction in *B. subtilis*, we analyzed mutant strains of *B. subtilis* that lack *pbuG* or *pbuO*. PbuG is a previously described hypoxanthine/guanine permease (412), while PbuO is a protein paralogous to PbuG annotated as a putative purine permease (see SubtiWiki: http://subtiwiki.uni-goettingen.de/index.php) (194). The hypoxanthine inducing effect disappeared when pbuO alone, or in combination with pbuG, was deleted (Fig. 9).

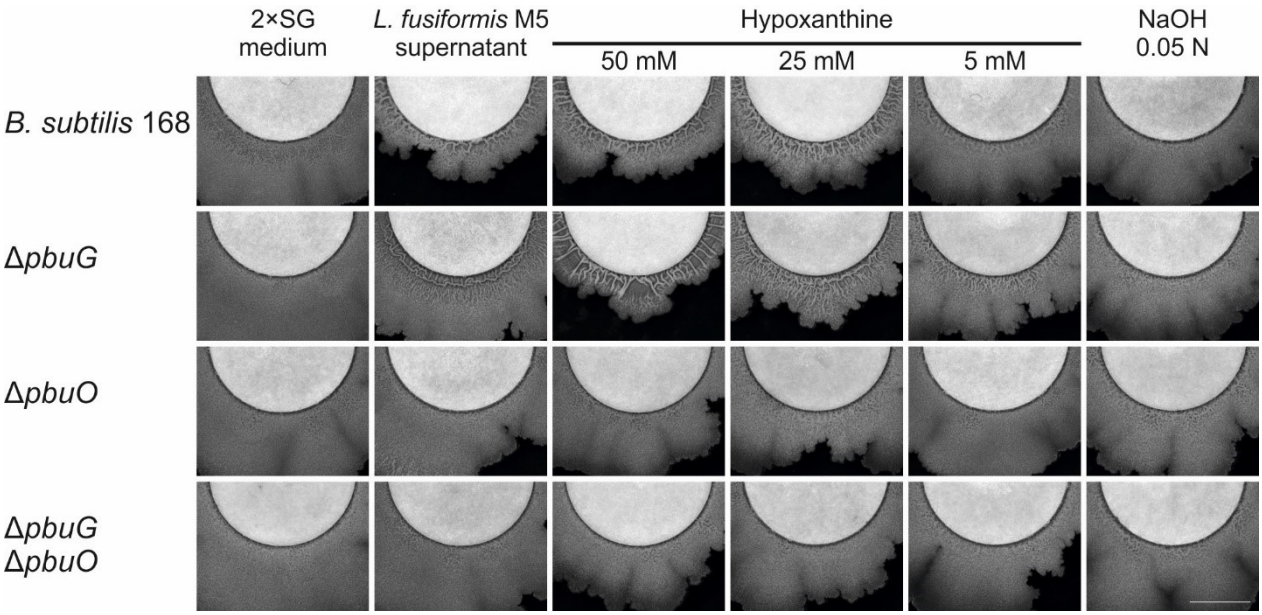

Figure 9: Effect of hypoxanthine and cell-free supernatant of *L. fusiformis* M5 on the development of biofilm colonies of *B. subtilis* 168 and knock-out mutants of *pbuO* and *pbuG* permease genes. Colonies were inoculated with 2 µL of culture at 5 mm from white cotton discs impregnated with 50 µL cell-free supernatant of *L. fusiformis* M5 or 2×SG medium. The discs were reimpregnated with 25 µL of the corresponding medium or supernatant every 24 h. Bright-field images of colony areas close to the cotton discs are shown after 72 h of incubation. The scale bar represents 5 mm.

These final results demonstrated that hypoxanthine uptake is important for induction of wrinkle formation and PbuO is mainly responsible for the observed effect under our experimental conditions.

Discussion.

In this work, we identified a chemical sensing mechanism between *B. subtilis* and other soil bacteria that promotes architectural complexity in colony biofilms. We devised a screening system that allowed us to analyze a collection of soil bacteria, selecting those that could form stable multispecies communities with *B. subtilis*. Using this screening system, we identified *L. fusiformis* M5 as a bacterium capable of inducing an increase of colony wrinkle formation in *B. subtilis* via hypoxanthine as chemical cue.

Hypoxanthine is a purine that plays an important role in the pentose phosphate salvage pathway, which is a mechanism for cells to interconvert nucleosides and nucleobases according to their metabolic needs (413, 414). In *B. subtilis*, hypoxanthine is particularly relevant in this

pathway because it can be taken up by cells and used as a substrate by phosphoribosyl-transferases in order to produce inosine monophosphate (IMP), which in turn is converted to adenosine monophosphate or guanine monophosphate (412) (also see SubtiWiki Pathways: http://subtiwiki.uni-goettingen.de/apps/pathway.php?pathway=2) (194). The role of hypoxanthine in eukaryotic cell metabolism has been extensively investigated. In humans, it has been studied in the context of diseases such as gout, Lesch–Nyhan disease, and endothelial cell injury of cardiovascular diseases. Although these conditions have different etiologies and clinical evolution, they have in common an excessive accumulation of hypoxanthine and uric acid, whose catabolism leads to oxidative-stress-induced apoptosis (415)(416)(417). In bacteria, hypoxanthine has been mainly studied in relation to DNA damage and mutagenesis due to spontaneous deamination of adenine, which yields hypoxanthine and leads to AT-to-GC transitions after DNA replication (418). In *B. subtilis*, hypoxanthine has been studied both related to purine metabolism (412), and DNA damage and repair (419). To the best of our knowledge, this is the first time that hypoxanthine has been reported as a mediator of interactions in *B. subtilis* biofilms.

L. fusiformis is a free-living bacterium that can be isolated from soil and has been studied due to its production of interesting secondary metabolites and bioremediation potential (420–422). Here, we have identified a strain of *L. fusiformis* able to produce and excrete hypoxanthine in sufficient levels to induce the formation of wrinkles in biofilm colonies of *B. subtilis*. We found no evidence that this phenomenon is dependent on the signal transduction of a single Kin kinase (Fig. 5), and the expression levels of the matrix-component-related operons *epsA-O* and *tapA-sipW-tasA* remained equal when *B. subtilis* was exposed to the supernatant of *L. fusiformis* M5 (Fig. 6). Importantly, we cannot discard the possibility of changes in the expression of other genes. For example, changes in the expression of motility-related genes might be responsible for the apparent differences in colony expansion observed in our wrinkle formation assays. Another possibility is that an alternative exopolysaccharide biosynthetic pathway could be affected. Recently, *ydaJKLMN* was reported as a new operon important for the production of a so far unidentified exopolysaccharide in *B. subtilis* (423). However, overexpression of the *ydaK-N* operon under a xylose-inducible promoter could promote wrinkle formation in a Δ*epsH* mutant strain (423), while the presence of the *eps* operon is essential for the induction of wrinkle development in the presence of hypoxanthine. Therefore, it seems unlikely that hypoxanthine-induced wrinkle formation would proceed by directly inducing the production of an alternative exopolysaccharide. In contrast, we could detect the presence of dead cells with Sytox Green at the site of wrinkle formation (Fig. 8). Additionally, a knock-out mutant of the hypoxanthine/guanine permease PbuO lost the ability to form wrinkled colonies; specifically, a Δ*pbuG* mutant strain showed slightly reduced or similar wrinkle formation as *B. subtilis* 168, while a Δ*pbuO* strain completely lost the ability to form wrinkled colonies when exposed to hypoxanthine. Based on these results, we suggest that hypoxanthine induces the formation of wrinkles in colony biofilms of *B. subtilis* not by inducing the expression of biofilm-related genes, but rather by metabolic effects derived from the excess of available hypoxanthine. In this regard, we note that an excess of hypoxanthine can cause oxidative stress and cell death in eukaryotic cells by increasing the formation of reactive oxygen species when hypoxanthine is metabolized to urate (415, 416). A similar catabolic pathway could be followed by hypoxanthine in *B. subtilis*, which possesses multiple hypoxanthine/xanthine oxidases known as PucA, B, C, D and E (see SubtiWiki Pathways: http://www.subtiwiki.uni-goettingen.de/apps/pathway.php?pathway=41) (194). Additionally, it has been shown that localized cell death can lead to the formation of

wrinkles in colonies of *B. subtilis* by providing an outlet for compressive mechanical forces that buckle the biofilm and promote the appearance of wrinkles (388). Thus, we hypothesize that the hypoxanthine provided by *L. fusiformis* induces oxidative stress and cell death in *B. subtilis*, which leads to the formation of wrinkles as a mechanical consequence. This is in accordance with the fact that the observed development of wrinkles only occurs in the interaction zone between colonies of these organisms (Fig. 1), or in the proximity of the cotton discs during our colony wrinkle formation assays (Figs. 5 and 9). This development of wrinkles does not happen in the rest of the *B. subtilis* colony, presumably due to a lower concentration of diffused hypoxanthine. Importantly, this induction of increased wrinkle formation in *B. subtilis* would therefore be a consequence of regular metabolic processes of *L. fusiformis*, rather than a canonical signaling mechanism intended to elicit a response in the receiver. Regarding hypoxanthine production by *L. fusiformis* M5, we have previously sequenced this strain, finding genes homologous to those known in other organisms to be responsible for hypoxanthine synthesis and export (424), although more research is needed to establish its production yield. However, we note that hypoxanthine can be produced by spontaneous deamination of adenine, such as that present in the surroundings of decaying cells (425). Unfortunately, our efforts to transform *L. fusiformis* M5 failed, preventing us to construct mutants with altered hypoxanthine production.

In recent years, biofilm research has grown from an incipient field to a major area of microbiological interest. Due to the high cell density of biofilms, social interactions are an inherent characteristic of these microbial populations, regardless of whether they are formed as single or multi-species communities (168, 180, 397, 426). The interactions between the organisms forming a biofilm are therefore an important aspect of this research field, since they shape the development of these communities, be it by intraspecies signaling, interspecies communications, or chemical cues derived from the metabolism of community members, such as the case presented here. Further study of the sociomicrobiology of biofilms will lead to an increased understanding of these communities as they form in nature, better enabling us to eliminate them when they are noxious to human activities, or to promote them when needed for biotechnological applications.

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CHAPTER 4

Draft genome sequence of the soil isolate *Lysinibacillus fusiformis* M5, a potential hypoxanthine producer.

Ramses Gallegos-Monterrosa, Gergely Maróti, Balázs Bálint, and Ákos T. Kovács.

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Lysinibacillus fusiformis strain M5 is a potential hypoxanthine producer that was isolated from clay soil. Here, we present the draft genome sequence that was annotated in order to facilitate future studies of *L. fusiformis* M5.

Lysinibacillus fusiformis is a Gram-positive endospore-forming soil bacterium that was recently reclassified from the *Bacillus* genus due to differences in its cell wall components (427). Although *L. fusiformis* has been suspected for it pathogenicity (428–430), other studies reported the isolation of this species from diverse environmental samples, and it has been proposed as a potential producer of industrially attractive metabolites (420, 431).

Screening of a library of isolates obtained from a Mexican clay soil collected at the warm and humid region of Tepoztlán, Morelos, resulted in the identification of *L. fusiformis* M5. It was selected for further study due to its ability to produce hypoxanthine (R. Gallegos-Monterrosa and Á. T. Kovács, unpublished data). Hypoxanthine is a common metabolite produced by bacteria as part of the purine salvage pathway (414, 432). This nucleobase and its concomitant enzymes have been extensively studied due to their role in cell metabolism and signaling, and as potential drug targets (433, 434).

We performed whole-genome sequencing of *L. fusiformis* strain M5 in order to facilitate the identification of genes involved in hypoxanthine production. Genomic DNA of *L. fusiformis* M5 was isolated with GeneMATRIX bacterial and yeast genomic DNA purification kit, according to the manufacturer's instructions (EURx, Gdańsk, Poland). A mate-pair library was generated using the Illumina Nextera mate-pair kit (catalog no. FC-132- 1001), with insert sizes ranging between 7 and 11 kb. DNA sequencing was carried out on an Illumina MiSeq machine using V2 sequencing chemistry, resulting in 2 × 250-bp reads. Raw data were preprocessed for *de novo* assembly according to the manufacturer's recommendations. Data processing of Nextera mate pair reads was performed using Illumina Sequencing Platforms (http://www.illumina.com/documents/products/technotes/technote_nextera_matepair_data_processing.pdf)

De novo assembly was performed with CLC Genomics Work-bench 8.0.2 (CLC bio), with contigs being subsequently arranged into scaffolds using SSPACE 3.0 (435). Gaps in scaffolds were closed with SPAdes version 3.1.1 (436), together with an in-house R script (B. Bálint, unpublished data). The assembly produced 7 contigs and a circularized plasmid that comprise 4,744,577 and 134,678 bases, respectively, with G + C contents of 37 and 36%, respectively. Automated annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (437); 4,753 genes were identified, including 74 tRNA and 22 rRNA regions. Around 96% of the identified genes corresponded to hypothetical proteins (4577 coding open reading frames [ORFs]).

Genes coding for proteins possibly involved in hypoxanthine production were identified among the annotated genes, namely, *pbuE*, a putative hypoxanthine transporter; and *ade*C and *yer*A, putative adenine deaminases involved in the purine salvage pathway. Genome comparison confirmed the presence of homologous genes (identity, ≥95%) in the genomes of *L. fusiformis* RB-21 (GenBank accession no. CP010820.1) and *L. fusiformis* SW-B9 (GenBank accession no. JRBA00000000.1) (410). Based on genomic BLAST, *L. fusiformis* M5 shows closest homology to *L. fusiformis* strain H1k (GenBank accession no. AYMK00000000.1).

Accession number(s).

This whole-genome shotgun project has been deposited in GenBank under the accession no. MECQ00000000. The version described in this paper is the first version, MECQ00000000.1

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CHAPTER 5

Unraveling the predator-prey relationship of *Cupriavidus necator* and *Bacillus subtilis*.

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Cupriavidus necator is a non-obligate bacterial predator of Gram-negative and Gram-positive bacteria. In this study, we set out to determine the conditions, which are necessary to observe predatory behavior of *C. necator*. Using *Bacillus subtilis* as a prey organism, we confirmed that the predatory performance of *C. necator* is correlated with the available copper level, and that the killing is mediated, at least in part, by secreted extracellular factors. The predatory activity depends on the nutrition status of *C. necator*, but does not require a quorum of predator cells. This suggests that *C. necator* is no group predator. Further analyses revealed that sporulation enables *B. subtilis* to avoid predation by *C. necator*. In contrast to the interaction with predatory myxobacteria, however, an intact spore coat is not required for resistance. Instead resistance is possibly mediated by quiescence.

Introduction.

Over the past decades it has become increasingly evident that the composition and dynamics of microbial communities have a profound effect on Earth's ecosystems. Conversely, these consortia are shaped by environmental cues and organismic interactions. Predator-prey relationships are of particular interest in this context due to their evolutionary implications (438). Surprisingly, our knowledge about predator-prey interactions in the bacterial world is still very limited (439, 440). Even abundant soil bacteria, among them *Streptomyces* spp., have long been neglected with regard to their predatory potential (441).

In this study, we set out to verify and to explore the predator- prey relationship of two common soil-dwelling microbes, namely *Cupriavidus necator* and *Bacillus subtilis*. The former bacterium is distinguished by an extraordinary metal resistance, and its growth responds favorably to high concentrations of Cu^{2+}, which is tolerated up to 800 µM (442). *C. necator* was reported to prey upon a wide range of Gram-negative and Gram-positive bacteria (443). The prey spectrum is not restricted to soil bacteria, but also includes human commensals and pathogens, as well as other predatory bacteria such as *Agromyces ramosus* (442). The actinomycete *A. ramosus* is known to kill its prey upon cell-to-cell contact (444). Previous studies revealed that a contact-mediated attack on *C. necator* triggers a counter attack of the latter, which ultimately leads to

lysis of the *A. ramosus* mycelium (445). It has been proposed that the counter attack involves the secretion of a copper-binding peptide, which is toxic to the actinomycete and which might also be used for the killing of other prey bacteria, including *B. subtilis* (445, 446).

Since these initial studies by Casida *et al.*, however, the predatory nature of *C. necator* was increasingly put into question. Although a growth stimulation of *C. necator* had been demonstrated in the presence of other bacteria using the so-called indirect phage analysis technique, the assumed killing of the prey organisms had not been monitored (447). Ultimate proof for predation was hence missing. Further doubts were raised after the taxonomic reclassification of *Wautersia eutropha* (formerly *Ralstonia eutropha* and *Alcaligenes eutrophus*, respectively) into *C. necator* (448). None of the newly assigned *C. necator* strains had ever been associated with predatory behavior despite the fact that some of them, in particular strain H16, had been thoroughly investigated (449). Reevaluation of the predatory activity of *C. necator* was therefore imperative.

B. subtilis was chosen as a model for the analysis of a possible predator-prey relationship. Not only had *B. subtilis* been described as a prey bacterium of *C. necator* (442), but it is also commonly used to address basic questions concerning the development of prey resistance (142, 143, 450, 451). In theory, *B. subtilis* can resort to a variety of possible defense strategies to evade predation (452, 453). Some resistance traits are specific for certain *B. subtilis* strains and they provide protection only against selected predators. An example would be the release of inhibitory or toxic secondary metabolites, such as the antibiotic bacillaene, which is used to hold off the predatory bacterium *Myxococcus xanthus* (450). Other conceivable defense mechanisms, among them motility and biofilm formation, are frequent features of *B. subtilis* strains and can be expected to confer unspecific protection (454). Lastly, most strains of *B. subtilis* are capable to sporulate under stressful conditions, which dramatically improves their potential to resist predation by protozoa (142), nematodes (143), as well as myxobacteria (450).

Given the diversity of predation strategies that are used by bacteria (439, 455) and the observations that had previously been made for *C. necator* (442, 445), we were interested in clarifying whether the putative predatory activity of this species depends on specific triggers, among them nutrient limitation and access to Cu^{2+}, and whether prey killing is exclusively mediated by extracellular factors or requires cell contact. By analyzing prey preference we further sought to identify defense mechanisms that confer resistance against *C. necator* predation. Taken together, our analyses revealed that *C. necator* is truly a predatory bacterium, which uses a hunting strategy distinct from other bacteria. Predatory success does not depend on outnumbering the prey and does also not necessarily involve prey contact. Although predation is positively correlated with the Cu^{2+} concentration, it can also be observed at very low levels of this transient metal. Sporulation of *B. subtilis* grants protection against *C. necator*, but an intact spore coat is surprisingly not required.

Material and Methods.

Bacterial strains and cultivation conditions.

Cupriavidus necator N-1 (DSM 545) and *C. necator* H16 (formerly *Ralstonia eutropha* H16; DSM 428) were cultivated in Lysogeny Broth (LB) or in H-3 mineral medium (0.1% aspartic acid, 0.23% KH_2PO_4, 0.257% Na_2HPO_4, 0.1% NH_4Cl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $NaHCO_3$, 0.001% $CaCl_2 \cdot 2H_2O$,

and 0.5% SL-6 trace element solution). In specified experiments the growth media were supplemented with $CuCl_2 \cdot 2H_2O$, which was added as a filter-sterilized solution to give final concentrations ranging from 0.3 µM to 1.0 mM. The *Bacillus subtilis* strains used in this study are listed in Table 1. All *B. subtilis* strains were routinely grown in LB medium at 30°C. For selection, media were supplemented with chloramphenicol (12.5 µg ml^{-1}), tetracycline (10.0 µg ml^{-1}), or kanamycin (10.0 µg ml^{-1}), respectively.

Table 1: *B. subtilis* strains used in this study.

Strain	Description, genotype	Source or reference
168	laboratory strain, *trpC2*	Bacillus Genetic Stock Center
NCIB 3610	wild isolate	(456)
DK1042	NCIB 3610 *comI*^{Q12l}	(368)
RO-NN-1	wild isolate	(457)
PS216	wild isolate	(458)
ATCC 6633	wild isolate, subtilin producer	ATCC collection
TB48	168 *trpC2 amyE*::Phy-*gfp* Cm	(372)
TB34	DK1042 *amyE*::Phy-*gfp* Cm	This study
TB268	RO-NN-1 *amyE*::Phy-*gfp* Cm	This study
TB269	PS216 *amyE*::Phy-*gfp* Cm	This study
TB270	ATCC6633 *amyE*::Phy-*gfp* Cm	This study
SWV215	168 *trpC2 spo0A*::Km	(459)
RL1265	PY79 *spoIIAC*::Km	(91)
RL50	168 *trpC2 cotA*::Cm	(460)
RL52	168 *trpC2 cotC*::Cm	(460)
DL1032	NCIB 3610 Δ*epsA-O*:tet, Δ*tasA*:Km	(371)
GC260	PY79 *gerR*::Km	(461)
AH2835	*cotE*::C006D	(462)
AD18	*spoIVA*::Neo	(463)
PE277	PY79 *safA*::Tet	(464)
PE697	PY 79 *spoVID*::Km	(465)
TB193	NCIB3610 Δ*motA amyE*::Phy-*gfp* Cm	(33)
TB421	DK1042 *spo0A*::Km *amyE*::Phy-*gfp* Cm	This study
TB422	DK1042 *spoIIAC*::Km *amyE*::Phy-*gfp* Cm	This study
TB423	DK1042 *tasA*::Km *amyE*::Phy-*gfp* Cm	This study
TB424	DK1042 *gerR*::Km *amyE*::Phy-*gfp* Cm	This study
TB425	DK1042 *cotA*::Cm	This study
TB426	DK1042 *cotC*::Cm	This study
TB567	DK1042 *cotE*::Cm	This study
TB568	DK1042 *safA*::Tet *amyE*::Phy-*gfp* Cm	This study
TB569	DK1042 *spoIVA*::Neo *amyE*::Phy-*gfp* Cm	This study
TB570	DK1042 *spoVID*::Km *amyE*::Phy-*gfp* Cm	This study

Strain constructions.

B. subtilis strains used for the predation assays were all endowed with chloramphenicol resistance genes via transforming genomic DNA obtained from *B. subtilis* strain TB48 (372) using natural competence (466). In these strains, the P_{hy}-*gfp* (Cm) cassette is recombined into the *amyE* locus of *B. subtilis* that was validated by the lack of a-amylase activity and the presence of green fluorescence in the transformed strains. Further mutations (see Table 1) were subsequently introduced into *B. subtilis* TB34 (NCIB 3610 natural competent derivative DK1042 with Cm

antibiotic marker) resulting marker exchanged mutants. In the case of mutations harboring Cm markers (i.e., *cotA*, *cotC* and *cotE*), the mutations were transferred directly into DK1042.

Correlation of optical densities with viable cell count data.
The statistical relationship between colony-forming units (CFU) and optical density was determined according to a previously described protocol (467).

CFU-based predation assay.
Unless otherwise stated, *C. necator* N-1 was used in the analysis of predator-prey interactions. The assay was conducted as previously described (467) with the following minor changes: *C. necator* was initially grown for 24 h in 5 ml LB medium or, alternatively, for 3 days in 15 ml H-3 medium with or without $CuCl_2 \cdot 2H_2O$. *B. subtilis* cells were harvested from a 10 ml LB culture that had been incubated at 30°C for 24 h. At this time point, the *B. subtilis* cells had already reached the stationary phase (data not shown). Prior to the coincubation experiment, the cell concentration of *C. necator* was adjusted to 2×10^8 cells ml^{-1} and the cell concentration of *B. subtilis* was adjusted to 1×10^8 cells ml^{-1}.

Evaluation of the predation efficiency.
The predatory activity was quantified as previously described (467).

Contact dependence of predatory behavior.
C. necator was grown in H-3 medium supplemented with $CuCl_2 \cdot 2H_2O$ (50 µM) to an OD_{600} of 2.5. After centrifugation (2400×g, 5 min), the cell pellet was washed with PBS buffer and directly mixed with *B. subtilis*. In parallel, the supernatant was filter-sterilized and mixed with *B. subtilis*. Control experiments included *B. subtilis* suspensions treated with 370 µl of PBS buffer or H-3 medium supplemented with $CuCl_2 \cdot 2H_2O$ (50 µM).

B. subtilis spore preparation.
B. subtilis was cultivated for 3–5 days on Schaeffer's sporulation medium (0.8% Difco Nutrient Broth supplemented with 1 mM $MgSO_4 \cdot 7H_2O$, 13.4 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 0.13 mM $MnSO_4 \cdot H_2O$) until more than 80–90% of the population sporulated. Spores were purified following a previously described protocol (468). Briefly, cultures were centrifuged (1020×g, 8 min, 4°C) and washed two times with 10 mM PBS buffer (pH 7.4). Samples were incubated at −20°C for 3 × 24 h and the washing step was repeated every day. Afterwards, the spores were stored at −20°C.

Decoating of spores and lysozyme resistance assays.
Decoating of wild type and mutant spores was performed as previously described (469). Briefly, a spore suspension of *B. subtilis* NCIB 3610 (1 ml; 10^{10}-10^{11} spores ml^{-1}) was mixed with the same volume of decoating solution (0.1 M NaCl; 0.1 M NaOH; 1% sodium dodecyl sulfate and 0.1 M dithiothreitol) and incubated for two hours at 70°C. Afterwards, the mixture was centrifuged (3500 r.p.m., 5 min) and washed two times with PBS buffer. Decoated spores were then used in the CFU-based predation assay. For lysozyme resistance assays, 100 µl of lysozyme solution (1 mg ml^{-1}) was mixed with an equal volume of *B. subtilis* spores and incubated for 20 min at 37°C (469). Later, serial dilutions of the mixture were made and plated on LB agar plates. After two days of incubation at 30°C, CFU were counted.

Statistical analysis.

Data was analyzed with a paired-sample *t* test and nonparametric statistical tests, including Mann-Whitney *U* test and Wilcoxon median test. All statistical analyses were done using SPSS software (version 22.0, IBM, USA).

Results.

Influence of nutrition status and prey type.

To assess the impact of the nutrition status on the putative predatory activity of *C. necator*, the bacterium (i.e., strain N-1) was grown either in nutrient-rich (LB) or in nutrient-poor (H-3 mineral salt) medium prior to the addition of the prey culture. Previous studies on myxobacteria had unveiled that predatory performance can be species-dependent and sometimes even strain-specific (440, 450, 470, 471). We thus tested *C. necator* against five *B. subtilis* strains, including the two domesticated strains Marburg 168 and ATCC 6633, the ancestral strain NCIB 3610, as well as the two wild isolates PS216 and RO-NN-1. Since no lysis of *B. subtilis*-covered agar plates was observed under any condition tested, predator and prey were cultured together in PBS buffer for 24 h. Afterwards the mixed populations were streaked out on growth-selective agar media and the respective CFU number was determined following a previously described protocol (467). The results of this predation assay indicated that *C. necator* was more efficient in killing its prey after it had been grown in the low nutrient H-3 medium (Fig. 1A). Since the observation was consistently made irrespective of the *B. subtilis* strain tested, we concluded that nutrient deficiency is correlated with predatory performance. The type of prey was also found to significantly affect the outcome of the predator-prey interaction. As expected, the domesticated strains of *B. subtilis* were found to be more susceptible to *C. necator* predation than the wild isolates. This initially suggested that the former might have lost resistance traits due to lack of selection pressure in the laboratory. Contrasting previous observations with the predatory bacterium *Myxococcus xanthus* (450), however, the ancestral strain NCIB 3610 exhibited almost no resistance against *C. necator*, even when the latter had been grown in the rich LB medium. It was hence evident that strain-specific factors contribute to prey survival, and that resistance cannot be generally associated with ancestral strains.

The consumption of *B. subtilis*, which was evaluated based on the increase of the predator population in comparison to prey-free control experiments, was strongly affected by the nutrition status of *C. necator*. After the predatory bacterium had been grown in the nutrient-rich LB medium, significant feeding was only observed on the laboratory strain 168, whereas the other *B. subtilis* strains were not consumed (Fig. 1B). This result was surprising considering the efficient killing of strains ATCC 6633 and NCIB 3610 under the same conditions and demonstrated that killing of bacteria is not necessarily an indication for predation. In cases where the assay was carried out with starved *C. necator* cells, however, significant killing was always accompanied by efficient prey utilization. Despite this consistency, we observed quantitative differences concerning the growth stimulation of the predator. Again, it appeared that *C. necator* preferred feeding on strain 168 over the other *B. subtilis* strains. Two conclusions were drawn from these data. First, *C. necator* will only prey on other bacteria in the absence of alternative nutrient sources that are more easily exploitable (even though exceptions such as strain 168 may occur). Second, the predatory performance is strain-specific. Further analyses encompassing different prey species even showed that predatory activity is not restricted to strain N-1, but can also be observed in *C. necator* H16 (Fig. S1, all supplementary material for this chapter is available in annexum B).

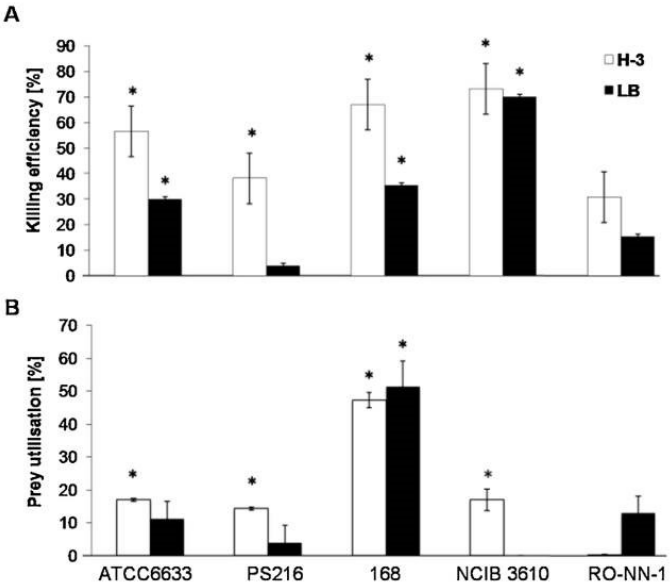

Figure 1: Results from the CFU-based predation assay. (A) Mean killing efficiency ($\bar{e}$ ± 95% confidence interval) of *C. necator* against different *B. subtilis* strains. Asterisks denote significant differences between killing efficiencies (Mann-Whitney U-test: * = P < 0.05; d.f. = 2). (B) Mean prey utilization ($\bar{u}$ ± 95 % confidence interval) of *C. necator* when preying on different *B. subtilis* strains. Asterisks denote significant differences in the prey consumption (Wilcoxon test: * = P < 0.05; d.f. = 2).

Impact of copper(II).

It had previously been proposed that elevated concentrations of Cu^{2+} foster the predatory activity of *C. necator* (445). To verify this assumption, we evaluated the effect of different Cu^{2+} concentrations in H-3 mineral medium (0.01 µM; 12.5 µM; 25 µM; 50 µM; 100 µM; 200 µM and 400 µM) on the killing efficiency of *C. necator* in subsequent co-incubation experiments with *B. subtilis* 168. To remove any bias due to Cu^{2+}-associated toxicity, the prey survival was related to monocultures, in which the *B. subtilis* cell suspension was mixed with a control solution that was obtained after processing predator-free H-3 medium with the respective Cu^{2+} concentration according to the standard assay protocol. This analysis confirmed that the predatory activity of *C. necator* increased after exposure to Cu^{2+} in a concentration- dependent manner (Fig. 2). The maximum killing efficiency was observed at a Cu^{2+} concentration of 50 µM. We did not observe significant changes in the number of surviving *B. subtilis* cells beyond this threshold. It was hence clear that Cu^{2+} has a strong impact on the predatory activity of *C. necator*. Furthermore, it became obvious that the predation-enhancing effect of Cu^{2+} is limited and that a defined fraction of the *B. subtilis* population will survive, possibly due to resistance. Lastly, *C. necator* also preys on *B. subtilis* at low Cu^{2+} levels, although its killing efficiency is reduced under these conditions.

Group predation and proximity to prey.

Some predatory bacteria are assumed to hunt collectively. They pool lytic enzymes and/or antibiotics to degrade the cell wall of their prey (472–475). In order to test whether the predatory activity of *C. necator* depends on numerical superiority over its prey, we varied the predator-prey ratio (PPR) in co-cultivation experiments with *B. subtilis* 168. This analysis revealed that *C. necator* does not need to be present in large numbers for effective predation. Maximum killing efficiencies were already observed at a PPR of 5:1. Even when the experiment started with an excess of the prey bacterium, the cell number of *B. subtilis* severely declined during co-

cultivation with *C. necator* (Fig. 3). We hence reasoned that *C. necator* is capable of individual predation and does not rely on cooperative feeding.

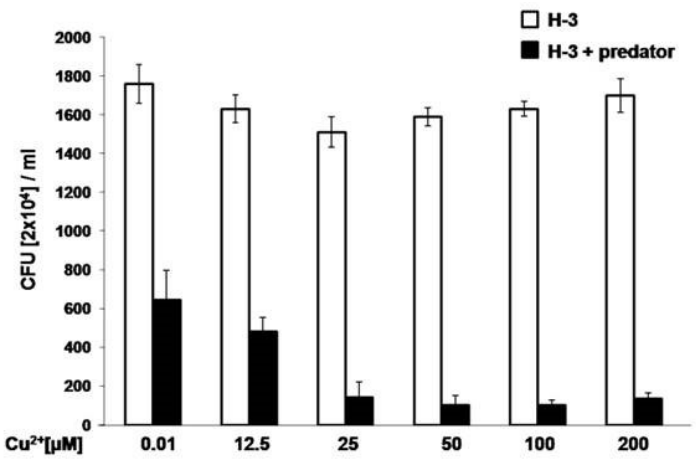

Figure 2: Copper(II) dependence of predatory behavior. Prey reduction of *B. subtilis* 168 after co-cultivation with *C. necator* in the presence of different Cu^{2+} concentrations.

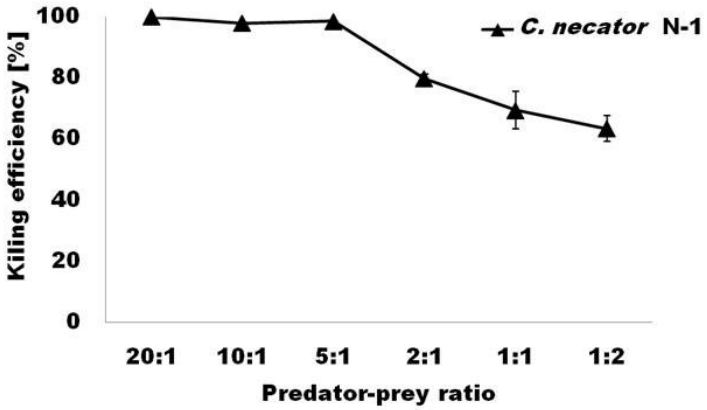

Figure 3: Frequency dependence of predatory behavior. Different predator-prey ratios versus the mean killing efficiency ($\bar{e}$ ± 95% confidence interval) of *C. necator* are shown against *B. subtilis* 168.

To clarify whether the killing of *B. subtilis* 168 requires physical contact or whether it is mediated by secreted extracellular factors, such as enzymes and antibiotics, a defined number of prey cells was individually exposed to the cell fraction and a cell-free supernatant of a *C. necator* culture. In case of the harvested cells, the predation assay was carried out in PBS buffer, as previously described, whereas the H-3 medium-derived supernatant was directly mixed with the suspension of strain 168 in PBS buffer. Control experiments were also conducted in the presence of Cu^{2+} (50 µM) to assess the effects of this transient metal on the growth of strain 168. The number of *B. subtilis* CFUs that was obtained after 24-h incubation revealed that the nutrient-poor H-3 medium still has a minor growth-promoting effect when compared to PBS buffer, which lacks organic nutrients (Fig. 4). The addition of Cu^{2+} (50 µM) led to a negligible decrease of the CFU number. To our surprise, both the cell fraction and the culture supernatant of *C. necator* caused an almost complete eradication of the prey population. Because its supernatant has strong

antimicrobial effects, *C. necator* can kill *B. subtilis* 168 without making physical contact. This indicates the release of a molecule or enzyme that is toxic to strain 168.

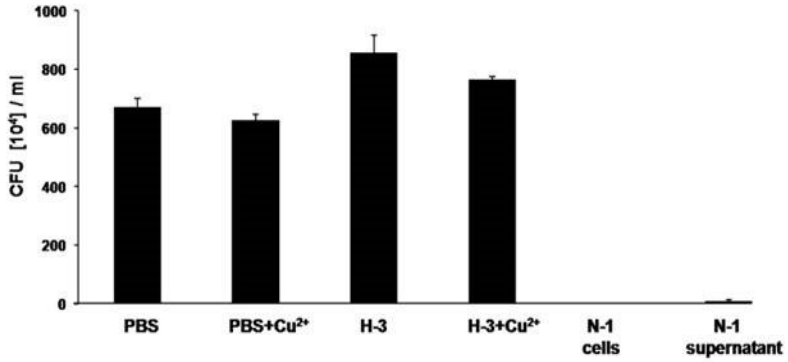

Figure 4: Contact dependence of predatory behavior. Cells and supernatant from *C. necator* cultures were tested in the CFU-based predation assay against *B. subtilis* 168. PBS buffer, H-3 medium supplemented with 50 µM of copper was mixed with *B. subtilis* and served as a control

Potential resistance factors of *Bacillus subtilis* against predation.

B. subtilis is a flagellated bacterium and, thus, capable of active movement (476). Motility is required for surface spreading of *B. subtilis* (160, 385), but also important during complex development, including biofilm formation (33). Two integral membrane proteins, MotA and MotB, are essential components of the flagellar motor (477, 478). To evaluate the impact of motility on predation resistance, we analyzed the survival rate of a *B. subtilis* Δ*motA* mutant in the CFU-based predation assay. However, there was no evidence for an increased susceptibility to bacterial predation when compared to the wildtype strain (data not shown), indicating that active motion does not provide protection against *C. necator*.

Another characteristic feature of Bacillus spp. is the formation of endospores. This morphological adaptation can be triggered by nutritional limitation (479), but also by other stressful conditions including predation (142, 143, 450). In order to assess the predation resistance of *B. subtilis* spores, we grew the sporulation-competent strain NCIB 3610 for 24, 48, or 72 h, and exposed the respective cultures to *C. necator*. Surviving *B. subtilis* were quantified after plating on growth-selective agar media and heat treatment at 80°C to eliminate the vegetative cells. Consistent with our previous analysis (Fig. 1), *C. necator* exhibited very high killing efficiencies against 24 h- and 48 h-old cultures of strain NCIB 3610. On the other hand, the 72 h-old culture was hardly affected by the predator. In parallel experiments, the heat treatment was omitted, but this did not significantly alter the number of *B. subtilis* CFU on the agar plates (Fig. 5). Subsequent analyses confirmed that cultures harvested after 24 h or 48 h consisted mainly of vegetative cells, whereas 72 h-old cultures were largely dominated by spores. This strongly suggested that sporulation confers resistance against predation. Further evidence supporting this assumption was obtained after testing isolated spores from strain NCIB 3610 in the CFU-based predation assay. The co-cultivation with *C. necator* did not reduce the spore number in comparison to control experiments lacking the predatory bacterium (data not shown). Finally, the predation resistance of sporulation-deficient *spo0A* and *sigF* mutants was evaluated. The DNA-binding protein Spo0A is a global transcriptional regulator which, once phosphorylated, activates several genes that are required for early spore development in *B. subtilis* (480). SigF, which is transcriptionally activated by Spo0A, is known as the first forespore specific sigma factor (481). Both *B. subtilis* mutant strains were effectively killed by *C. necator* and the outcome of the co-

cultivation experiment was consistent for 24 h-, 48 h-, and 72 h-old cultures of every mutant (Fig. 5).

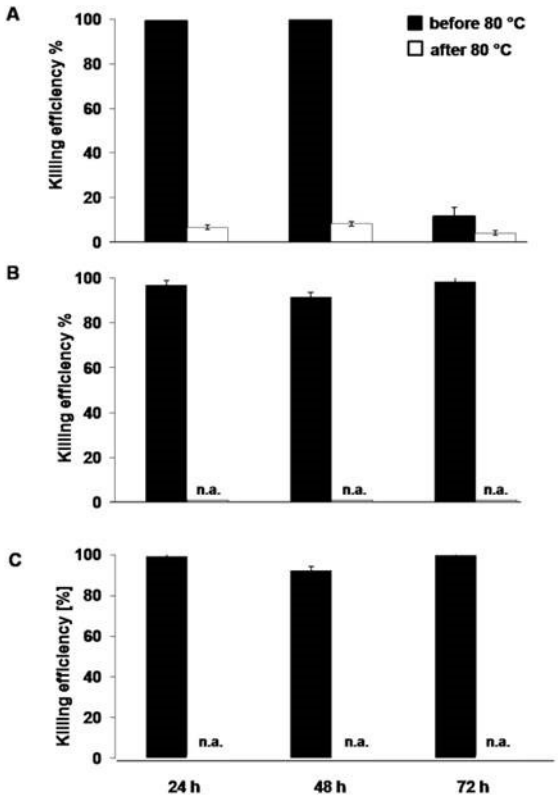

Figure 5: Time dependence of predatory behavior. (A) Killing efficiency of *B. subtilis* NCIB 3610 and the non-sporulating (B) *spoA* and (C) *sigF* mutants (n.a., not applicable for statistical analysis).

Spore components mediating resistance against predation.

Even though the precise mechanism of spore persistence is not known yet, the spore coat is generally recognized as the first line of defense against toxic molecules. Therefore, it might also have a role in protecting *B. subtilis* from a secreted killing factor of *C. necator*. The spore coat consists of at least 70 different proteins, some of which have been associated with specific resistance functions (482). It is hence plausible that a spore might even resist desiccation, extreme heat, UV radiation, or predation when single spore proteins are absent. This raised the question whether a fully maturated endospore is essential for *B. subtilis* to survive an attack by *C. necator* or whether the predation resistance is due to defined spore components. In order to answer this question, NCIB3610-derived mutant strains defective in the production of specific spore proteins were chosen, their spores were isolated and subsequently tested in the CFU-based predation assay (Fig. 6).

Initial experiments were carried out with spores lacking the outer coat proteins CotA, CotC, and CotE, respectively. While CotA is needed for the biosynthesis of a melanin-type brown pigment (460, 482, 483), CotE was found to be indispensable for the assembly of the outer coat (484). Spores from *cotE* mutants still retain an intact inner coat, but they do not possess an outer coat. No specific function has been assigned to the CotC protein yet (460, 485, 486). None of the mutant spores tested showed an increased sensitivity to predation when compared to wildtype spores. We thus inferred that the outer coat is not important for resisting bacterial predation.

Next, we examined the fate of *safA* and *spoVID* mutant spores during a 24-h co-incubation with *C. necator*. The *safA* mutant spores lack an inner coat, whereas the outer coat is still present (482). Spores from *spoVID* mutants exhibit a fully mature cortex, but they typically lack both inner and outer coat (465, 487). Again, however, no change in predation resistance was apparent for any of these mutant spores (Fig. 6), suggesting that the entire spore coat is expendable as a defense against *C. necator*. To verify this, we chemically removed the coat of spores from the wildtype strain NCIB 3610. Such decoated spores typically do not tolerate treatment with lysozyme or sodium hypochlorite (469). Although we were able to confirm the lack of these properties of decoated spores, we did not observe an increased sensitivity to predation by *C. necator* (Fig. 6).

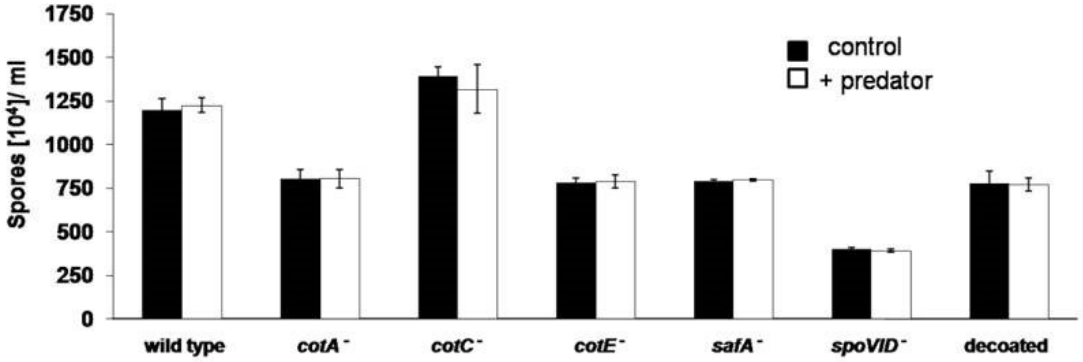

Figure 6: Survival of wild-type, mutant, or decoated spores of *B. subtilis* with or without exposure to *C. necator*.

Discussion.

After some preliminary studies in the 1980s (442, 445), the predatory behavior of *C. necator* has not received further attention, which is surprising considering the widespread occurrence of this bacterium in nature and its industrial usage as a bioplastic producer (488). The aim of this study was to verify the predatory activity of *C. necator* and to determine how specific factors affect prey killing and consumption. Another goal was to identify molecular factors and mechanisms by which prey bacteria can resist this micropredator. Initially, we observed that the nutrition status and also the prey type significantly influenced the predatory performance. A starved *C. necator* population was more likely to feed on *B. subtilis* than a culture that had previously been grown in a nutrient-rich medium. Furthermore, there was significant variation in the killing and utilization of prey on the subspecies level, which suggests some degree of specialization. Similar observations were made when the predatory myxobacterium *Myxococcus xanthus* was feeding on *B. subtilis* strains (450). In the corresponding study, the different survival rates of the prey bacteria could be traced to a strain-specific production of defensive molecules (450). The same explanation might also hold true for the two *B. subtilis* strains PS216 and RO-NN-1, which were found to be largely resistant against predation by *C. necator*. Another possibility is that the resilience of PS216 and RO-NN-1 is due to an earlier onset of sporulation in the two strains.

Previous studies revealed that Cu^{2+} stimulates the growth of *C. necator* (445)(442). Furthermore, it has been suggested that Cu^{2+} is necessary for predation (445). During our own analyses we noted that *C. necator* already shows significant predatory activity in the presence of Cu^{2+} levels as low as 0.01 µM, even though maximum killing efficiencies were only reached at a Cu^{2+}

concentration of 50 µM. In natural soil, the Cu^{2+} level is typically around 0.01 µM (489). This means that ordinary soil provides sufficient Cu^{2+} to support the predatory behavior of *C. necator*.

Bacterial predation can be distinguished by the mechanisms that are used to achieve a killing of prey bacteria (455). In general, the hunting strategies of predatory bacteria presume a physical contact with their prey, but exceptions are known as well (471). The supernatant of *C. necator* was found to exhibit strong antibacterial effects. Secreted molecules are thus likely major contributors to the killing of other bacteria. Still, it is possible that cell contact is needed for efficient prey consumption. The numerical proportion between predator and prey cells can also have a strong impact on the outcome of co-incubation experiments. In particular, bacteria practicing group predation, such as *Lysobacter* spp., must outnumber their prey to achieve appreciable killing efficiencies (467). In this study, we found that *C. necator* maintains predation at comparatively low predator-to-prey ratios. Although the killing efficiency was shown to benefit from a modest increase of the *C. necator* number, which might indicate a collaborative hunting behavior, it is evident that large consortia are not needed for predation. We thus conclude that *C. necator* does not pursue the so-called wolfpack strategy (455).

Lastly, the predatory performance also depends on the resistance of the prey. Bacteria, in general, and *B. subtilis*, in particular, have evolved a variety of strategies in order to reduce predation pressure (452). Our analyses confirmed that resistance mechanisms must not be common to all members of a species, but instead can be rather strain-specific. Sporulation might represent a conserved means of *B. subtilis* protection, which not only prevents predation by *C. necator*, but also by myxobacteria (450), protozoa (142) and nematodes (143). While sporulation-deficient strains of *B. subtilis* were readily killed by *C. necator*, an intact spore coat was surprisingly not found to be crucial for the spore resistance properties. Both chemically decoated wild-type spores and coat-defective spores from selected mutants (e.g., *cotE*$^{-}$, *safA*$^{-}$, *spoVID*$^{-}$) were not destroyed by the predatory bacterium. This observation clearly contrasts previous findings of *B. subtilis* spore resistance against *Tetrahymena thermophila* and *Caenorhabditis elegans* (142, 143). The lack of essential coat morphogenetic proteins results in lysozyme sensitivity (486, 490, 491), which might explain an increased susceptibility of the spore to enzymatic digestion. Obviously, this mechanism is not relevant for predation resistance against *C. necator*, and we hence speculate that a metabolically inactive state (i.e., persister-like cell state) is sufficient for protection.

Conclusions.

The bacterium *C. necator* pursues a hitherto unique predation strategy, which is mainly distinguished by its relation to copper(II). Nutrient deficiency or the presence of preferred prey organisms may serve as triggers for predatory behavior. The subsequent attack does not require large numbers of *C. necator*. The killing of prey bacteria is likely mediated by secreted molecules. Further analyses are necessary to determine the chemical nature of these compounds. Some *B. subtilis* strains can reduce predation pressure by *C. necator*. This resistance has been traced to sporulation.

Acknowledgements.

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CHAPTER 6

Variance of cell-cell communication networks governs adaptation to distinct life-styles in *Bacillus subtilis*.

Ramses Gallegos-Monterrosa, Tino Barchewitz, Sonja Köppenhöfer, Balázs Bálint, Péter Bihari, Gergely Maróti, and Ákos T. Kovács.

[The manuscript presented in this chapter is under preparation as a letter/short communication for future submission for publication]

Microbes commonly display great genetic plasticity, which has allowed them to colonize all ecological niches on Earth (397, 492, 493). The Gram-positive model bacterium *Bacillus subtilis* is a soil-dwelling organism that can be isolated from a wide variety of environments. An interesting characteristic of this bacterium is its ability to form biofilms that display complex population heterogeneity: individual cells develop diverse phenotypes in response to different environmental conditions within the biofilm (89, 112). Here, we scrutinized the impact of the number and variety of cell-cell communication modules of *B. subtilis* on adaptation and evolution. We examine how the Rap family of phosphatase regulators impacts sporulation in diverse niches using a library of single and double *rap-phr* mutants in competition under 4 distinct growth conditions. Using specific DNA barcodes, population dynamics were followed, revealing the impact of individual Rap phosphatases in the adaptability of *B. subtilis*.

In recent years, *B. subtilis* has become a model organism for the study of biofilms and population heterogeneity; a major reason for this is that *B. subtilis* cells commonly display diverse phenotypes within an isogenic population (54, 89). Even when grown in liquid mixed cultures, where environmental conditions are assumed to be homogeneous, *B. subtilis* cells can be found as single flagellated cells or as non-flagellated chained cells due to stochastic variation in the expression of motility-related genes (98). This population heterogeneity further increases when *B. subtilis* develops a biofilm, i.e., cells commit to particular functions, such as biofilm matrix production, exoenzyme secretion, or spore generation (54, 135). The development of these different cell types is partially due to variation in the environmental conditions that exist in diverse parts of the biofilms, which can then be seen as a collection of ecological microniches, each with its own type of specialized inhabitant (21, 25, 494).

B. subtilis possess a complex regulatory network that allows the cells in its biofilms to generate this heterogeneous population. This network is mainly controlled by the master transcriptional regulators Spo0A, DegU, and ComA. The activity of these regulators depends on their phosphorylation level; which is controlled by the activity of specific kinases that can sense a wide array of environmental and intracellular signals, and phosphorylate their corresponding response regulators accordingly. DegU and ComA are activated by kinases DegS and ComP respectively,

while Spo0A can be activated by 5 different kinases that act through a phosphorelay formed by the response regulators Spo0F and Spo0B. Furthermore, the regulatory network includes multiple cross-talk mechanisms and regulatory feedback loops that contribute to its modulation by constantly monitoring the general metabolic state of each particular cell within the biofilm (89, 112, 358).

The population-heterogeneity regulatory network of *B. subtilis* is further controlled by a family of response regulator aspartyl-phosphate (Rap) phosphatases and their cognate phosphatase-regulator (Phr) peptides. The cytoplasmic Rap proteins exert their regulatory function by inhibiting the activity of their target regulator (Spo0F, DegU, or ComA) via dephosphorylation or by directly blocking DNA binding. The Rap proteins are in turn inhibited by their cognate Phr peptides, which are produced as pre-Phr proteins that are exported to the extracellular milieu and cleaved to produce mature 5-6 amino acid Phr peptides. The Phr peptides are imported back into the cell upon reaching threshold concentrations and bind to their cognate Rap phosphatase, inducing conformational changes that inhibit its activity (186, 187). The *rap* and *phr* genes are usually found as pairs in the same loci, with the *phr* genes following and slightly overlapping the corresponding *rap* genes, and expression of both genes being transcriptionally coupled (193–195).

The Rap-Phr regulatory pairs are highly prevalent in the *Bacillus* genus, with ca. 2700 *rap* genes recently reported to be distributed among 346 *Bacilli* genomes; from those, ca. 80 different putative *rap-phr* alleles were found in the *B. subtilis* group alone (199). Only a small minority of the *B. subtilis* Rap phosphatases has been characterized, finding that they have high redundancy in their regulatory function: most of them target Spo0F, ComA, or both; and only one (RapG) is known to act on DegU (187). Interestingly, *B. subtilis* shows great genomic plasticity regarding *rap-phr* gene pairs; 127 recently compared strains of the *B. subtilis* group showed to have multiple and diverse *rap-phr* gene pairs, with an average of 11 *rap* genes per strain (199). This genetic variation among *B. subtilis* strains is not superfluous: since the Rap phosphatases modulate the activity of the main regulators of population heterogeneity, it has been proposed that the Rap-Phr pairs serve to adjust this regulatory network of *B. subtilis* to the needs of particular ecological niches (201, 495). As an example, it has been shown that *B. subtilis* strains isolated from gastrointestinal tracts of diverse animals have diverse sporulation initiation rates, with some being able to start sporulating already during logarithmic growth phase. This variation is correlated to the presence or absence of specific Rap-Phr pairs, and thus it has been suggested that the precise combination of *rap-phr* gene pairs matches the particular sporulation needs of a given niche (201).

Since the Phr peptides function as quorum sensing molecules (186), bacterial social interactions and evolutionary dynamics can lead to the gain or loss of specific *rap-phr* gene pairs in a particular genome, and to function diversification of the Rap-Phr systems (199, 200). This idea is supported by the fact that up to 75% of *rap-phr* gene pairs are located in sections of the genome related to mobile genetic elements (such as transposons), suggesting that Rap-Phr systems are commonly acquired by horizontal gene transfer among *Bacillus* strains (199); moreover, at least one *B. subtilis* Rap phosphatase (RapI) is known to promote the propagation of the mobile genetic element that contains it (496). The genetic variation in Rap-Phr systems among *B. subtilis* strains complicates the understanding of the role that the whole set of Rap phosphatases plays in modulating the population-heterogeneity regulatory network of any

particular *B. subtilis* strain. Furthermore, the best-known Rap-Phr systems have usually been studied independently from each other, in diverse genetic backgrounds, and using different experimental conditions (186, 187). Likewise, previous investigations have focused on different aspects of Rap-Phr regulation, e.g.: RapA and RapB have been mainly studied for their role in sporulation regulation (224), while RapC and RapF are known to regulate competence development (191, 192). Conspicuously, Rap-Phr systems have not been thoroughly investigated in regards to biofilm formation (187); to the best of our knowledge, only RapP has been previously shown to affect their formation (198, 216).

We were interested in determining the impact that each Rap-Phr system has on the population heterogeneity of *B. subtilis*, particularly on sporulation, and how absence or presence of different Rap phosphatases would affect the adaptability of *B. subtilis* to different growth conditions. We used *B. subtilis* DK1042 (hereafter WT), which is a transformable strain derived from the wild-type isolate NCIB 3610. Strains NCIB 3610 and DK1042 have not undergone the domestication process that other commonly used *B. subtilis* strains have. This domestication can lead to loss of genetic functions and regulation changes, including modifications to Rap-Phr systems (115, 497). WT has 11 *rap* genes encoded in its genome (*rapA* to *rapK*), additionally, it also possesses 1 more Rap-Phr system (*rapP-phrP*) encoded in its pBS32 plasmid (187, 216). We created single knock-out mutant strains of all the *rap-phr* genes, and double knock-out mutant strains that lack two *rap-phr* gene pairs in all possible combinations. In all cases both the *rap* and *phr* genes were deleted from the genome or plasmid. All created strains and WT were further tagged with a pre-defined specific DNA barcode: a randomly-generated 12 bp nucleotide sequence that was integrated into the *amyE* locus of each strain. We used an experimental competition approach to analyze how the different Rap-Phr system combinations would impact the adaptability of all mutant strains (and WT as control) to 2 different growth conditions: shaken liquid cultures, where cells would multiply in a planktonic state; or static liquid cultures, where cells would form a pellicle biofilm on the air-liquid interface. All studied strains (78 mutants + WT) were mixed together in equal ratios, and the mix was used to inoculate bottles with 100 ml of MSgg medium (for planktonic cultures), or microplate wells with 2 ml of MSgg medium (for pellicle cultures). We introduced further variability in our studied conditions by using 2 different incubation times: 2 or 5 days, at 30°C. After each incubation period, we collected spores from these cultures by incubating aliquots (from the shaken cultures), or the dispersed pellicles (from the static cultures), at 80°C for 20 minutes. We used the spores to reinoculate bottles or microplate wells that were incubated in the same conditions. These cycles were performed during 9 transfers for each culture condition. We obtained total DNA from all cultures after the 1st and 9th transfer, and from the mix used to start the experimental competition; this DNA was used to PCR amplify the *amyE* locus containing the strain-specific DNA barcodes. Using high-throughput sequencing, we were able to examine the population dynamics of all the used strains throughout the competition experiment by analyzing their representation ratio in the competing population.

We observed that all 4 studied growth conditions presented different selection regimes, which resulted in different strains being favored during the competition (Fig. 1). Due to the applied culture reinoculation regime, only cells that had formed mature spores were transferred to the next culture iteration. We expected that this would reinforce the competitive selection of each culture condition, and thus amplify the effects of small advantages that the absence or presence of particular Rap-Phr systems would confer to specific strains regarding spore

Figure 1: Heat map representation of the population dynamics of *B. subtilis rap-phr* mutants in competition. Boxes represent the population percentage of strains. Text columns at far-left and far-right indicate which *rap-phr* genes have been deleted (A indicates a Δ*rapA* mutant, AB indicates a Δ*rapA*Δ*rapB* mutant, and so on), WT indicates *B. subtilis* DK1042. Text rows on top indicate type of culture (planktonic or pellicle), incubation period (2d= 2 days, 5d= 5days), transfer number of represented population (t1 or t9), and mix and replicate number. Competition populations were started from 2 population mixes (A and B), with 6 replicates per mix. The first two box columns indicate the population representation of tested strains in the competition starter mixes. Both panels show the same data, although using different scales (shown in the top-right corner of each panel): **a)** logarithmic increment of percentage **b)** linear increment of percentage.

formation. Indeed, we observed that at the first culture transfer, no strain had increased their total population representation beyond 10% in most experimental replicates (from initial average population representations of 1.26%), in contrast, at the ninth culture transfer the majority of the experimental replicates showed at least one strain that represented more than 30% of the total population. Interestingly, strains that increased their population representation after the first culture transfer did not necessarily maintain this trend throughout the competition experiment, in both planktonic and pellicle forming conditions. This is probably caused by the evolutionary adaptation to the experimental conditions of other strains in the same population and genetic drift due to fitness advantages, which may confer them a selective advantage independent from the Rap-Phr systems; however, we note that our experimental setup offers very limited time for evolutionary adaptations to entirely explain these population dynamics. Alternatively, initial population mix conditions, where all strains are present in similar ratios, may confer slight advantages to specific strains that are overcome by others during the competition. In both planktonic and pellicle forming conditions incubation time was a critical selective parameter: populations that were incubated for 2 days showed greater variation in their final population composition than populations incubated for 5 days. Spore formation in *B. subtilis* begins with the detection of starvation conditions, however, this is not an homogeneous process in a population: cells use a bet-hedging strategy to avoid sporulation synchronization (86). *B. subtilis* strains that lack specific Rap-Phr systems show temporal differences in sporulation initiation of several hours compared to strains that have those Rap phosphatases (201). The 2-day incubation period seemed to be insufficient to trigger wide-spread sporulation, and thus only early spores of most strains were transferred into the next culture cycle. In contrast, the 5-day incubation seemed to allow specific strains to sporulate efficiently and thus be overrepresented at the start of each sequential culture cycle.

Our experimental methodology and population dynamics analysis allows for detailed examination of the role of each Rap-Phr system regarding sporulation. Since cell population determines the maximum number of possible spores, we also examined how the *rap-phr* mutations affect growth after 16 hours of incubation (Fig. 2). We observed drastically different effects on growth depending on the mutated *rap* genes; interestingly, some *rap* mutations have a consistently negative effect on growth, but this effect was rescued in specific double *rap* mutants, e.g.: a Δ*rapI* mutation has a strong negative effect on growth by itself or combined with any other *rap* mutation except Δ*rapG*; a Δ*rapG*Δ*rapI* double mutant was able to grow almost as efficiently as WT. On the other hand, single rap mutations that showed a mild effect or no effect on growth, such as Δ*rapA* and Δ*rapK*, had a drastic growth defect when combined in a double mutant strain. Likewise, we examined the effect of the *rap* mutations during pellicle formation after 2 and 5 days of incubation (Figs. S1 and S2, all supplementary material for this chapter is available in annexum C). We again observed drastic differences in the effects of single and double rap mutations upon the capacity of *B. subtilis* to form pellicles; interestingly, there was no correlation between growth and pellicle formation (compare Figs. 2, S1, and S2). Some strains that showed poor 16-hour growth, such as Δ*rapB*Δ*rapI,* were able to form stronger pellicles than strains that grew more efficiently, such as Δ*rapB*Δ*rapH*. Furthermore, mutations in diverse Rap phosphatases that target the same regulator can have drastically diverse effects on *B. subtilis* competitiveness, both individually and epistatically: RapA and RapB regulate Spo0F; however, Δ*rapA* or Δ*rapA*Δ*rapB* strains became nearly extinct in all tested conditions, while a Δ*rapB* strain increased its population representation, especially when combined with mutations in other Rap proteins that also target Spo0F, such as Δ*rapB*Δ*rapE and* Δ*rapB*Δ*rapH* mutants. It is

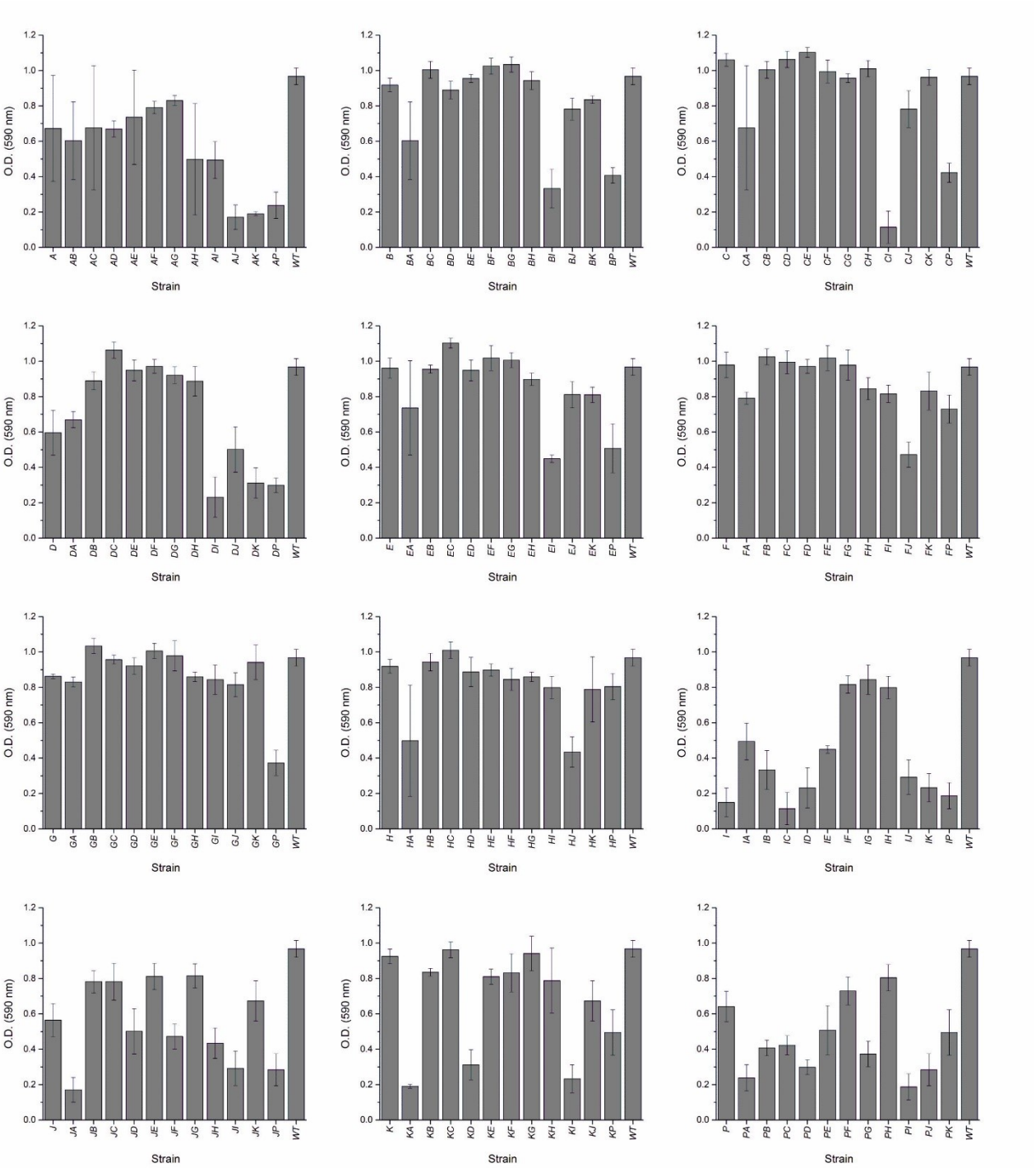

Figure 2: Growth of *B. subtilis* DK1042 and *rap-phr* mutants (as $O.D._{590}$ increment over 16 hours). X-axis indicates which *rap-phr* genes have been deleted (A indicates a Δ*rapA* mutant, AB indicates a Δ*rapA*Δ*rapB* mutant, and so on), WT indicates *B. subtilis* DK1042. Bars represent the average of 4 independent replicates. Error bars represent standard deviation.

worth noting that growth of a given mutant strain does not directly correlates with fitness in our competition experiments; e.g. a Δ*rapA*Δ*rapG* mutant strain shows better growth than a Δ*rapA*Δ*rapF* strain; however, the later shows better competitiveness and increased population representation ratio under all our studied conditions already at the first culture transfer. Importantly, the population obtained from the first culture cycle does not depend on viable spores yet, all the subsequent cultures do. All mutant strains that showed a drastic decrease in

population representation after the first culture cycle also showed poor growth (Fig. 3). Thus, the regulatory and fitness impact of any individual Rap phosphatase cannot be understood solely by knowing its target transcriptional regulator; rather, the whole set of Rap-Phr proteins must be considered in order to explain the regulation of population heterogeneity in *B. subtilis*.

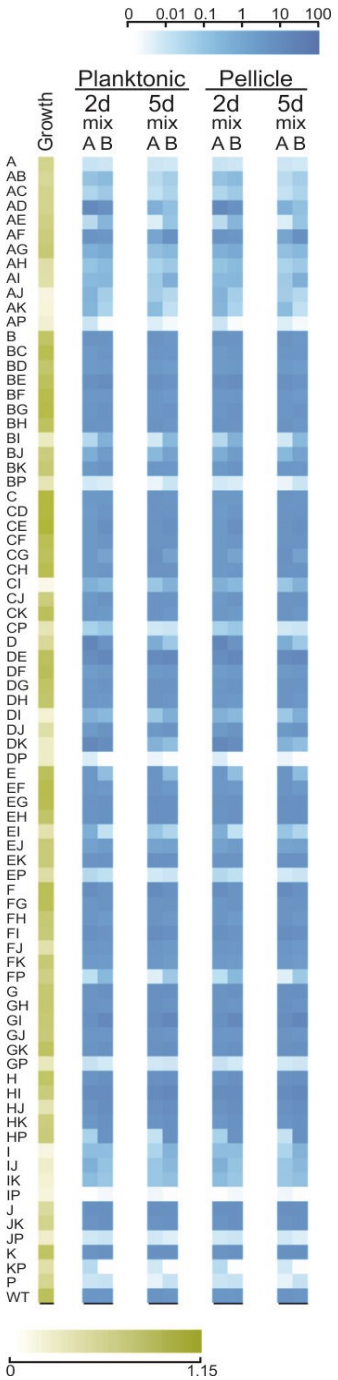

Figure 3: Heat map comparing growth of *B. subtilis rap-phr* mutants (16 hours) to their population percentage after the first culture cycle (t1). Yellow boxes represent 16-hour growth on MSgg medium as increase in $O.D._{590}$. The yellow intensity scale bar (bottom) indicates $O.D._{590}$ increment. Blue boxes represent the population percentage of all tested strains. The blue intensity scale bar (top) indicates increment of percentage. Each blue box represents the average percentage of 6 population replicates per starter mix. Text rows on top indicate type of culture (planktonic or pellicle), incubation period (2d= 2 days, 5d= 5days), and starter mix (A or B). Text columns at far-left indicate which *rap-phr* genes have been deleted (A indicates a Δ*rapA* mutant, AB indicates a Δ*rapA*Δ*rapB* mutant, and so on), WT indicates *B. subtilis* DK1042.

Finally, since Spo0A, ComA, and DegU are the master regulators of population heterogeneity in *B. subtilis*, we reasoned that mutations in their genes (or the phosphorelay genes *spo0F* and *spo0A*) could be a mechanism for *B. subtilis* to adapt to diverse environmental conditions. In such case, mutations in these genes that provide strong fitness advantages could spread in a population irrespectively of mutations in *rap-phr* gene pairs. We isolated clones from 10 replicate populations obtained at the end of the competition experiment, the chosen populations were selected randomly and they represent at least 2 replicates of each growth condition. The obtained clones were resequenced in order to detect mutations that could underlie evolutionary adaptations to the growth conditions. 7 of the resequenced clones showed diverse mutations (frameshift deletions or codon-substitutions); these changes were not clearly related to any single growth condition (see Table S1). Interestingly, a clone obtained from pellicle growth conditions showed a codon-substitution mutation in *degS*; while a clone from planktonic growth populations showed a frameshift deletion mutation in *comP*. These mutations could imply changes to the phosphorylation rates of DegU and ComA, which partially control the population heterogeneity regulatory network of *B. subtilis*. Furthermore, 3 clones (2 from pellicle growth conditions and 1 from planktonic growth conditions) showed codon-substitution mutations in the genes of proteins that are involved in spore germination by sensing available nutrients in the environment. Additionally, one clone (from pellicle growth conditions) showed a codon-substitution mutation in *yhaX*, which is a gene involved in spore maturation. These mutations in sporulation- and germination-related genes could underlie adaptive changes of *B. subtilis* to the cyclic growth-sporulation conditions used during the experimental competition. We also observed numerous mutations (single and double nucleotide changes) in the SPβ prophage region and in other prophage-like element regions of 9 out of 10 resequenced clones. The accumulation of mutations in prophage elements of *B. subtilis* and other bacterial species has been previously reported in the context of experimental evolution, and may be a common bacterial adaptive mechanism for specific lifestyles such as biofilm formation (405, 498). In particular, sporulation cycles seem to consistently promote this accumulation of prophage-element mutations in diverse *B. subtilis* strains and under different growth conditions (405, 499), further suggesting an adaptive evolutionary role. However, more investigations are needed to determine the fitness impact of these mutations. Importantly, no mutations were detected in any of the *spo0F, spo0B, spo0A, comA*, and *degU* genes of these clones. Furthermore, we compared the corresponding proteins from 100 *B. subtilis* reported genomes of both environmental isolates and laboratory strains by simple sequence alignment. This comparison revealed that these proteins are highly conserved among *B. subtilis* strains (>99% sequence identity in all compared genomes, tBLASTn (410), Figs. S3 to S7). Taken together, these results suggest that mutations in the master regulators of population heterogeneity might not be the major mechanism for *B. subtilis* to adapt to new ecological niches.

In this work we have examined how variability in the number and function of Rap-Phr pairs allows *B. subtilis* to better adapt to diverse environmental conditions. Our experimental competition approach, paired with high-throughput sequencing, allowed us to analyze the population dynamics during the competition and assess the impact of each Rap-Phr system. Furthermore, by using single and double *rap-phr* mutants we are able to better understand the epistatic effects that the presence or absence of Rap-Phr systems may have upon population heterogeneity of *B. subtilis*. Recent investigations indicate that Rap-Phr systems are readily transferred among *B. subtilis* strains; possibly helped by the natural development of competence by this bacterium, and by the ability of some Rap-Phr systems to promote their own genetic

mobility (199, 200). Our results indicate that variability in Rap-Phr systems among *B. subtilis* mutant strains strongly impacts their ability to compete in diverse environments, and that Rap-Phr systems are particularly important if adaptation depends on differentiation of particular cell types, such as spores. Over evolutionary time this could explain the genomic diversity of Rap-Phr systems and the ecological success of this widely spread bacterium. We conclude that the diversity of Rap-Phr regulatory systems of *B. subtilis* allows it to fine-tune its genetic regulatory network in order to quickly adapt to new ecological niches by acquiring those that provide the bacterium with increased fitness. Further, this exchange of elements of a regulatory family of proteins could be a general mechanism for genetically-related bacteria to more efficiently and quickly adapt to new environments.

Methods.

Strains and Media.

All strains used in this study are listed in Table 1. When fresh cultures were needed, strains were pre-grown overnight in Lysogeny broth (LB) medium (LB-Lennox, Carl Roth; 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, and 5 g L^{-1} NaCl) at 37°C and shaken at 225 r.p.m. LB medium was used for all *B. subtilis* and *Escherichia coli* transformations. MSgg medium (5 mM potassium phosphates buffer (pH 7), 100 mM MOPS, 2 mM $MgCl_2$, 700 µM $CaCl_2$, 100 µM $MnCl_2$, 50 µM $FeCl_3$, 1 µM $ZnCl_2$, 2 µM thiamine, 0.5% glycerol, 0.5% glutamate, 50 µM L-tryptophan, and 50 µM L-phenylalanine, adapted from (91)) was used for the competition experiment and to examine growth kinetics and pellicle formation. GCHE medium (1% glucose, 0.2% glutamate, 100 mM potassium phosphate buffer (pH: 7), 3 mM trisodium citrate, 3 mM $MgSO_4$, 22 mg L^{-1} ferric ammonium citrate, 50 mg L^{-1} L-tryptophan, and 0.1% casein hydrolysate) was used to induce natural competence in *B. subtilis* (373). Gallegos Rich medium was used to grow *Lactococcus lactis* MG1363, in order to purify pMH66: 21 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 8.3 g L^{-1} NaCl, 3 g L^{-1} soya peptone, 2.6 g L^{-1} glucose, and 2.5 g L^{-1} $MgSO_4 \cdot 7H_2O$ (500). Media were supplemented with Bacto agar 1.5 % when media were needed for preparing plates. Antibiotics were used at the following final concentrations: kanamycin, 10 µg mL^{-1}; chloramphenicol, 5 µg mL^{-1}; erythromycin-lincomycin (MLS), 0.5 µg mL^{-1} and 12.5 µg mL^{-1} respectively; ampicillin, 100 µg mL^{-1}; spectinomycin, 100 µg mL^{-1}; tetracycline, 10 µg mL^{-1}.

Table 1: Strains and plasmids used in this study.

Name	Characteristics	Reference
B. subtilis		
NCIB 3610	Prototroph, wild-type	BGSC
DK1042	NCIB 3610 *comI*^{Q12l}	(368)
TB499	DK1042 *rapA::km*^{r}	This study
TB396	DK1042 *rapC::km*^{r}	This study
TB315	DK1042 *rapD::km*^{r}	This study
TB339	DK1042 *rapE::spec*^{r}	This study
TB341	DK1042 *rapF::spec*^{r}	This study
TB404	DK1042 *rapG::spec*^{r}	This study
TB405	DK1042 *rapH::spec*^{r}	This study
TB272	DK1042 *rapI::km*^{r}	This study
TB274	DK1042 *rapJ::km*^{r}	This study
TB557	DK1042 *rapK::km*^{r}	This study
TB435	DK1042 *rapP::mls*^{r}	This study

TB588	DK1042 Δ*rapA*	This study
TB575	DK1042 Δ*rapB*	This study
TB410.1	DK1042 Δ*rapC*	This study
TB513	DK1042 Δ*rapD*	This study
TB407	DK1042 Δ*rapE*	This study
TB408.2	DK1042 Δ*rapF*	This study
TB412	DK1042 Δ*rapG*	This study
TB409.1	DK1042 Δ*rapH*	This study
TB444	DK1042 Δ*rapI*	This study
TB411.2	DK1042 Δ*rapJ*	This study
TB587	DK1042 Δ*rapK*	This study
TB445	DK1042 Δ*rapP*	This study
TB577	DK1042 Δ*rapB, rapA::km*^{r}	This study
TB518	DK1042 Δ*rapC, rapA::km*^{r}	This study
TB555	DK1042 Δ*rapD, rapA::km*^{r}	This study
TB517	DK1042 Δ*rapE, rapA::km*^{r}	This study
TB523	DK1042 Δ*rapF, rapA::km*^{r}	This study
TB544	DK1042 Δ*rapG, rapA::km*^{r}	This study
TB522	DK1042 Δ*rapH, rapA::km*^{r}	This study
TB545	DK1042 Δ*rapI, rapA::km*^{r}	This study
TB516	DK1042 Δ*rapJ, rapA::km*^{r}	This study
TB647	DK1042 Δ*rapK, rapA::km*^{r}	This study
TB519	DK1042 Δ*rapP, rapA::km*^{r}	This study
TB582	DK1042 Δ*rapB, rapC::km*^{r}	This study
TB578	DK1042 Δ*rapB, rapD::km*^{r}	This study
TB271	DK1042 Δ*rapB, rapE::spec*^{r}	This study
TB275	DK1042 Δ*rapB, rapF::spec*^{r}	This study
TB583	DK1042 Δ*rapB, rapG::spec*^{r}	This study
TB276	DK1042 Δ*rapB, rapH::spec*^{r}	This study
TB727	DK1042 Δ*rapB, rapI::km*^{r}	This study
TB586	DK1042 Δ*rapB, rapJ::km*^{r}	This study
TB579	DK1042 Δ*rapB, rapK::km*^{r}	This study
TB584	DK1042 Δ*rapB, rapP::mls*^{r}	This study
TB521	DK1042 Δ*rapC, rapD::km*^{r}	This study
TB542	DK1042 Δ*rapC, rapE::spec*^{r}	This study
TB454	DK1042 Δ*rapC, rapF::spec*^{r}	This study
TB436	DK1042 Δ*rapG, rapC::km*^{r}	This study
TB453	DK1042 Δ*rapC, rapH::spec*^{r}	This study
TB548	DK1042 Δ*rapI, rapC::km*^{r}	This study
TB455	DK1042 Δ*rapC, rapJ::km*^{r}	This study
TB561	DK1042 Δ*rapC, rapK::km*^{r}	This study
TB547	DK1042 Δ*rapP, rapC::km*^{r}	This study
TB503	DK1042 Δ*rapE, rapD::km*^{r}	This study
TB504	DK1042 Δ*rapF, rapD::km*^{r}	This study
TB546	DK1042 Δ*rapG, rapD::km*^{r}	This study
TB520	DK1042 Δ*rapH, rapD::km*^{r}	This study
TB550	DK1042 Δ*rapI, rapD::km*^{r}	This study
TB502	DK1042 Δ*rapJ, rapD::km*^{r}	This study
TB564	DK1042 Δ*rapD, rapK::km*^{r}	This study
TB549	DK1042 Δ*rapP, rapD::km*^{r}	This study
TB456	DK1042 Δ*rapE, rapF::spec*^{r}	This study
TB451	DK1042 Δ*rapG, rapE::spec*^{r}	This study

TB457	DK1042 Δ*rapE, rapH::spec*^{r}	This study
TB285	DK1042 Δ*rapI, rapE::spec*^{r}	This study
TB458	DK1042 Δ*rapE, rapJ::km*^{r}	This study
TB558	DK1042 Δ*rapE, rapK::km*^{r}	This study
TB283	DK1042 Δ*rapP, rapE::spec*^{r}	This study
TB452	DK1042 Δ*rapG, rapF::spec*^{r}	This study
TB543	DK1042 Δ*rapH, rapF::spec*^{r}	This study
TB472	DK1042 Δ*rapI, rapF::spec*^{r}	This study
TB495	DK1042 Δ*rapJ, rapF::spec*^{r}	This study
TB559	DK1042 Δ*rapF, rapK::km*^{r}	This study
TB293	DK1042 Δ*rapP, rapF::spec*^{r}	This study
TB433	DK1042 Δ*rapG, rapH::spec*^{r}	This study
TB474	DK1042 Δ*rapI, rapG::spec*^{r}	This study
TB434	DK1042 Δ*rapG, rapJ::km*^{r}	This study
TB566	DK1042 Δ*rapG, rapK::km*^{r}	This study
TB443	DK1042 Δ*rapG, rapP::mls*^{r}	This study
TB473	DK1042 Δ*rapI, rapH::spec*^{r}	This study
TB496	DK1042 Δ*rapJ, rapH::spec*^{r}	This study
TB560	DK1042 Δ*rapH, rapK::km*^{r}	This study
TB284	DK1042 Δ*rapP, rapH::spec*^{r}	This study
TB551	DK1042 Δ*rapI, rapJ::km*^{r}	This study
TB562	DK1042 Δ*rapI, rapK::km*^{r}	This study
TB552	DK1042 Δ*rapI, rapP::mls*^{r}	This study
TB565	DK1042 Δ*rapJ, rapK::km*^{r}	This study
TB292	DK1042 Δ*rapJ, rapP::mls*^{r}	This study
TB563	DK1042 Δ*rapP, rapK::km*^{r}	This study
TB614.BC	DK1042 *amyE*::barcode (*cat*^{r})	This study
E. coli		
MC1061	Cloning host; K-12 F$^-$ λ$^-$ Δ(*ara-leu*)7697 [*araD139*]B/r Δ(*codB-lacI*)3 *galK16 galE15 e14*$^-$ *mcrA0 relA1 rpsL150*(*Str*^{r}) *spoT1 mcrB1 hsdR2*(r$^-$ m$^+$)	(406)

Plasmid	**Characteristics**	**Reference**
pMAD_rapB	pMAD thermosensitive plasmid, *rapB-5´, rapB*-3´	provided by Stephanie Trauth, (Ilka Bischofs´ Laboratory)
pBluescript Sk(+)	Cloning vector. *Amp*^{r}*, lacZ*	Stratagene
pTB120	pBluescript Sk(+) *lacZ::lox66-neo*^{r}*-lox71*	This study
pTB233	pBluescript Sk(+) *lacZ::lox66-spec*^{r}*-lox71*	This study
pTB234	pBluescript Sk(+) *lacZ::lox66-mls*^{r}*-lox71*	This study
pTB250	pTB120 *rapI-5´-lox66-neo*^{r}*-lox71-phrI*-3´	This study
pTB251	pTB120 *rapJ-5´-lox66-neo*^{r}*-lox71-rapJ*-3´	This study
pTB252	pTB120 *rapK-5´-lox66-neo*^{r}*-lox71-phrK*-3´	This study
pTB295	pTB120 *rapD-5´-lox66-neo*^{r}*-lox71-rapD*-3´	This study
pTB310	pTB233 *rapE-5´-lox66-spec*^{r}*-lox71-phrE*-3´	This study
pTB311	pTB233 *rapF-5´-lox66-spec*^{r}*-lox71-phrF*-3´	This study
pTB349	pTB233 *rapH-5´-lox66-spec*^{r}*-lox71-phrH*-3´	This study
pTB380	pTB120 *rapA-5´-lox66-neo*^{r}*-lox71-phrA*-3´	This study
pTB382	pTB120 *rapC-5´-lox66-neo*^{r}*-lox71-phrC*-3´	This study
pTB383	pTB233 *rapG-5´-lox66-spec*^{r}*-lox71-phrG*-3´	This study
pTB414	pTB234 *rapP-5´-lox66-mls*^{r}*-lox71-phrP*-3´	This study
pMH66	pNZ124-based Cre-encoding plasmid, *Tet*^{r} *Ts*	(407)

pTB16	pDG782 derivate plasmid. *amyE* integration vector for *B. subtilis*. km^r	(160)
pNW33n	cat^r, *Geobacillus-E. coli* shuttle vector	BGSC
pTB666.1 to pTB666.80	pTB16 derivate plasmids. *amyE-5´-*cat^r (barcode)-*amyE-3´*. The barcodes are 12-bp random nucleotide sequences	This study

Note: All *B. subtilis* strains with at least one deletion of a *rap-phr* pair were tagged with a barcode using a pTB666 plasmid. The resulting barcoded strains have no additional modifications compared to their parental strain (see TB614.BC as an example, and Table S2).

Strain and Plasmid construction.

To create the single and double *rap-phr* mutant strains, plasmids were first designed that allowed to create clean-deletion mutants of all *rap-phr* gene pairs. All plasmids used in this study are listed in Table 1, and they were created using standard molecular biology techniques. Briefly, upstream and downstream DNA fragments of ~600 bp flanking the *rap-phr* genes to be mutated were PCR amplified from genomic DNA of *B. subtilis* NCIB 3610. Afterwards, these DNA fragments were sequentially cloned into plasmids pTB120, pTB233, or pTB234, all of which are pBluescript SK(+)-derived plasmids containing an antibiotic resistance cassette (kanamycin, spectinomycin, and MLS, respectively) flanked by *Cre*-recombinase recognition sites *lox66* and *lox71*. Thus, the obtained plasmids contain an antibiotic resistance cassette flanked by the upstream and downstream regions of a given *rap-phr* gene pair. All plasmids were created and maintained in *E. coli* MC1061.

B. subtilis mutants of a single *rap-phr* pair were created via transformation of DK1042 with the corresponding plasmid containing the flanking regions of the target *rap-phr* pair. Double *rap-phr* mutants were created by transforming clean-deletion mutants of single *rap-phr* pairs with genomic DNA of strains that had the desired *rap-phr* mutation still with the corresponding antibiotic resistance cassette. All *B. subtilis* strains generated in this work were obtained via natural competence transformation (373). Briefly, overnight cultures of the receiver strains grown in LB medium were diluted to a 1:50 ratio with GCHE medium, these cultures were incubated at 37°C for 4 h with shaking at 225 r.p.m. After this incubation period, 5–10 µg of genomic or plasmid DNA were mixed with 500 µL of competent cells and further incubated for 2h before plating on LB plates added with selection antibiotics. *B. subtilis* clean-deletion mutants of single *rap-phr* gene pairs were obtained by using the Cre recombinase expressed from plasmid pMH66 to eliminate their corresponding antibiotic resistance cassette, and subsequently curating pMH66 via thermal loss of the plasmid (408). Briefly, strains were transformed with 10 µg of pMH66, selecting transformants via incubation at 37°C on LB plates added with tetracycline. Candidates were then screened for their capacity to grow at 37°C on LB plates added with the antibiotic to which their parental strains (prior to transformation with pMH66) were resistant, those that were not able to grow were further incubated on LB plates at 43°C for 18 h to induce the loss of pMH66. Candidates that were then unable to grow at 37°C on LB plates added with tetracycline were considered to have lost pMH66.

In order to track each strain during the competition experiment, *B. subtilis* DK1042 and all single and double *rap-phr* mutants were marked with a randomly-generated DNA 12 bp barcode in their *amyE* locus. To do this, plasmid pTB666 was created by cloning a chloramphenicol resistance cassette (*cat*) into pTB16 (160), substituting its original kanamycin resistance cassette. The *cat* cassette was amplified from pNW33n using primers oTB118 and oTB119 (see Table 2). Primer oTB119 has a 12 nt-long random sequence after the binding site of the primer, the DNA

barcode. Thus, pTB666 carries a barcoded *cat* which is flanked by the 5´- and 3´-end of the *B. subtilis amyE* gene. 80 different clones of *E. coli* carrying pTB666 were isolated during creation of this plasmid. Each version of pTB666 from these clones was isolated and sequenced with oBC_rev in order to identify it. The various versions of pTB666 were used to transform *B. subtilis* DK1042 and all single and double *rap-phr* mutants using natural transformation as described above.

Successful construction of all used strains and plasmids was validated via PCR, sequencing, and restriction pattern analysis; and by the lack of amylase activity on LB plates added with 1% starch for the case of barcoded strains (374). All PCR primers used in this study are listed in Table 2. Primer pairs were used to amplify the indicated loci in order to confirm the proper mutation of the corresponding gene.

Table 2: Primers used in this study.

Primer	**Target locus**	**Sequence (5´→3´)**
oRGM2	*neo-lox66*	TACCGTTCGTATAATGTATGCTATACGAAGTTATAGATCAATTTGATAATTACTAATAC
oRGM7	*neo-lox71*	TACCGTTCGTATAGCATACATTATACGAAGTTATTAGAGCTTGGGTTACAGGCATGG
oRGM14	*mls-lox71*	TACCGTTCGTATAGCATACATTATACGAAGTTATAGAAACGCAAAAAGGCCATCCGTCAG
oRGM15	*mls-lox66*	TACCGTTCGTATAATGTATGCTATACGAAGTTATCCTACCGCGGGCGGCCGCACTCTTCC
oRGM16	*spe-lox71*	TACCGTTCGTATAGCATACATTATACGAAGTTATCTCGAGATCCCCCTATGCAAGGG
oRGM17	*spe-lox66*	TACCGTTCGTATAATGTATGCTATACGAAGTTATTAAAAAATTTAGAAGCCAATGAAATC
oRGM20	*rapI 5´*	ATCCTCGAGTGGTTCCTCCAAGGAGAATG
oRGM21	*rapI 5´*	ACGCTGCAGGTGACTAAGTCGTACGG
oRGM22	*rapI 3´*	ATCGGATCCAGTTGCTGCAGATCGGGTAG
oRGM23	*rapI 3´*	ACGGAGCTCACCATTGTTTGGTCGTTCTG
oRGM24	*rapI*	TTGGTGCTACTAGCAGTG
oRGM25	*rapI*	GGGCAGCAAACTCATAGTTC
oRGM26	*rapJ 5´*	ATCGGTACCTATGCCCTCTATCCGAGAGC
oRGM27	*rapJ 5´*	ACGGAATTCTGCGCGAATGAGCTTGTACC
oRGM28	*rapJ 3´*	ATCGGATCCAAAGAAGCTTGCCGAGCAG
oRGM29	*rapJ 3´*	ACGGAGCTCGTCAAGACGGGAAATAATC
oRGM30	*rapJ*	CCTCCAATGCTCCACGGAAG
oRGM31	*rapJ*	GGATAGATCGGGCAAATCC
oRGM32	*rapK 5´*	ATCGGTACCTCTTCTGTTACCGCTGAGTC
oRGM33	*rapK 5´*	ACGGAATTCAACTTCAGAAGCGATCTTAC
oRGM34	*rapK 3´*	ATCGGATCCACATCCAGGTAGCTGAAAGG
oRGM35	*rapK 3´*	ATGCGGCCGCAAACAGGATCGAGACTATTTG
oRGM36	*rapK*	GCGGTCTTTTATGTATGAAATC
oRGM37	*rapK*	GGATAGACAGGGAAGTGTAG
oRGM44	*rapD 3´*	GGATCCAAAAGCCGCTTTTTTTATCATG
oRGM45	*rapD 3´*	ACGGAGCTCTGACTGAAGCGTACAGATCG
oRGM46	*rapD*	TTGCTGCTTCAGCAGGTCTC
oRGM47	*rapD*	GCGTCTCAGAGCTTTCAAAC
oRGM48	*rapE 5´*	ATGGGCCCGGCCAATCAGCTGGATCTTC
oRGM49	*rapE 5´*	AGCTGCAGATCTTCATTCCCACTTCAG

oRGM50	*rapE* 3´	ATGGATCCTGTAACTCTCGCACCTACTC
oRGM51	*rapE* 3´	ACGAGCTCATGTTATTAGCGCCTTTGCC
oRGM52	*rapE*	TTTGCTGTGAGCCGGTGTAG
oRGM53	*rapE*	GCAATGCCAGCTTGATCTTC
oRGM54	*rapF 5´*	ATGGGCCCGATTCGCTGTAAACGCGTAG
oRGM55	*rapF 5´*	CTGAATTCGTATGCTGAATCGGCGTATG
oRGM56	*rapF* 3´	ATGGATCCGAAGTTGCACAACGAGGAATG
oRGM57	*rapF* 3´	ACGAGCTCCGGCGGCATCACGTCTAAAG
oRGM58	*rapF*	ACGGAAGAGCAATCGTTGTC
oRGM59	*rapF*	GGCCGTCCGGTTTATGTCAC
oRGM62	*rapG* 3´	ATGGATCCCGGACCATCAAACCCACTCAC
oRGM63	*rapG* 3´	ATGCGGCCGCACGGCGATTTGAATACACTTG
oRGM64	*rapG*	TGCAGTGCGGCGATTTCTTC
oRGM65	*rapG*	TATTGCGATCGGCACGCTTG
oRGM66	*rapH 5´*	ATGGGCCCTTGATACGACGGGAAATGAG
oRGM67	*rapH 5´*	CTGCAGCGCGAAGACGGTATGGCTTGAC
oRGM68	*rapH* 3´	GGATCCATTCCCCTTACAAACTTAGTG
oRGM69	*rapH* 3´	GCTCTAGAATCCGGAAGCGTTACTTCAC
oRGM70	*rapH*	CCGCTGTCAGATCCATTTGC
oRGM71	*rapH*	CCTGCTCACTCCTTACTCAC
oRGM72	*rapA 5´*	ATGGTACCCAGTATCGATGCACCTGTTG
oRGM73	*rapA 5´*	ATGAATTCCGGCTTCAGCGACGTGGAAC
oRGM74	*rapA* 3´	GTTCTAGATGCGGCACGCAATCAAAC
oRGM75	*rapA* 3´	CTGAGCTCAGGCTTCAGCTGCCTCATAC
oRGM76	*rapA*	CGCGGCATTCTGTTATATGG
oRGM77	*rapA*	TCCAGTCCTGATGCTTTCTC
oRGM84	*rapC 5´*	ATGGTACCGACGACGATCAACGGTTTGG
oRGM85	*rapC 5´*	ATCTGCAGTTGACCGACCGCTGAAGAAG
oRGM86	*rapC* 3´	ATGGATCCCTAATGCGGAAGCACTCGAC
oRGM87	*rapC* 3´	ATGAGCTCGGATTTGCATGCCGATGAAG
oRGM88	*rapC*	AATCGAGCGCCTTGAGAAGC
oRGM89	*rapC*	TCGGGAATCGATGACATGAC
oRGM90	*rapD 5´*	ATGGTACCTTCCGAAAGCGCCGCCTATC
oRGM91	*rapD 5´*	ATCTGCAGCGGAATACCACTCGTCTAAC
oRGM92	*rapG 5´*	ATGTCGACCGCACATTGTGAGCGCTACC
oRGM93	*rapG 5´*	ATCTGCAGTGATGGCAAGGTACCAATCG
oRGM94	*rapP 5´*	ATGGGCCCTCCCAATCGTTTGGAGAAAG
oRGM95	*rapP 5´*	ATGAATTCTGGGATTAAATCCGAAAC
oRGM96	*rapP* 3´	ATGGATCCACTTATAAGGTCGCAGATAG
oRGM97	*rapP* 3´	ATGAGCTCGGGCTGCATATAAATAATAAG
oRGM98	*rapP*	TCCAACGTGCAGTGGAAAGG
oRGM99	*rapP*	CTTCACTCAAGAAGAACAAG
oTB118	Cm^R	GATCAGATCTCCGGCGTAGAGGATCTGG
oTB119	Cm^R (barcode)	CACGAAGCTTGCNNNNNNNNNNNNTATCATCGGCAATAGTTACCC
oTB120	*amyE*	GAGGAAGCGGAAGAATGAAG
oTB121	*amyE*	TTCGGTAAGTCCCGTCTAGC
oBC_fw	barcode locus	TGCGGTGATTGTTAGGTTGAGGCCGTTGAG
oBC_rev	barcode locus	AGTCAGTCAGCCAGGAGGCTTACTTGTCTG
oBC1	barcode locus (fw)	AATGATACGGCGACCACCGAGATCTACACATCGTACGTGCGGTGATTGTT AGGTTGAGGCCGTTGAG
oBC2	barcode locus (fw)	AATGATACGGCGACCACCGAGATCTACACACTATCTGTGCGGTGATTGTT AGGTTGAGGCCGTTGAG

oBC3	barcode locus (fw)	AATGATACGGCGACCACCGAGATCTACACTAGCGAGTTGCGGTGATTGTTAGGTTGAGGCCGTTGAG
oBC4	barcode locus (fw)	AATGATACGGCGACCACCGAGATCTACACCTGCGTGTTGCGGTGATTGTTAGGTTGAGGCCGTTGAG
oBC5	barcode locus (rv)	CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCAGGAGGCTTACTTGTCTG
oBC6	barcode locus (rv)	CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGTCAGCCAGGAGGCTTACTTGTCTG
oBC7	barcode locus (rv)	CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGTCAGCCAGGAGGCTTACTTGTCTG
oBC8	barcode locus (rv)	CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGTCAGCCAGGAGGCTTACTTGTCTG
oBC9	barcode locus (rv)	CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGTCAGCCAGGAGGCTTACTTGTCTG
oBC10	barcode locus (rv)	CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAGTCAGCCAGGAGGCTTACTTGTCTG
oBC11	barcode locus (rv)	CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGTCAGCCAGGAGGCTTACTTGTCTG
oBC12	barcode locus (rv)	CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAGTCAGCCAGGAGGCTTACTTGTCTG
oBC13	barcode locus (rv)	CAAGCAGAAGACGGCATACGAGATGTCGTAGTAGTCAGTCAGCCAGGAGGCTTACTTGTCTG
oBC14	barcode locus (rv)	CAAGCAGAAGACGGCATACGAGATTAGCAGACAGTCAGTCAGCCAGGAGGCTTACTTGTCTG
oBC15	barcode locus (rv)	CAAGCAGAAGACGGCATACGAGATTCATAGACAGTCAGTCAGCCAGGAGGCTTACTTGTCTG
oBC16	barcode locus (rv)	CAAGCAGAAGACGGCATACGAGATTCGCTATAAGTCAGTCAGCCAGGAGGCTTACTTGTCTG

Experimental competition.

The experimental competition was done using the barcoded versions of *B. subtilis* DK1042 and the single and double *rap-phr* mutants. The experiment was initiated from 4 different starter mixes. Each mix was obtained by mixing overnight cultures of all the competing strains in similar ratios after adjusting their $O.D._{600}$ to 1.0. Each starting mix was used to inoculate 100 ml bottles (0.5 ml of mix + 9.5 ml of MSgg medium) and 2 ml microplate wells (100 µl of mix + 1900 µl of MSgg medium). The experimental competition used 4 growth conditions: planktonic growth (10 ml culture in bottles shaken at 200 rpm) or pellicle development (static 2 ml in 24-well microplate), and incubation for 2 or 5 days. 24 replicate populations were used for each growth condition (6 replicates from each starting mix). All cultures were incubated at 30°C throughout the experiment.

After each incubation period, spores obtained from each population replicate were used to inoculate the next iteration of the same population. For this, pellicles obtained from the microplate cultures were collected in Eppendorf tubes with 1 ml of MSgg medium and sonicated until the pellicles were completely dispersed; afterwards, 100 µl aliquots from the dispersed pellicles, or 500 µl aliquots from the planktonic culture bottles were incubated at 80°C for 20 minutes in order to kill al vegetative cells. After the incubation period, the heath-treated aliquots were used to inoculate new 100 ml bottles or 2 ml microplate well using the same volumes as during the initiation of the experiment. This regime was followed during 9 culture reinoculation

cycles. Additionally, DNA was obtained from aliquots of the starter mixes and from aliquots of each population replicate obtained before the heath treatment during the 1st, 3rd, 5th, 7th, and 9th culture reinoculations. The DNA extraction was performed with the GeneMatrix Bacterial and Yeast Genomic DNA Purification Kit (EURx Ltd., Poland) with the following modifications to the manufacturer's instructions: step 2, added 10 µl of lysozyme (10 mg ml^{-1}); step 3, extended the incubation time to 25 min; step 6, extended the incubation time to 45 min.

48-plex high-throughput barcode sequencing.

The *B. subtilis amyE* locus containing the barcodes was PCR amplified from the DNA samples obtained from the competition experiment using primers oBC1 to oBC4 and oBC5 to oBC16. These primers contain distinct 5-bp (for oBC1-4 primers) or 7-bp (for oBC5-16 primers) sequences that allowed us to identify up to 48 individual replicate populations per Illumina sequencing run. Data analysis was carried out using the R statistics environment (501). PCR products, each represented by one R1-R2 Illumina sequence pair, were looked up for the presence of the 79 barcodes that differentiate between the 79 bacterial strains used in the study (see Table S2 for the barcode sequences of each strain). We linked a PCR product to a given barcode if at least one of its paired-end reads displayed 100% sequence identity over the entire length of the barcode. PCR products that gave ambiguous results (multiple hits against more than one barcodes) were excluded from the study. Figures 1 and 3 were prepared with Genesis (502).

Note: In this dissertation chapter, only the results of 12 population replicates (from starter mix A and B) at transfer points 1 and 9 are shown and discussed. Once the remaining sequencing data becomes available it will be fully shown and discussed in the version of this manuscript that will be submitted for publication. We note that the data presented here already includes the starting and end points of our experiments, and therefore we believe the discussed conclusions are valid.

Growth kinetics.

We examined the ability of all barcoded strains to grow on MSgg medium in order to assess the impact of the *rap-phr* mutations. Overnight LB liquid cultures of all barcoded strains were adjusted to $O.D._{600}$ 0.2. 10 µl of the O.D. adjusted cultures were added to 190 µl of MSgg liquid medium in 200 µl microplate wells. These cultures were incubated at 30°C for 17 hours with shaking. Cell growth was measured as $O.D._{590}$ change every 15 min using a Tecan Infinite 200Pro microplate reader (Tecan Group Ltd., Switzerland).

Pellicle formation.

We examined the ability of all barcoded strains to form pellicles on MSgg medium. Overnight LB liquid cultures of all barcoded strains were adjusted to $O.D._{600}$ 0.1. 20 µl of the O.D. adjusted cultures were added to 2 ml of MSgg liquid medium in 2 ml microplate wells. These cultures were incubated at 30°C for 5 days. After 2 days of incubation and at the end of the incubation period the obtained pellicles were examined with an Axio Zoom V16 stereomicroscope (Carl Zeiss, Germany) equipped with a Zeiss CL 9000 LED light source, a PlanApo Z 0.5× objective, and AxioCam MRm monochrome camera (Carl Zeiss, Germany).

Resequencing of selected clones.

10 populations from the end of the competition experiment were selected randomly (representing at least 2 replicates of each growth condition). Aliquots of the selected populations were used to inoculate LB plates that were incubated overnight at 30°C in order to

isolate clones from each population. Overnight LB liquid cultures of the isolated clones were used to extract genomic DNA using the GeneMatrix Bacterial and Yeast Genomic DNA Purification Kit (EURx Ltd., Poland). Paired-end fragment reads (2 × 150 nucleotides) were generated using an Illumina NextSeq sequencer. Primary data analysis (base-calling) was carried out with "bcl2fastq" software (v2.17.1.14, Illumina). All further analysis steps were done in CLC Genomics Workbench Tool 9.5.1. Reads were quality-trimmed using an error probability of 0.05 (Q13) as the threshold. In addition, the first ten bases of each read were removed. Reads that displayed ≥80% similarity to the reference over ≥80% of their read lengths were used in mapping. Non-specific reads were randomly placed to one of their possible genomic locations. Quality-based SNP and small In/Del variant calling was carried out requiring ≥8× read coverage with ≥25% variant frequency. Only variants supported by good quality bases (Q ≥ 20) were taken into account and only when they were supported by evidence from both DNA strands.

Protein sequence comparison of ComA, DegU, Spo0A, Spo0B, and Spo0F.

We used tBLASTn (410) to compare the protein sequences of Spo0A, Spo0B, Spo0F, ComA and DegU from DK1042 against 100 reported genomes of *B. subtilis*. The tBLASTn was done against the *B. subtilis* group (taxid: 1423) of the reference genomic sequences (refseq_genomic) database of NCBI. Figures S3 to S7 were prepared with Jalview (503).

GENERAL DISCUSSION

The history of microbiology, although short compared to other sciences, has been one of enthusiastic discovery of an extensive world that exists alongside our own macroscopic dimensions (8, 504). The realm of the smallest organisms has proved to be complex and very dynamic, and due to the incredible large variety of microorganisms and quick evolutionary changes, it could be in fact more sophisticated than the macroscopic world. This statement is no mere literary license, the latest estimations put the number of microbial species in the trillions (505), while macroscopic species are usually estimated to be in the low millions (up to 20 millions) (506). Further, many microbes have reproductive cycles of a few hours, while most macroscopic species require several days before they can reproduce. These short reproductive cycles foster genetic diversity and evolutionary adaptation to shifting environments, thus keeping microbes in constant change (507, 508).

Part of the complexity of the microbial world stems from the ability of microorganisms to sense the state of their environment, and respond accordingly. Although this has been systematically studied for decades, it is only recently that scientist have been able to explore how entire communities of microorganisms respond to environmental changes, and especially how microorganisms interact with their peers and with members of other species. As showcased during this dissertation, microbial interactions can take on many forms; from cooperative interactions among members of the same species to form a biofilm, to predatory relations where one bacterium can hunt and consume another. In this final section I offer an extended discussion on the studies presented in this dissertation, from the general perspective of cellular interactions, and with emphasis on how these studies represent different facets of this topic.

1. Scouting and shaping the environment.

One of the first recognized characteristics of bacteria and other microbes was their capacity to sense their surroundings and react to them. Early bacteriologists, such as Robert Koch and Louis Pasteur, already recognized the ability (or lack thereof) of diverse substances to sustain microbial growth during their early attempts to develop stable laboratory cultures of bacteria (6, 7). These experiences (along with the posterior sophistication of laboratory media) showed that bacterial species have a wide variety of preferences and capacities to utilize different substances as nutrients. Later studies have also demonstrated that bacteria have a wide variety of signal receptors that enables them to perceive their surroundings, a phenomenon known as chemosensing. Furthermore, most bacteria do not simply wait for the proper nutrients to become available; they also have motility mechanisms that allow them to actively explore their surroundings, looking for their preferred food source. This mechanism is known in general as chemotaxis (509, 510).

Chemosensing is the first step for the establishments of microbial interactions. Bacteria can detect (and respond to) not only nutrient sources, but a wide variety of compounds that are important for cell life; e.g., they can sense signals that indicate the presence of toxic products, related bacterial cells, non-bacterial organisms, and general cues that give them information

about the environment such as dwindling nutrients or stress factors from neighbor cells (509, 511–514).

The microbial relationships that can arise from the detection of environmental signals are varied, especially because they can be unilateral, where not all partners react in a similar fashion or only one of them receives a benefit or is affected. An example of this case is commensalism; a type of relationship between organisms where one partner, known as the commensal, receives a benefit such as nutrients, while the partner that provides the resource, known as the host, receives no benefit nor harm from the commensal (515, 516). Examples of commensalism abound among microbial interactions; microbial communities often portray cases of species that can use metabolites produced by other organisms as nutrients. In the case of communities with high cell density (such as biofilms) these interactions may be particularly relevant if the involved metabolite is toxic for the producing partner. These relationships were both partners receive different types of benefits (nutrients or waste-removal) are known as mutualism (174). Interestingly, interactions are not always stable among microbes; many species of free-living bacteria can use various carbon sources to support their metabolism, and therefore the nature of the relationship among them often depends on environmental factors such as nutrient abundance and environment structure (517, 518). As an example, *Pseudomonas putida* R1 and *Acinetobacter* C6 can both utilize benzyl alcohol as their sole carbon and energy source; when grown together in a chemostat with lows amounts of the alcohol these microbes will compete for the nutrient; however, when grown as biofilms, the organisms establish structured surface-attached consortia, in which *Acinetobacter* C6 remains close to the bulk liquid with high concentrations of benzyl alcohol, and *P. putida* R1 utilizes benzoate leaking from *Acinetobacter* C6 (519). This case highlights how flexible microbial interactions can be as compared to interactions between macroscopic organisms, where the roles of predator-prey or parasite-host tend to be more stable.

As highlighted in chapter 1 and exemplified in chapter 3, bacteria rely on environmental cues to make the decision to change their lifestyle towards biofilm formation, and these cues oftentimes are provided by other organisms. While working with microbial communities it can be hard to ascertain if the metabolites that an organism produces are simple waste products that the cell needs to excrete; or if the products are secreted in order to provide the cell with an additional benefit, such a resource scavenging or competition killing. In any case, cells that detect these cues can interpret and react to them in different ways. Biofilm formation seems to be a common reaction to environmental insults in many bacterial species; this is not surprising when one considers the increased survivability of cells living in a biofilm (20, 46). However, the formation and maturation of biofilms is rarely a straightforward process that can be traced back to a single trigger signal.

The interaction studied in chapter 3 between *Lysinibacillus fusiformis* M5 and *Bacillus subtilis* 168 is a good example of how intricate the study of sociomicrobiology can be. During this study we originally set up to look for interacting partners that would produce biofilm-promoting signals recognized by *B. subtilis*; this expectation can be easily glanced from our experimental approach: we screened our soil samples looking for partners that could form stable communities with *B. subtilis,* and that would produce diffusible compounds able to induce increased architectural complexity in colonies of *B. subtilis*. Upon identifying *L. fusiformis* M5 we carried forward our investigations by analyzing the role that the Kin histidine-sensor kinases would have in

this process. As thoroughly described in chapter 1, the Kin kinases are closely involved in biofilm formation by sensing diverse environmental and intracellular signals, and activating the main promotor of biofilm matrix production Spo0A. Our rationale was that at least one of the kin kinases would sense the signals produced by *L. fusiformis* M5 that induce biofilm matrix production. However, our results suggested that the Kin kinases were not involved in the observed phenomenon. Identification of the signaling molecule as the purine hypoxanthine, and posterior analysis of its metabolic effect on *B. subtilis,* finally suggested that the observed induction of increased architectural complexity may be a consequence of toxicity and cell death derived from the excess of hypoxanthine in *B. subtilis*. Therefore, although the phenotypic change observed in *B. subtilis* was the expected one, i.e.: increased architectural complexity in colony biofilms; the mechanism that promotes this behavior did not proceed through the expected increased expression of biofilm-matrix genes. Rather, a metabolic response of *B. subtilis* 168 to environmental alterations brought forward by *L. fusiformis* M5 seems to be responsible for a phenotypic change similar to the one that may be produced by an overproduction of biofilm matrix.

Our study does not reveal the effects of *B. subtilis* upon *L. fusiformis* M5; both of these bacterial species are known inhabitants of soil environments, and thus it is not unlikely that they would find themselves sharing the same ecological niche. *B. subtilis* is a known producer of diverse antimicrobial compounds (87), and indeed we commonly observed that this organism would kill interaction partners during our screening of soil samples. On the other hand, hypoxanthine is a valuable cellular metabolite, and thus it seems unlikely that *L. fusiformis* would freely excrete large amounts of this purine to the medium, even if its cells produce abundant amounts of it. However, a possibility to explain the observed interaction between these organisms is that *B. subtilis* could produce antimicrobials that would kill at least some portion of the *L. fusiformis* M5 cells, thus releasing their hypoxanthine to the intercellular milieu. This would have the unintended consequence of providing *B. subtilis* with toxic amounts of hypoxanthine and cause cell stress and death. This hypothesis could explain the observed survival of *L. fusiformis* M5 when co-cultured with *B. subtilis*, and the increased formation of wrinkles in the *B. subtilis* colony areas with presumably high concentrations of hypoxanthine: the interaction areas when the 2 species were cultured as neighboring colonies, and the areas close to the discs infused with the tested compounds. Furthermore, this would also explain a phenomenon observed during our investigations: although the cell-free supernatant of *L. fusiformis* M5 induces an increased formation of wrinkles in *B. subtilis* colonies, this effect was not as pronounced as the one observed when co-culturing both bacteria. This could be because the cell-free supernatant of *L. fusiformis* M5 would contain only the excess of hypoxanthine provided by cells lysed during their normal growth cycle or during the purification process; while the co-culturing would increase *L. fusiformis* M5 cell death and lysis caused by *B. subtilis*. Investigating if hypoxanthine is readily produced and exported by *L. fusiformis* M5 would shed light upon this last point. A mutant strain unable to synthetize or export this purine could be used to test the hypothesis: if the mutant is still able to induce the formation of wrinkles in *B. subtilis* it would suggest that *L. fusiformis* M5 cell lysis (possibly caused by *B. subtilis*) is necessary to liberate the hypoxanthine. Although genetic systems need to be developed to work with *L. fusiformis* M5, this dissertation offers the sequenced genome of this organism (see chapter 4) as a stepping stone towards this goal.

As can be realized from the case discussed above and from the review on the multiple signals that can influence biofilm formation, unraveling how microbes interact in real ecological niches

requires careful investigation of how cells sense and influence each other, including the products that they release to the environment. In particular, better knowledge of the cues that induce beneficial biofilm development may allow a simplified and economical promotion of their formation without the need of establishing exogenous microbial communities in complex ecological niches, which may involve risks for the native community.

2. Living with the family – kin relationships and differentiation.

As amply described during this dissertation, biofilm formation is a complex process that, once accomplished, is characterized by high cell densities and elaborate structures. Successful formation and maintenance of a biofilm is dependent on intricate interactions between its forming members, which need to be able to both detect and produce diverse communication signals (46). Proper interpretation of this signaling network leads to the production of multiple substances that further complicates the interaction among the cells. Since the production of these substances has a metabolic cost associated to it, and some of them can be reutilized as nutrients, the sociomicrobiology of biofilms goes beyond communication networks, even when dealing with populations of one single bacterial species (168, 277).

Social theory and microbiology have come together in recent years as social scientists recognize the usefulness of microbial research models to probe social inquiries; at the same time, microbiologists have recognized the value of social theory when trying to explain microbial interactions in large populations (12). In the case of bacterial biofilms, a particularly attractive topic for sociomicrobiologists is the production of the diverse compounds and polymers that are needed to produce the biofilm matrix. The production of these matrix components usually requires an important metabolic investment from the producing cell: it has to procure the nutrients required for the production of the component, as well as produce the enzymes required for its synthesis and secretion. However, once this has been accomplished, the secreted matrix component usually becomes a "public good", i.e.: any other cell in the vicinity may benefit from it. If all the cells in the biofilm produce the same public good at similar rates, then this is not a problem, since all would be considered as cooperators in the production of the matrix component; however, this is normally not the case (11, 174). For example, *B. subtilis* needs to produce different matrix components in order to form a mature biofilm. Further, it also needs to produce different enzymes that are necessary to provide the biofilm with nutrients obtained from the supporting surface. As a consequence, *B. subtilis* generates a wide population heterogeneity in order to fulfill these requirements (89, 112). The interesting aspect of this behavior, from a social theory point of view, is that the production of these diverse biofilm components involves equally diverse metabolic costs to the cells, but they all benefit from the same public goods. Thus, those cells that provide the metabolically cheapest public good would receive a larger benefit from forming part of the biofilm population, since they can devote more resources to reproduction. Following this idea, it would be in the best interest of all cells to produce the cheapest possible public good or minimal amounts of their corresponding public good. Taking this idea to its logical conclusion, cells that do not contribute at all to produce the matrix components (which may be called "social cheaters") would be maximally benefitted from the efforts of the rest of the population.

The problem however is that, in order to produce a mature biofilm, all public goods must be provided in appropriate amounts. Furthermore, if all cells produce the same cheapest public good, the biofilm may not form properly, and thus the population would be exposed to environmental aggressions. This would risk the collapse and death of the whole population, no matter how much benefit individual cells may have received from economizing metabolic costs (11, 166, 226). Therein lays a central question for social theory and sociomicrobiology: how do cells balance what is best for them as individuals, with that which may be best for the community?

In the case of *B. subtilis* biofilms, their complex population heterogeneity and spatiotemporal arrangement makes them a particularly interesting study model for bacterial interactions in single-species populations. To begin with, there is a large amount of available *B. subtilis* strains, many of which have been at least partially characterized. Importantly, these strains show wide differences regarding their ability to form biofilms. A very simple classification divides them into wild-type and laboratory strains; the main difference being that laboratory strains are usually considered to have been domesticated through cultivation under laboratory conditions, while the wild-type strains resemble more closely the characteristics of strains that may be isolated from soil samples (115, 217, 362). Domesticated strains have traditionally been considered as poor biofilm formers due to mutations in key biofilm-related genes that affect the production of public goods, such as the exopolysaccharide matrix component, and other social traits, like quorum sensing molecules such as Phr peptides (115). The classification of *B. subtilis* strains into two categories ignores a spectrum of continuous change that can be further modified by environmental conditions.

As shown in chapter 2, we compared the biofilm forming capabilities of several *B. subtilis* 168 variants. This strain has been commonly used by research laboratories to investigate a wide array of microbiological topics (85, 362). Strain 168 is normally considered as a domesticated strain: it has been grown under laboratory conditions for many generations and continuously selected for increased fitness in such conditions. Further, researchers have selected for desirable characteristics during this domestication process, such as increased competence development and ease of genetic manipulation (115, 217, 362). This selection has sometimes been purposeful, such as in the original selection of lineages with increased transformability after X-ray mutagenesis (359); but it can also be unintentional, e.g., while choosing candidate clones to obtain a specific *B. subtilis* mutant, a researcher may unwittingly select for mutations that improve transformability and are carried along in the selected clone. The domestication process of *B. subtilis* 168 has also endowed it with mutations that impact biofilm-formation. A variant of strain 168 studied by Anna L. McLoon *et al*. found that it had mutations in the *sfp, epsC, swrA*, and *degQ* genes; and that it completely lacked the *rapP-phrP* regulatory pair (normally carried by a plasmid). The examined variant showed poor biofilm capabilities, both as colonies and as pellicles. When the mutations were repaired by replacing them with a wild-type allele (from *B. subtilis* strain NCIB 3610), and the *rapP-phrP* gene pair introduced in the *amyE* locus, the biofilm forming capabilities of strain 168 were restored (115). Although the phenotypic characteristics of various *B. subtilis* strains had been described before, this report was the first to establish the specific genetic differences between a domesticated strain of *B. subtilis* (168) and a wild-type one (NCIB 3610). As a consequence of this study, strain 168 was established as a deficient biofilm former among biofilm researchers, and discarded as a model. However, strain 168 is still commonly used to probe other questions related to *B. subtilis* physiology, and we observed a

wide variation among the appearance of its colonies in those reports. Further, our own laboratory experience with *B. subtilis* 168, and conversations with fellow researchers comparing growth methods for *B. subtilis* suggested the existence of a broad variety of phenotypes that could be displayed by this strain.

Our comparison of several variants of *B. subtilis* 168 showed that classifying strains into categories, such a domesticated and wild-type, is subjective and dependent on changing conditions. We showed that the medium and growth conditions are in fact more important for the formation of a biofilm that some of the previously reported genetic differences. Further, there is genetic variation regarding some of the biofilm-related genes previously reported to be mutated in strain 168, some variants actually have the wild-type allele of *epsC*, while still having the additional reported mutations in *sfp, swrA*, and *degQ*. Interestingly, all of the mutations reported in the original study of McLoon are related to the population heterogeneity regulatory network of *B. subtilis*, and thus impact the social interactions among its own cells: *epsC* codifies for an epimerase necessary for the production of the exopolysaccharide matrix component, and its expression is controlled by Spo0A (520); both *degQ* and *swrA* are involved in DegU regulation, which controls the mobility/sessile lifestyle switch (98, 521); *sfp* is required for proper production of surfactin, which influences both motility and biofilm matrix production (520); and *rapP-phrP* plays a regulatory role on both Spo0A and ComA (198). This highlights the importance of cell-cell interactions in the shaping of any community: small changes in the production of public goods and regulatory mechanisms will lead to drastically changed phenotypes. However, it is worth noting that these changes are dependent on environmental factors. As discussed previously, the production of all biofilm matrix components usually implies a metabolic cost, but it is important to consider that these costs may not always be the same. We have shown how certain rich media, and even specific types of nutrients (such as carbon sources), seem to alleviate the apparent deficiencies of *B. subtilis* 168. In particular, it is noteworthy that some 168 variants were able to colonize plant roots as efficiently as the wild-type strain NCIB 3610. The relationship between plants and the organisms living in their rhizosphere is of increasing scientific interest, both due to commercial interests (crop protection), and to the complexity of these interactions (522). Plants are able to produce complex carbohydrates in their root systems, which can be used to select for specific microbial colonizers (320, 512). Thus, it may be that *B. subtilis* strains with some of the genetic markers for domestication are still able to colonize and form biofilms on plant roots of soil ecosystems, just like the so-called "wild-type" strains. If this is the case, then the whole "wild-type vs domesticated" strain classification should be discarded, as it would only be useful in regard to specific growth conditions and environments. In any event, our comparison of various *B. subtilis* 168 variants has shown that researchers should be careful when establishing classifications or disregarding organisms as research models. In particular for complex phenomena such as biofilm formation, multiple conditions need to be tested before giving a verdict. To paraphrase a famous quote: if you judge a fish for its ability to climb a tree, it will spend its whole life thinking it is inadequate.

The impact of *B. subtilis* genetic variability on sociomicrobiology has another important facet: ecological adaptation and evolution. The population heterogeneity regulatory network of this bacterium is partially controlled by the Rap-Phr family of aspartate phosphatases and their cognate peptidic regulators. This family of regulatory systems is simultaneously wide spread among *B. subtilis* strains and highly conserved, with a recent study finding more than 80 different *rap-phr* clusters in the *B. subtilis* taxonomical group, and a shared sequence homology of ~45%.

B. subtilis strains normally have 11 *rap-phr* gene pairs, and most of them regulate Spo0A or ComA activity (187, 199). However, this apparent regulatory redundancy is not superfluous; rather, studies suggest that differences in *rap-phr* content mas be responsible for important phenotypic differences among otherwise genetically similar *B. subtilis* strains (201, 495). As an example, it has been shown that sporulation rates can vary greatly among *B. subtilis* strains isolated from different environments. Cláudia R. Serra *et al.* compared the rates of sporulation initiation between reference *B. subtilis* strain NCIB 3610 and strains isolated from the gut microflora of diverse animals. Her team found that a strain obtained from chickens (called BSP1) would normally initiate sporulation during exponential growth phase, thus achieving high rates of mature spore formation earlier that other strains. Importantly, all studied strains achieved similar levels of spore formation after long incubation times. This behavior is at first perplexing, since spore formation is a non-reversible cell process that implies a temporal arrest of cell growth and important reallocation of metabolic resources. This behavior, especially if it occurs when cells could still grow and reproduce, seems counter-intuitive to the general idea of always maximizing cell growth as a direct indicator of fitness. However, when one considers that this is a chicken gut isolate, and that the gastrointestinal tract of these animals is rather short compared to other animals, then it makes sense (from an adaptive point of view) that cell lineages that initiate sporulation at a relative early stage may actually increase their fitness for this environment. This is supported by 2 reasons: first, as the cells finish their transit through the intestinal tract of the chicken and are expelled with the feces, they would suddenly find themselves in a much different environment that desiccates very fast and could possibly kill them, thus spores have a better chance of survival; second, if these strains of *B. subtilis* colonize the gastrointestinal tract of chickens as a normal phase of their life cycle, then they also need to sporulate efficiently in order to survive the digestive process of the animal and reach the sections of the gastrointestinal tract where they can germinate and reproduce. Interestingly, strain BSP1 lacks 3 different *rap-phr* gene pairs that are present in strain NCIB 3610 (*rapE*, *rapI* and *rapK*, with their respective *phr* genes). Genetic examination showed that these differences were mainly responsible for the observed differences in sporulation initiation timing among various *B. subtilis* strains (201).

The study of Serra, and other investigations with similar findings about *rap-phr* genetic variability among *B. subtilis* strains living under diverse environmental conditions, lead to the general hypothesis that Rap-Phr regulator pairs may serve as a mechanism for quick adaptation to new environments by fine-tuning the population heterogeneity of this bacterium (201, 495). This idea is supported by recent findings showcasing how the wide diversity of *rap-phr* gene pairs can be generated and maintained by the natural competence development of *B. subtilis* in combination with mobile genetic elements that contain these *rap-phr* gene pairs (199, 200). Furthermore, *B. subtilis* is a soil bacterium widely distributed among soil ecosystems, and thus likely to face a broad set of adaptive challenges. Therefore, the existence of a flexible mechanism to control its population heterogeneity regulatory network could be a great advantage for this bacterium when colonizing new ecological niches.

One lingering problem for understanding how the whole family of Rap phosphatases plays its regulatory role in *B. subtilis* is that these proteins have previously been studied independently from one another. This complicates the direct comparison of results, since they are usually obtained with different experimental conditions and strains. I present in chapter 6 the first comprehensive study of how the whole set of Rap-Phr systems impacts competitiveness and adaptability of one single *B. subtilis* strain. Furthermore, by using experimental competition with

various growth conditions, we were able to infer the roles of independent Rap proteins on spore formation, and provide insights into how the whole network may function in other *B. subtilis* strains.

Our study reveals new differences among Rap systems that were previously known to target the same transcriptional regulator or phosphorelay protein. For example, the RapA and RapB proteins are known regulators of Spo0F, they both are able to dephosphorylate this protein, thus preventing the activation of the master biofilm matrix transcriptional regulator Spo0A (187). Our study reveals that RapA and RapB have a differential impact on adaptability: mutants lacking *rapA* tend to become extinct under our experimental conditions, while mutants in *rapB* usually increased their population representation. Interestingly, this effect was in general more pronounced in the planktonic conditions than in the pellicle forming conditions. These results suggest that important differences exist between RapA and RapB regulation, despite their apparently similar activity. The results regarding differential selectivity between the studied conditions suggest different regulatory capacities, i.e. RapA may be more efficient than RapB in its regulation of Spo0F, and thus a *rapA* mutant strain would have excessive and early activation of its Spo0A-regulated genes. This may lead to an intense production of biofilm matrix components, at the cost of cell growth. Small initial differences between *rapA* mutants and other strains would then be quickly amplified during or sequential culturing approach due to the low number of *rapA* mutant spores being transferred in each step. This effect could be slightly alleviated during our pellicle forming conditions due to the initial advantage that an early biofilm formation would provide to *rapA* mutant strains. Thus, what were previously thought to be regulators with synonymous function are revealed as condition-dependent proteins. Additionally, the use of single and double *rap-phr* mutants combined with lineage tracking throughout the experimental competition allows for a detailed examination of the impact on competitiveness of each Rap phosphatase in a single *B. subtilis* strain. Further, we have also examined how each of these mutations (single and in double mutant combination) impacts independent growth. This led to the clear realization that growth does not equal fitness: some *rap* mutants, such as *rapD*, showed a decreased growth rate as compared to the wild-type strain; however, *rapD* mutants consistently showed an increase in population representation throughout all our tested conditions. These results support the observations of Serra *et al.* about sporulation and fitness: sacrificing growth rate in order to efficiently generate mature spores can be a good adaptive strategy in specific environmental conditions.

Besides the conclusions that can be drawn about the Rap phosphatases of *B. subtilis*, chapter 6 also offers insights about the ecological impact of the used growth conditions. In nature, environmental conditions change constantly, which forces their microbial inhabitants to constantly adapt to them and promotes genetic variation (397, 398). In laboratory conditions microbes usually face a very different situation: stable and optimal growth conditions with an abundance of nutrients. As mentioned above, organisms grown under these conditions can become domesticated, losing some genetic traits required for life in natural environments, and adapting to their new growth conditions. Furthermore, they are usually grown in axenic conditions, thus avoiding competition beyond their own kin. In our experimental setup we have provided the genetic diversity by competing all 79 tested strains at once. We observed important differences regarding selectivity of planktonic and pellicle forming conditions: static growth (pellicle formation) showed consistency in its selectivity, providing similar strains with increased population representation at the end of the competition experiment; on the contrary,

shaken growth conditions (planktonic) showed higher variation regarding the strains that increased their representation in all population replicates. These results may be due to the different type of selective pressure that each growth condition offers. Shaken cultures do not provide any benefit to cells that reach the air-liquid interface or try to produce a pellicle, thus all strains able to produce spores upon nutrient depletion may be selected for the next growth cycle. On the contrary, static cultures would quickly deplete their oxygen as cells grow, thus favoring strains that can colonize their surface in order to keep growing and eventually form spores. Additionally, it is important to consider the quorum sensing aspect of the Phr regulatory peptides: mixed growth conditions would constantly diffuse any produced molecule, thus delaying the accumulation of active concentrations of Phr peptides and allowing their cognate Rap phosphatases to act for extended periods of time. This would have the consequence of repressing the activity of the master regulators of population heterogeneity longer than usual, decreasing the chances of concerted sporulation from any single rap mutant strain. Although our study is focused on *B. subtilis* and its Rap proteins, analysis of the tested growth conditions can offer insights about the expected behavior of other quorum sensing systems in similar growth conditions, and their impact on bacterial interactions in general.

3. Community interactions – microbial life in a crowded world.

Microbiologists have traditionally used axenic cultures to study microorganisms. However, in natural settings microbes rarely live out their lives exclusively exposed to members of their own species; rather, they usually share their ecological niches with other microorganisms. This exposure to other microbes is especially pronounced in soil environments due to their ecological richness in microbial species (62, 63, 398). The recent development of advanced microscopy techniques and bioinformatic tools allows scientists to study multispecies bacterial communities and the relationships that occur in them. This has revealed a social world among microbes as fascinating and complex as the macroscopic one.

One of the most easily recognized relationships between two animal species is that of predator and prey. Zoologists have long studied predator-prey animal relationships, recognizing the ecological impact that these interactions can have upon other species not directly involved in it, and sometimes the environment itself. An interesting example of this is how the presence of grey wolves in the Yellowstone national park (studied after reintroduction of the species in 1995) forced the local elks (prey population) to change its grazing behavior; this in turn changed the distribution of diverse tree species, which allowed an unexpected biodiversity to flourish. This phenomena is known in ecology as top-down ecosystem control (523, 524) (see http://ethology.eu/how-wolves-change-rivers/). Even when focusing just in the predator and prey, the diversity in these relationships becomes apparent. Both prey and predator engage in multiple strategies to survive, e.g.: prey may rely on herd protection to confuse a predator, camouflage for single individuals to remain hidden, or aggressive defense by fighting and scaring off the predator. Likewise, predators can use a number of strategies to bring down their prey, e.g.: camouflage to stalk and approach their prey, venom to avoid a dangerous fight, and pack collaboration to improve the likelihood of successful hunting (525, 526).

Predator-prey relationships also exist among microbes. The first reported cases of bacterial predation was observed in myxobacteria more than 75 years ago (527). Since then, researchers

have described many different bacterial predatory species that utilize diverse strategies to hunt their prey, and also a wide array of strategies that cells use to avoid being predated upon (528, 529). An interesting characteristic of bacterial interactions is that their relationships are flexible and may change depending on the environmental conditions. As shown with the previously discussed case of competition/commensalism between *Acinetobacter* C6 and *P. putida* R1, the availability of nutrients and environmental structure can determine how microbes interact.

In chapter 5 we have analyzed the predator-prey relationship that can develop between *Cupriavidus necator* and *B. subtilis* in diverse environmental circumstances. Both bacteria are commonly found in soil environments (442), and thus not unlikely to find themselves sharing the same ecological niche. The ability of *C. necator* to prey on other bacteria had been previously discussed by researchers, although later results conflicted with this idea and suggested that *C. necator* was in fact not a bacterial predator (443, 448, 449). By testing different growth conditions and prey bacteria, we have established that *C. necator* exhibits predatory behavior only during particular starvation conditions, and that this predation does not require concerted efforts from the *C. necator* cells. An interesting observation is that *C. necator* cells grown in rich media would later kill co-incubated bacteria (such as *B. subtilis*), but not as a mechanism to obtain nutrients and foster its own growth. This is a curious phenomenon, and may indicate that *C. necator* produces antimicrobial compounds as a general mechanism to fend off competitors; and that this mechanism may be different from the one it utilizes to obtain nutrients from prey bacteria when grown under starvation conditions. Further, our findings contribute to the growing body of evidence about the flexibility of the social behavior of bacteria, especially when studied under growth conditions that may better represent the ones they face in nature.

B. subtilis is a bacterium well-known for its ability to produce spores that are highly-resistant to diverse environmental stresses, including predation (142, 143, 450, 451). Interestingly, we observed differences between *B. subtilis* strains regarding survivability to *C. necator* predation. These differences do not seem to be related to domestication, since both strain 168 and NCIB 3610 were highly susceptible to *C. necator* killing. We examined several *B. subtilis* factors known to be necessary for mature spore formation; however, none of them seem to be essential for *B. subtilis* resistance to *C. necator* killing. Importantly, cell-to-cell contact did not seem to be required for *C. necator* killing, which may be a factor explaining the observed results regarding spore resistance. In the case of predation by other organisms, such as amoeba, the presence of a proper spore coat is paramount for *B. subtilis* survivability. In these cases, the spore coat seems to offer direct physical protection to the cell from enzymes and other phagocytic activity coming from the predator. Thus, even minor spore coat compromise is sufficient to render the spore cell vulnerable to phagocytosis (142, 143, 450). Another relevant characteristic of a spore is its lack of metabolic activity, sometimes referred to as dormancy. This state offers protection to a cell due to their metabolic inactivity, which makes them resistant to diverse antimicrobials that target specific metabolic processes in order to kill cells. Thus dormancy can function as a circumstantial mechanism for survival even if a dormant cell lacks a spore coat (530, 531). Besides its normal sporulation process, large and dense communities of *B. subtilis*, such as its biofilms, may have concrete regions where the cells lack enough nutrients to form a mature spore, in which case the cells could remain in a state of dormancy (389). Differences in overall metabolic activity at the population level could thus explain our observed results regarding different survivability between *B. subtilis* strains, which may produce different ratios of dormant cells that can survive any antimicrobial produced by *C. necator*. Tellingly, the same reasoning could be applied to

tested strains that were incapable of initiating the process of sporulation and proved to be highly sensitive to *C. necator*: these cells, deprived of the sporulation mechanism, may be forced to continue their metabolic activity and thus remain vulnerable to antimicrobials produced by the predator.

A facet of microbial interactions that has gained much attention from the scientific community in recent years is the topic of microbiomes. A microbiome is the total collection of microbial species that live on or within any multicellular organism (532, 533). This definition has been recently expanded to include the organisms living in specific ecological niches as well, such as soil (534). Soil microbiomes are then the most extensive and varied collection of microorganisms on Earth. They may also be amongst the most dynamic, since soil is usually exposed to quickly changing conditions: from temperature changes through the day and season, to weather that can quickly alter humidity and structure of the soil through rain, wind, and erosion. Thus, the organisms that form part of these microbiomes can be expected to engage in complex and dynamic interactions in order to not only survive, but prosper. Furthermore, direct microbial interactions also have important consequences for tertiary organisms, which may be macroscopic. The type of root-colonizing biofilms used in chapter 2 to assess the biofilm forming capabilities of *B. subtilis* strains are actually of the utmost importance for plants. Specific fungal and bacterial species can be very beneficial for the host plant; for instance, certain fungi can produce phytohormones that promote plant development (522, 535). In the particular case of *B. subtilis*, biofilms of this bacterium can function as a biocontrol mechanism, offering protection to the plant against invasion by pathogenic organisms (67, 93). Colonization of the root systems by a beneficial microorganism is such an important phenomenon for plants that they actively try to control it by producing diverse exudates that promote selective colonization (320, 387, 512).

The continued study of the characteristics that allow *B. subtilis* to efficiently colonize and protect plant root systems is a research field with direct biotechnological applications in agriculture. Likewise, multi-species microbial interactions directly impact many other aspects important for human life, from health, to food production, to comfort goods; the study of sociomicrobiology is the latest paradigm shift already altering our understanding of how life works. Although much more research is needed in this novel field, scientific enthusiasm and dedication is sure to provide humanity with knowledge and tools to solve the challenges of the future.

4. Concluding remarks.

This dissertation presents an overview of the different cellular interactions that can take place in a biofilm formed by *B. subtilis*. The extensive background knowledge already gained by microbiologist about this bacterium makes it an ideal model to investigate different aspects of sociomicrobiology. From chemosensing and metabolic responses, to community structure and multispecies relations, the present work offers the reader a glimpse of the broad topics that can be probed with *B. subtilis* biofilms, either as a single-species population or as part of a microbial community.

The development of high-throughput sequencing technologies and high-resolution microscopy techniques, accompanied by ever more powerful informatic tools, has made it possible for microbiologists to examine how microorganisms live in natural environments. These investigations

have shown that some of the general notions about microbes gained with traditional laboratory techniques are inadequate reflections of microbial life and evolution as it happens in nature. For example, protozoan predation of bacteria, and specially the strategies used by bacteria to survive predation, has put forward the notion that human pathogens may have originally developed virulence mechanisms as a strategy to survive in open environments. This in turn has changed the strategies used to investigate virulence mechanisms of human pathogens, looking for their evolutionary origins in order to find better strategies to counter them (536–538).

The realization of the commonality of biofilms as possibly the most prevalent bacterial lifestyle makes them a priority for sociomicrobiological research. As more insights are gained about how diverse bacterial species interact with one another to establish sessile communities, biofilms reveal themselves as a lifestyle that offers stability and protection. From the point of view of a bacterial cell, forming part of a biofilm may be akin to living on a familiar island, with the other option being sailing into an open ocean, full of possibility and danger. Life within these islands fosters cellular interactions that lead to profound changes, both for the cell and the community. As microbes compete, adapt, collaborate, parasite, or become commensals with each other, the resulting community is far more than the sum of its parts; it becomes a veritable microbial city, full of individual stories to investigate.

To conclude, the study of biofilms from a sociomicrobiological point of view, such as the cases presented here, will help us to understand microbial life as it occurs outside laboratories. This knowledge will enable mankind to better benefit from microbial interactions, and even predict the behavior of those individual cells that do leave their biofilm in order to explore new environments and establish new communities.

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ANNEXUM A

Supplementary material for Chapter 2.

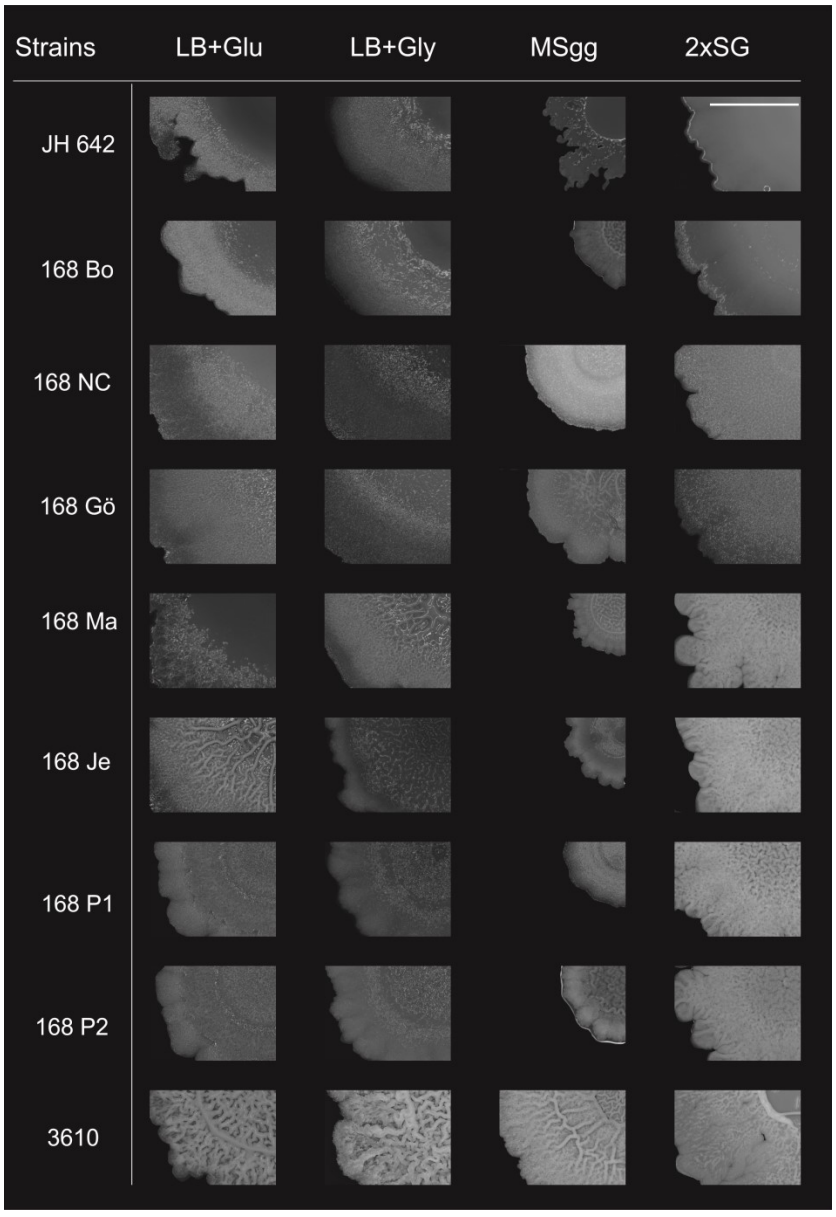

Figure S1: Magnified detail of complex colonies of *B. subtilis* strain 3610 and 168 variants on different media after 72 hours of incubation, arranged by increasing colony wrinkleality on 2xSG medium. The scale bar shown at the top right represents 5 mm. Strain abbreviations are described in Table 1.

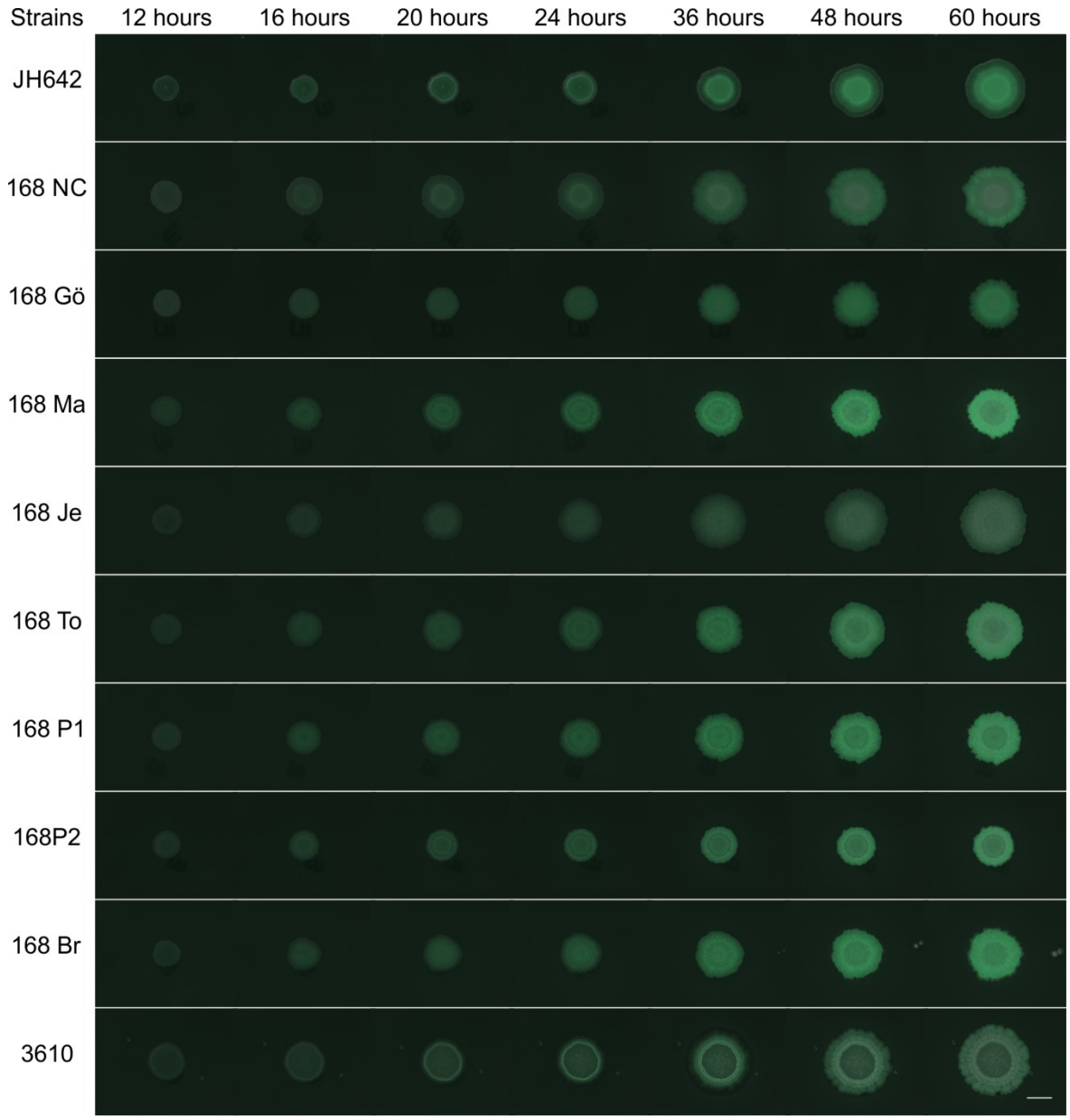

Figure S2: Colonies of *B. subtilis* strain 3610 and 168 variants carrying a transcriptional P_{eps}-GFP fusion at different time points. Overlays of fluorescence (false-colored green) and transmitted light (gray) images are shown. The colonies are representative of the observed phenotype for each strain or variant. The scale bar shown at the bottom right represents 5 mm. Strain abbreviations are described in Table 1.

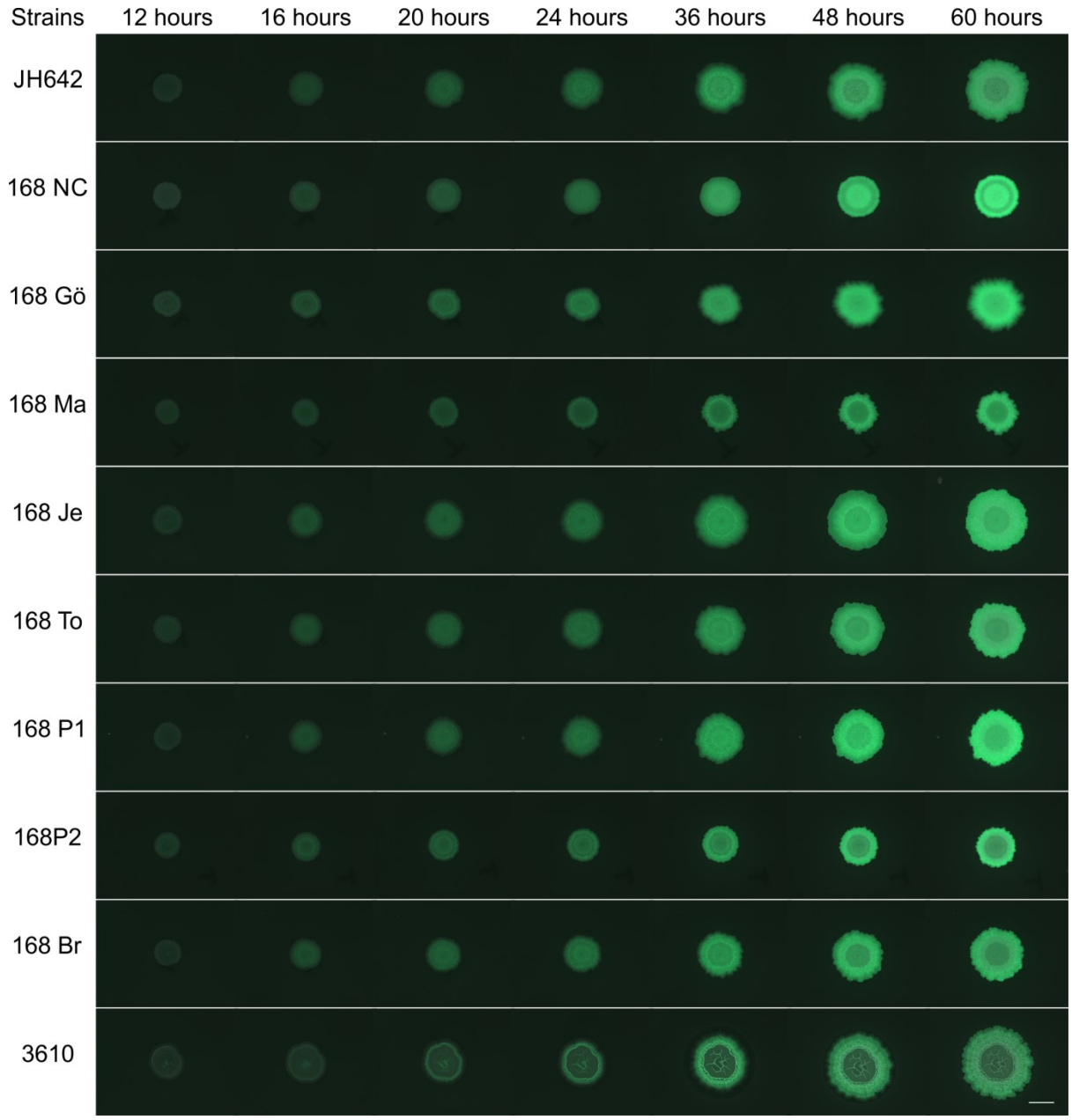

Figure S3: Colonies of *B. subtilis* strain 3610 and 168 variants carrying a transcriptional P_{tapA}-GFP fusion during different time points. Overlays of fluorescence (false-colored green) and transmitted light (gray) images are shown in all cases. The colonies are representative of the observed phenotype for each strain or variant. The scale bar shown at the bottom right represents 5 mm. Strain abbreviations are described in Table 1.

ANNEXUM B

Supplementary material for Chapter 5.

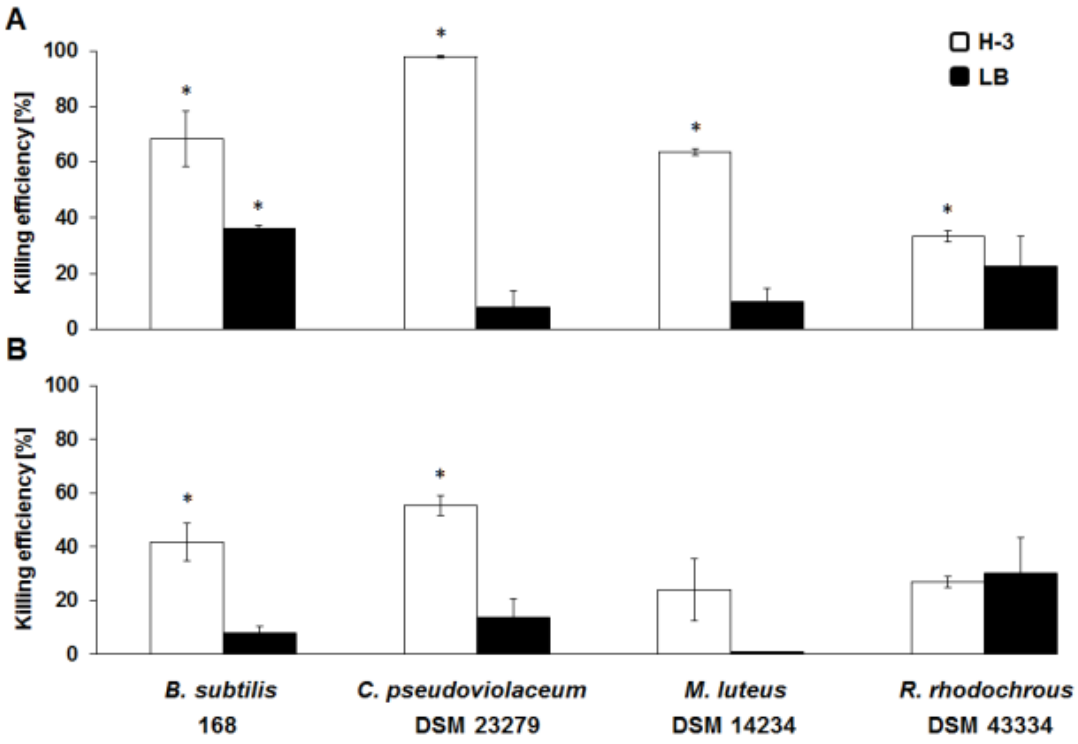

Figure S1: Results from the CFU-based predation assay. Mean killing efficiencies ($\bar{e}$ ± 95% confidence interval) of *C. necator* N-1 (A) and *C. necator* H16 (B) against different prey bacteria. Asterisks denote significant differences between the number of prey CFU of the control group (i.e., monocultures) and samples containing both predator and prey (Mann-Whitney U-test: * = $P < 0.05$; d.f. = 2). Prey species included *Bacillus subtilis*, *Chromobacterium pseudoviolaceum*, *Micrococcus luteus*, and *Rhodococcus rhodochrous*.

ANNEXUM C

Supplementary material for Chapter 6.

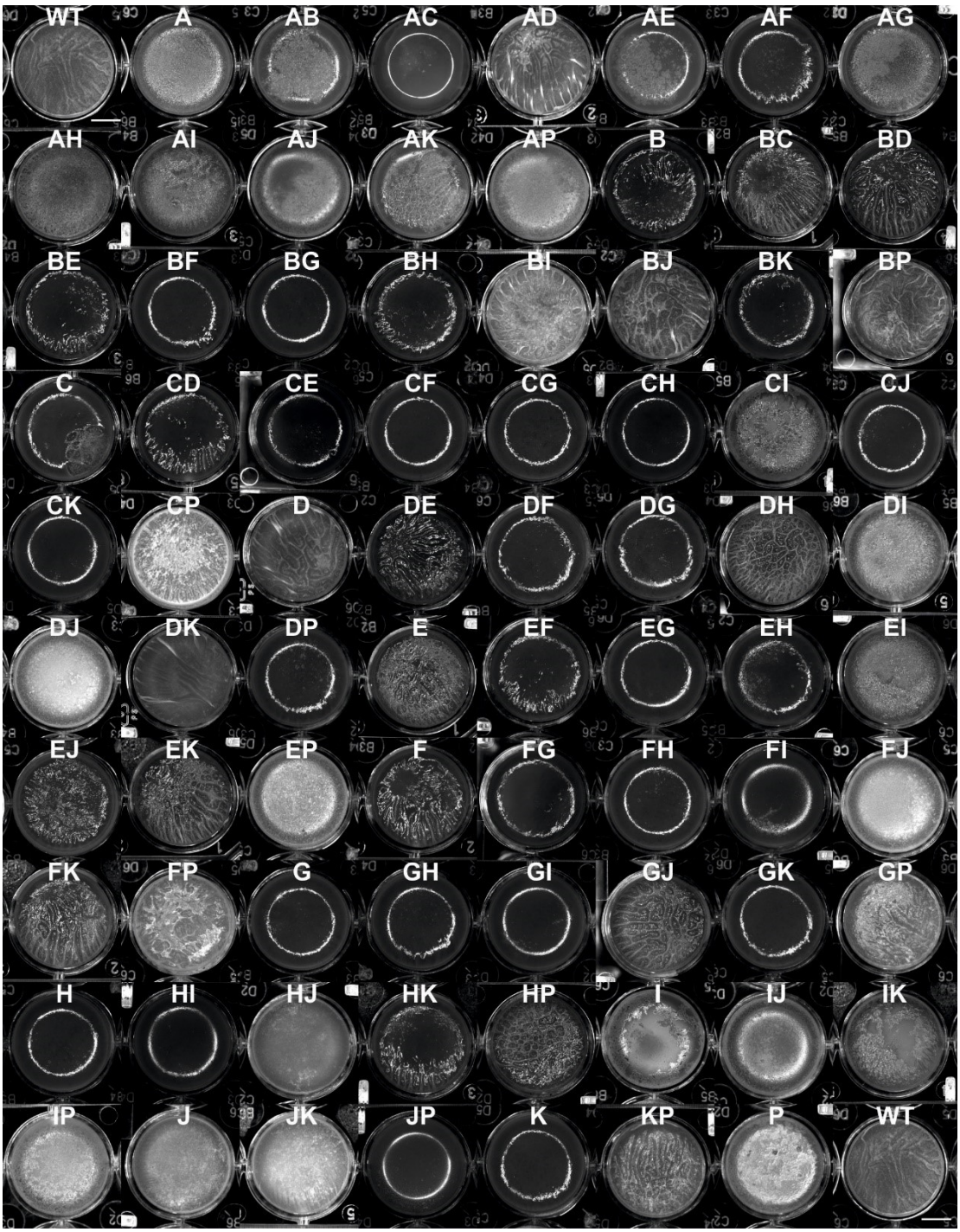

Figure S1: 2-day pellicles of *B. subtilis* DK1042 and *rap-phr* mutants. Bright-field images are shown after 2 days of incubation on MSgg medium at 30°C. WT indicates *B. subtilis* DK1042, A indicates a Δ*rapA* mutant, AB indicates a Δ*rapA*Δ*rapB* mutant, and so on. The images shown here have an adjusted contrast, so that the pellicles can be easily appreciated. The same image of *B. subtilis* DK1042 is presented twice (top-left and bottom-right) to facilitate pellicle comparison. The scale bars represent 5 mm.

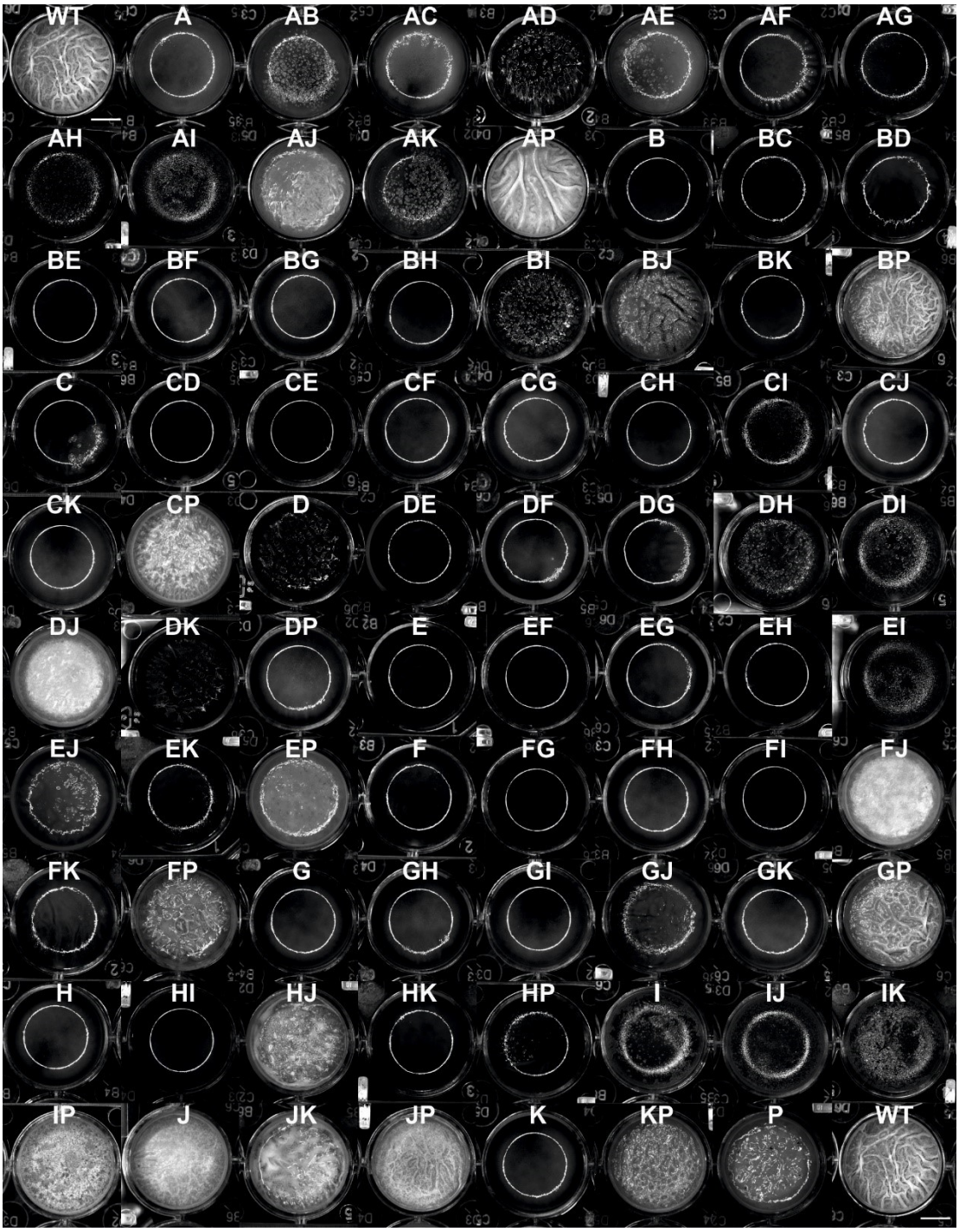

Figure S2: 5-day pellicles of *B. subtilis* DK1042 and *rap-phr* mutants. Bright-field images are shown after 5 days of incubation on MSgg medium at 30°C. WT indicates *B. subtilis* DK1042, A indicates a Δ*rapA* mutant, AB indicates a Δ*rapA*Δ*rapB* mutant, and so on. The images shown here have an adjusted contrast, so that the pellicles can be easily appreciated. . The same image of *B. subtilis* DK1042 is presented twice (top-left and bottom-right) to facilitate pellicle comparison. The scale bars represent 5 mm.

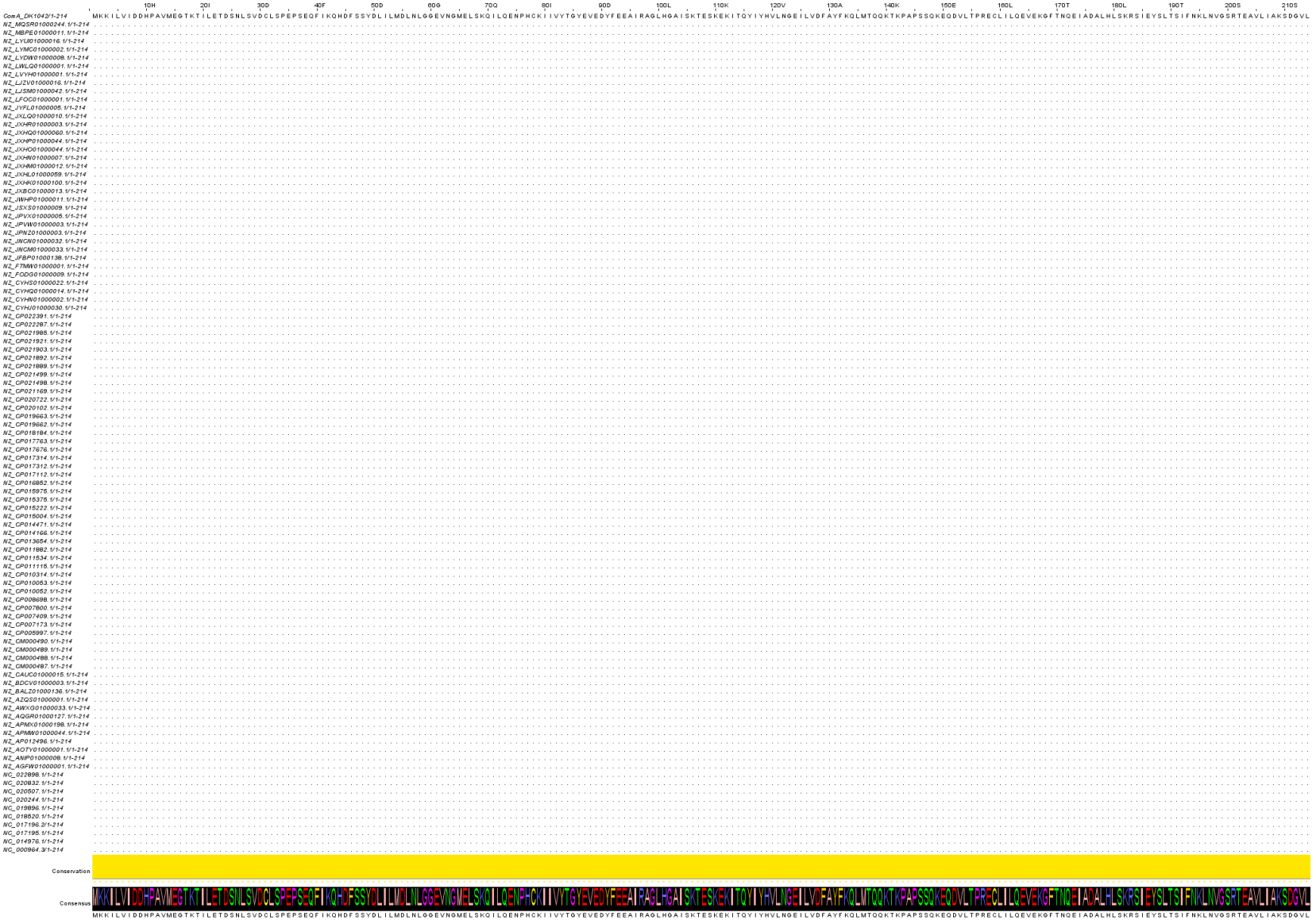

Figure S3: Sequence alignment of the ComA protein of *B. subtilis* DK1042 and matching translated regions of 100 *B. subtilis* reported genomes. Black dots represent amino acid matches, dissimilar amino acids are annotated in the alignment, the consensus sequence and codon conservation index are given at the bottom. The GenBank accession number of all compared strains is annotated in the far-left column.

Figure S4: Sequence alignment of the DegU protein of *B. subtilis* DK1042 and matching translated regions of 100 *B. subtilis* reported genomes. Black dots represent amino acid matches, dissimilar amino acids are annotated in the alignment, the consensus sequence and codon conservation index are given at the bottom. The GenBank accession number of all compared strains is annotated in the far-left column.

Figure S5: Sequence alignment of the Spo0A protein of *B. subtilis* DK1042 and matching translated regions of 100 *B. subtilis* reported genomes. Black dots represent amino acid matches, dissimilar amino acids are annotated in the alignment, the consensus sequence and codon conservation index are given at the bottom. The GenBank accession number of all compared strains is annotated in the far-left column.

Figure S6: Sequence alignment of the Spo0B protein of *B. subtilis* DK1042 and matching translated regions of 100 *B. subtilis* reported genomes. Black dots represent amino acid matches, dissimilar amino acids are annotated in the alignment, the consensus sequence and codon conservation index are given at the bottom. The GenBank accession number of all compared strains is annotated in the far-left column.

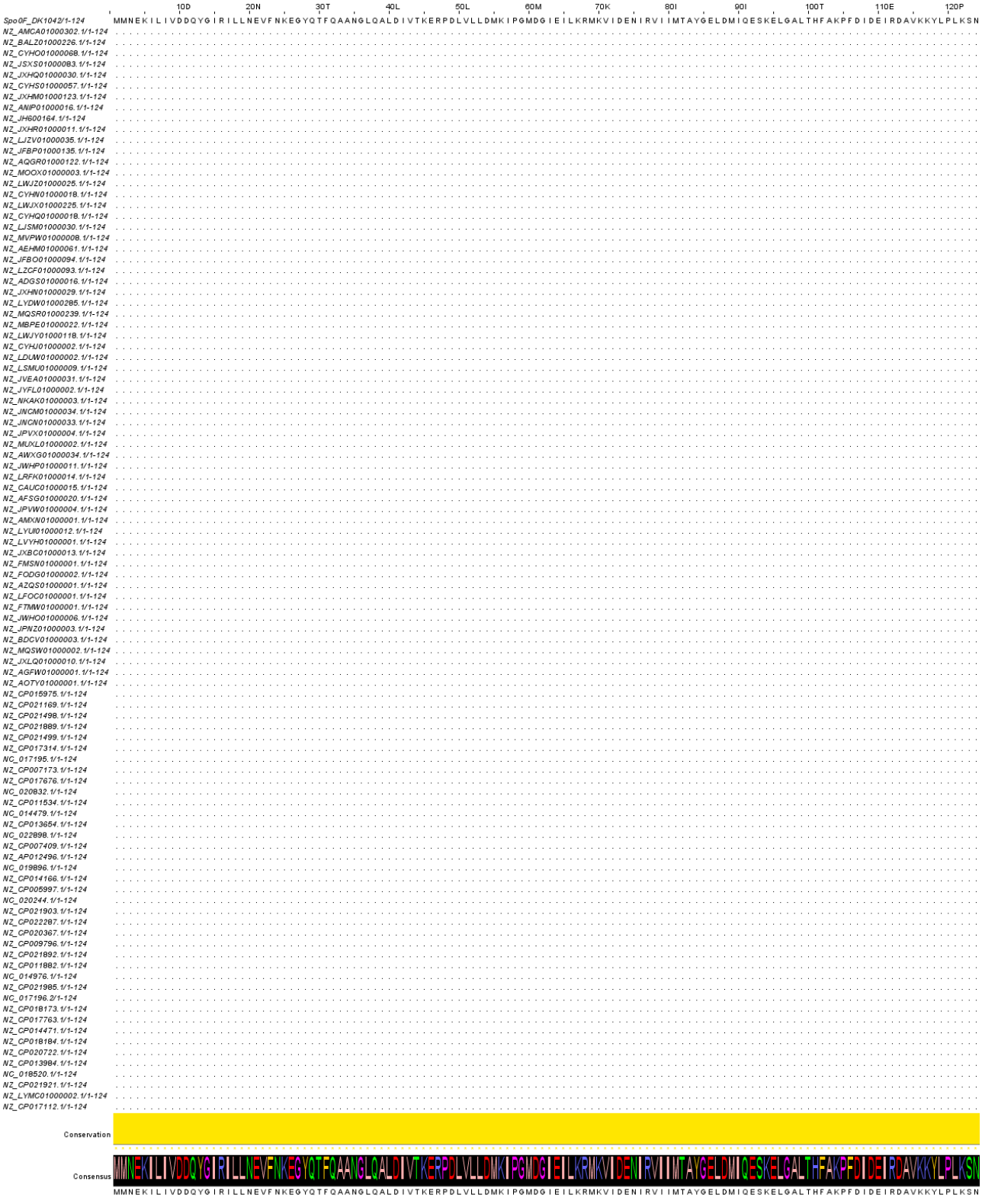

Figure S7: Sequence alignment of the Spo0F protein of *B. subtilis* DK1042 and matching translated regions of 100 *B. subtilis* reported genomes. Black dots represent amino acid matches, dissimilar amino acids are annotated in the alignment, the consensus sequence and codon conservation index are given at the bottom. The GenBank accession number of all compared strains is annotated in the far-left column.

Table S1: List of mutations detected in genomes of sequenced population clones.

Clone Isolated from Population										Gene	Function	Mutation
2 Days Pellicle Mix C Rep. 15.1	2 Days Pellicle Mix C Rep. 15.2	2 Days Pellicle Mix C Rep. 16.1	2 Days Pellicle Mix C Rep. 16.2	5 Days Pellicle Mix A Rep. 3	5 Days Pellicle Mix A Rep. 6	2 Days Planktonic Mix A Rep. 3	2 Days Planktonic Mix A Rep. 6	5 Days Planktonic Mix A Rep. 1	5 Days Planktonic Mix A Rep. 2			
				X						*ybxB*	Unknown, putative methyltransferase	Frameshift (deletion of base-pair 457)
			X							*gerKC*	Membrane receptor involved in spore germination	a.a. change (Val47Ala)
								X				a.a. change (Ile38Asn)
X										*yhaX*	SigE-dependent sporulation protein	a.a. change (Asp252Asn)
		X								*ymfD*	Exporter for the siderophore bacillibactin	Frameshift (deletion of base-pair 989)
			X			**X**		**X**		*rapE*	Rap-Phr system, regulates Spo0F~P	a.a. change (Ala56Thr)
			X			**X**		**X**		*yqcG*	Toxin, eliminates defective cells from developing biofilms, DNase activity	a.a. change (Glu159Lys)
							X			*comP*	Sensor kinase for ComX, Phosphorylates ComA	Frameshift (deletion of base-pair 1601)
				X						*gerAC*	Membrane receptor involved in spore germination	a.a. change (Asn252Lys)
					X					*degS*	Sensor kinase, Phosphorylates DegU	a.a. change (Tyr262Asp)

Table S2: DNA barcoded strains used in this study.

Name	Characteristics	DNA barcode
TB614.BC	DK1042 *amyE*::barcode (*cat*^{r})	CCCTAATGAGAA
TB588.BC	DK1042 Δ*rapA amyE*::barcode (*cat*^{r})	TTGGCCATTGTG
TB575.BC	DK1042 Δ*rapB amyE*::barcode (*cat*^{r})	TCTTCTGGAGCC
TB410.1.BC	DK1042 Δ*rapC amyE*::barcode (*cat*^{r})	AATTTCGAGTCG
TB513.BC	DK1042 Δ*rapD amyE*::barcode (*cat*^{r})	ATTGCTTTTTTT
TB407.BC	DK1042 Δ*rapE amyE*::barcode (*cat*^{r})	GGTAGGGCATTG
TB408.2.BC	DK1042 Δ*rapF amyE*::barcode (*cat*^{r})	CAGGGGTTGCAC
TB412.BC	DK1042 Δ*rapG amyE*::barcode (*ca*^{r})	GCATGCGAGTCG
TB409.1.BC	DK1042 Δ*rapH amyE*::barcode (*cat*^{r})	TCGGGTGATAGT
TB444.BC	DK1042 Δ*rapI amyE*::barcode (*cat*^{r})	TTGTTGAACACC
TB411.2.BC	DK1042 Δ*rapJ amyE*::barcode (*cat*^{r})	GGAAGGATTATG
TB587.BC	DK1042 Δ*rapK amyE*::barcode (*cat*^{r})	GGGTTACATATT
TB445.BC	DK1042 Δ*rapP amyE*::barcode (*cat*^{r})	TATTTCATGGAT
TB577.BC	DK1042 Δ*rapB, rapA*::*km*^{R} *amyE*::barcode (*cat*^{r})	GGAACGGGTCGT
TB518.BC	DK1042 Δ*rapC, rapA*::*km*^{R} *amyE*::barcode (*cat*^{r})	GGTGGGTGTGAG
TB555.BC	DK1042 Δ*rapD, rapA*::*km*^{R} *amyE*::barcode (*cat*^{r})	GGTAGGGGCCAG
TB517.BC	DK1042 Δ*rapE, rapA*::*km*^{R} *amyE*::barcode (*cat*^{r})	GATTGAGCCAGC
TB523.BC	DK1042 Δ*rapF, rapA*::*km*^{R} *amyE*::barcode (*cat*^{r})	CTGATACCGTTT
TB544.BC	DK1042 Δ*rapG, rapA*::*km*^{R} *amyE*::barcode (*cat*^{r})	GGCTCCGTTTAG
TB522.BC	DK1042 Δ*rapH, rapA*::*km*^{R} *amyE*::barcode (*cat*^{r})	TCATCTTCTGGT
TB545.BC	DK1042 Δ*rapI, rapA*::*km*^{R} *amyE*::barcode (*cat*^{r})	GACGGTCGGTGT
TB516.BC	DK1042 Δ*rapJ, rapA*::*km*^{R} *amyE*::barcode (*cat*^{r})	TTTAGTTTGGAC
TB647.BC	DK1042 Δ*rapK, rapA*::*km*^{R} *amyE*::barcode (*cat*^{r})	AGTCGTTGTACG
TB519.BC	DK1042 Δ*rapP, rapA*::*km*^{R} *amyE*::barcode (*cat*^{r})	ATTAGTTGTTAC
TB582.BC	DK1042 Δ*rapB, rapC*::*km*^{R} *amyE*::barcode (*cat*^{r})	GTCTTGGGGAGG
TB578.BC	DK1042 Δ*rapB, rapD*::*km*^{R} *amyE*::barcode (*cat*^{r})	TTTGGGGCCCGG
TB271.BC	DK1042 Δ*rapB, rapE*::*spec*^{R} *amyE*::barcode (*cat*^{r})	TCCGGGAATGAA
TB275.BC	DK1042 Δ*rapB, rapF*::*spec*^{R} *amyE*::barcode (*cat*^{r})	GGTTGTTCCTCT
TB583.BC	DK1042 Δ*rapB, rapG*::*spec*^{R} *amyE*::barcode (*cat*^{r})	GGGGGGGTGTTT
TB276.BC	DK1042 Δ*rapB, rapH*::*spec*^{R} *amyE*::barcode (*cat*^{r})	TCACAGACATTG
TB727.BC	DK1042 Δ*rapB, rapI*::*km*^{R} *amyE*::barcode (*cat*^{r})	CACATGACCAGA
TB586.BC	DK1042 Δ*rapB, rapJ*::*km*^{R} *amyE*::barcode (*cat*^{r})	GGTAGCTGGTCC
TB579.BC	DK1042 Δ*rapB, rapK*::*km*^{R} *amyE*::barcode (*cat*^{r})	TATAGGCCTTCG
TB584.BC	DK1042 Δ*rapB, rapP*::*mls*^{R} *amyE*::barcode (*cat*^{r})	AACACAAAGTAC
TB521.BC	DK1042 Δ*rapC, rapD*::*km*^{R} *amyE*::barcode (*cat*^{r})	GGGTTGTAGTGC
TB542.BC	DK1042 Δ*rapC, rapE*::*spec*^{R} *amyE*::barcode (*cat*^{r})	GCGCTAGTCCTA
TB454.BC	DK1042 Δ*rapC, rapF*::*spec*^{R} *amyE*::barcode (*cat*^{r})	GGTAGAGCTGTC
TB436.BC	DK1042 Δ*rapG, rapC*::*km*^{R} *amyE*::barcode (*cat*^{r})	GCGTAAGGGTAG
TB453.BC	DK1042 Δ*rapC, rapH*::*spec*^{R} *amyE*::barcode (*cat*^{r})	CTCTGCAACAAT
TB548.BC	DK1042 Δ*rapI, rapC*::*km*^{R} *amyE*::barcode (*cat*^{r})	GACACCCCCATC
TB455.BC	DK1042 Δ*rapC, rapJ*::*km*^{R} *amyE*::barcode (*cat*^{r})	TCAGTGAGGATG
TB561.BC	DK1042 Δ*rapC, rapK*::*km*^{R} *amyE*::barcode (*cat*^{r})	ATCGGAGTGGAG
TB547.BC	DK1042 Δ*rapP, rapC*::*km*^{R} *amyE*::barcode (*cat*^{r})	TATGGGCTGACG
TB503.BC	DK1042 Δ*rapE, rapD*::*km*^{R} *amyE*::barcode (*cat*^{r})	CGGCGTCTCGGG
TB504.BC	DK1042 Δ*rapF, rapD*::*km*^{R} *amyE*::barcode (*cat*^{r})	TTGAGCGCGGTG
TB546.BC	DK1042 Δ*rapG, rapD*::*km*^{R} *amyE*::barcode (*cat*^{r})	GGGAGAGGCAGG
TB520.BC	DK1042 Δ*rapH, rapD*::*km*^{R} *amyE*::barcode (*cat*^{r})	GGCAGGCTTGTA
TB550.BC	DK1042 Δ*rapI, rapD*::*km*^{R} *amyE*::barcode (*cat*^{r})	GGTATCGAAGGC
TB502.BC	DK1042 Δ*rapJ, rapD*::*km*^{R} *amyE*::barcode (*cat*^{r})	TAAGTCCGTTAG
TB564.BC	DK1042 Δ*rapD, rapK*::*km*^{R} *amyE*::barcode (*cat*^{r})	GCGTGATCCGGT

TB549.BC	DK1042 Δ*rapP, rapD*::*km*^{R} *amyE*::barcode (*cat*^{r})	GTTTTCCAATGC
TB456.BC	DK1042 Δ*rapE, rapF*::*spec*^{R} *amyE*::barcode (*cat*^{r})	GCTGACGGGGAA
TB451.BC	DK1042 Δ*rapG, rapE*::*spec*^{R} *amyE*::barcode (*cat*^{r})	TGTAGCGCTGGT
TB457.BC	DK1042 Δ*rapE, rapH*::*spec*^{R} *amyE*::barcode (*cat*^{r})	GACTTTAAGATG
TB285.BC	DK1042 Δ*rapI, rapE*::*spec*^{R} *amyE*::barcode (*cat*^{r})	TCTTTAGTGAAA
TB458.BC	DK1042 Δ*rapE, rapJ*::*km*^{R} *amyE*::barcode (*cat*^{r})	GGATTCCAAACG
TB558.BC	DK1042 Δ*rapE, rapK*::*km*^{R} *amyE*::barcode (*cat*^{r})	GCGGACGTCATG
TB283.BC	DK1042 Δ*rapP, rapE*::*spec*^{R} *amyE*::barcode (*cat*^{r})	AATGGTCAACTG
TB452.BC	DK1042 Δ*rapG, rapF*::*spec*^{R} *amyE*::barcode (*cat*^{r})	CCGGTTATGGCG
TB543.BC	DK1042 Δ*rapH, rapF*::*spec*^{R} *amyE*::barcode (*cat*^{r})	TGGGTGTTTGAT
TB472.BC	DK1042 Δ*rapI, rapF*::*spec*^{R} *amyE*::barcode (*cat*^{r})	TGTTTGTGCTTT
TB495.BC	DK1042 Δ*rapJ, rapF*::*spec*^{R} *amyE*::barcode (*cat*^{r})	GGTTAGGGTCCC
TB559.BC	DK1042 Δ*rapF, rapK*::*km*^{R} *amyE*::barcode (*cat*^{r})	CAATAATCGCTT
TB293.BC	DK1042 Δ*rapP, rapF*::*spec*^{R} *amyE*::barcode (*cat*^{r})	GTCACGCACGTT
TB433.BC	DK1042 Δ*rapG, rapH*::*spec*^{R} *amyE*::barcode (*cat*^{r})	GGGACGGTTTGT
TB474.BC	DK1042 Δ*rapI, rapG*::*spec*^{R} *amyE*::barcode (*cat*^{r})	GGGCAGCTGAGA
TB434.BC	DK1042 Δ*rapG, rapJ*::*km*^{R} *amyE*::barcode (*cat*^{r})	GTTACTTTAGCG
TB566.BC	DK1042 Δ*rapG, rapK*::*km*^{R} *amyE*::barcode (*cat*^{r})	TTGAGTCTTCGC
TB443.BC	DK1042 Δ*rapG, rapP*::*mls*^{R} *amyE*::barcode (*cat*^{r})	GTGTTATAGTAT
TB473.BC	DK1042 Δ*rapI, rapH*::*spec*^{R} *amyE*::barcode (*cat*^{r})	TCGCTAATTACA
TB496.BC	DK1042 Δ*rapJ, rapH*::*spec*^{R} *amyE*::barcode (*cat*^{r})	GTCAACAGATAT
TB560.BC	DK1042 Δ*rapH, rapK*::*km*^{R} *amyE*::barcode (*cat*^{r})	TTTAGTCCGGAA
TB284.BC	DK1042 Δ*rapP, rapH*::*spec*^{R} *amyE*::barcode (*cat*^{r})	GGTTGGGCGGGT
TB551.BC	DK1042 Δ*rapI, rapJ*::*km*^{R} *amyE*::barcode (*cat*^{r})	CCAAGGCCCGTG
TB562.BC	DK1042 Δ*rapI, rapK*::*km*^{R} *amyE*::barcode (*cat*^{r})	GCTCGAATCCCG
TB552.BC	DK1042 Δ*rapI, rapP*::*mls*^{R} *amyE*::barcode (*cat*^{r})	TGCCACGGAAAG
TB565.BC	DK1042 Δ*rapJ, rapK*::*km*^{R} *amyE*::barcode (*cat*^{r})	CTTGTTGAAACA
TB292.BC	DK1042 Δ*rapJ, rapP*::*mls*^{R} *amyE*::barcode (*cat*^{r})	GCATGCACCGTA
TB563.BC	DK1042 Δ*rapP, rapK*::*km*^{R} *amyE*::barcode (*cat*^{r})	TGGGCTGTGGCC

DECLARATION OF AUTHORSHIP

In accordance with the regulations established by the Course of Examination for Doctoral Candidates from the Faculty of Biology and Pharmacy of the Friedrich-Schiller-University Jena, I hereby declare that:

- I am familiar with the Course of Examination for Doctoral Candidates from the Faculty of Biology and Pharmacy of the Friedrich-Schiller-University Jena.

- I have composed and written this dissertation myself, I have not used any sections of text from a third party without identifying them as such, and I have acknowledged all used bibliographical sources within this work.

- People who assisted me with experiments, data analysis, and writing of the manuscripts are listed as co-authors of the respective manuscripts.

- I was not assisted by any professional doctoral consultant, and that no third parties have received either direct or indirect monetary benefits from me for work connected to the writing of this dissertation.

- This dissertation has not been previously submitted as a comprehensive work elsewhere, whether to the Friedrich-Schiller-University Jena or to any other university, nor has it been used as an examination paper for a state exam or other scientific examination. However, some of the manuscripts presented here have been published in scientific journals. Their bibliographical information is given at the beginning of each corresponding section.

M. Sc. Ramses Gallegos Monterrosa

Jena, April 5th, 2018.