The Nature of the Endospore Crust of *Bacillus subtilis* and its Utilization as a Platform for Protein Display.

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München, den 4.12.2019 Julia Brehm

Julia Brehm

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Abbreviations

ABTS:	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), substrate of laccases
AFM:	Atomic force microscopy
AIA:	Lectin from Artocarpus integrifolia (Jacalin)
BpuL:	*Bacillus pumilus* laccase (*cotA*)
***cge*/Cge:**	GerE-controlled genes/proteins
***cot*/Cot:**	Spore coat genes/proteins
CSL:	Oncorhynchus keta L-rhamnose binding lectin
EcoL:	*Escherichia coli* laccase (*cueO*)
GalNAc:	N-acetylgalactosamine
GFP:	Green fluorescent protein
GlcNAc:	N-acetylglucosamine
GMO:	Genetically modified organism
HPLC:	High-performance liquid chromatography
ManNAc:	N-acetylmannosamine
MESF:	Molecular equivalent of solvent fluorophore, number of GFP molecules per GFP bead
NeuNAc:	N-acetylneuramic acid
PEP:	Phosphoenolpyruvate
RFC10:	Request for comments 10, BioBrick cloning standard
RFC25:	Request for comments 25, BioBrick Freiburg standard for translational fusion
SASP:	Small acid-soluble protein
SEM:	Scanning electron microscopy
***sps*/Sps:**	Spore envelope polysaccharide synthesis genes/proteins
TEM:	Transmission electron microscopy
Vio:	Viosamine: 4-amino-4,6-deoxy-D-glucose
VioNAc:	N-acetyl-viosamine

List of Publications

Publications presented in this thesis:

Publication I:

Sporobeads: The Utilization of the *Bacillus subtilis* Endospore Crust as a Protein Display Platform. **Bartels J**, López Castellanos S, Radeck J, Mascher T. ACS Synth Biol. 2018 Feb 16; 7(2):452-461. doi: 10.1021/acssynbio.7b00285. Epub 2018 Jan 19

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The *Bacillus subtilis* endospore crust: protein interaction network, architecture and glycosylation state of a potential glycoprotein layer. **Bartels J**, Blüher A, López Castellanos S, Günther M, Richter M, Mascher T, Mol Microbiol. doi:10.1111/mmi.14381

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Author Contributions

Publication I: Bartels J, López Castellanos S, Radeck J, Mascher T. Sporobeads: The Utilization of the *Bacillus subtilis* Endospore Crust as a Protein Display Platform.

Julia Bartels, Jara Radeck and Sebastian López Castellanos constructed the vectors. Sebastian López Castellanos additionally evaluated the crust promoter strength. All other experiments were performed by Julia Bartels. Julia Bartels and Thorsten Mascher designed the study and wrote the manuscript. All authors read and approved the final manuscript.

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Julia Bartels and Sebastian López Castellanos constructed the strains. Markus Richter performed the HPLC experiments. Markus Günther and Anja Blüher assisted with the electron microscopy. All other experiments were performed by Julia Bartels. Julia Bartels evaluated the data. Julia Bartels and Thorsten Mascher designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Publication III: Popp PF, Dotzler M, Radeck J, Bartels J, Mascher T. The *Bacillus* BioBrick Box 2.0: expanding the genetic toolbox for the standardized work with *Bacillus subtilis*.

Thorsten Mascher, Jara Radeck, Philipp Popp, Mona Dotzler and Julia Bartels conceptualized the study and developed the experimental strategies for (individual parts of) the Bacillus BioBrick Box 2.0. Philipp Popp and Mona Dotzler cloned and evaluated all parts of the Bacillus BioBrick Box 2.0. Philipp Popp and Thorsten Mascher wrote the manuscript. All authors read and approved the final manuscript.

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Julia Brehm	Prof. Dr. Thorsten Mascher

Summary

Under starvation conditions, the soil bacterium *Bacillus subtilis* undergoes a complex program geared towards survival, which ultimately results in the formation of highly resistant yet dormant endospores. These spores contain packed DNA encased by a peptidoglycan cortex and concentric proteinaceous layers, protecting the spore from environmental influences. The crust is of special interest in this thesis, as it constitutes the outermost layer of the coat.

In this thesis, the goal is to display proteins of interest onto the surface of the spore, thereby creating biologically active particles, namely Sporobeads. For this purpose, it is only logical to utilize the spore's outermost layer: the crust. Moreover, this thesis aims to not only provide a proof of concept, but to also establish an easy-access vector system (Sporovectors) to easily produce Sporobeads for any given application. As scientific knowledge on the nature of the crust still has major gaps, this thesis aims to also reach a deeper understanding of this particular layer, which in turn might help to better understand and improve the application of the Sporobeads. Therefore, the interaction network of the six crust proteins (CotVWXYZ and CgeA), their role in the crust as well as the composition and the active players of the proposed glycosylation modification were also elucidated in this study. These two projects benefit from each other in a create-test-learn-redesign cycle. With the established methodology of the Sporobeads in place and the first insights into the crust proteins at hand, the basic research into the crust is more convenient. In turn, the nature of the crust helps explain the performance of the anchors and also provides a rationale for further improvement strategies.

To this end, the study showed that the utilization of the crust as a platform for protein display is feasible. The best anchors were CotY and CotZ, followed by CotX and CotV. CotW and CgeA were the least promising. The enzymes were stabilized on the spore surface during storage, and it was also possible to recycle the particles. The relative performance of the different anchors is partially explained by the protein interaction network and their roles in the crust structure. CotY and CotZ are the major structural pillar, whereas CotX and CotV play a more supportive role in structure propagation. CotW supports the assembly of the CotX/CotV structural pillar. CgeA seems to play a role in glycosylation, being least abundant. CotZ anchors the crust structure to the middle part of the spore, whereas CotX and CotY might already anchor it loosely to the poles of the spore.

CotX and CotV appear to be the most probable candidates for glycosylation, due to the conserved glycosylation motif in the CotX superfamily domain. The mode of glycosylation is quite complex, involving many players and presumably at least six different sugars divided into two independent yet probably cross-linked polysaccharide species: one related to rhamnose and one to galactose. The putative functions of the players involved seem to indicate that the rhamnose-related species might contain the rare sugar viosamine or VioNAc. Furthermore, the rhamnose-related polysaccharide variant could also potentially be capped by a unique sugar based on viosamine or VioNAc modified with a lysine side chain, similar to what happens in a relative *Bacillus anthracis*. This suggests that water dispersal is not the only role of this modification, but that said modification might act as additional protection against biological scavenging, due to its exceptional nature.

Lessons learned regarding the nature of the crust can, in turn, foster the development of potential improvement strategies. On the whole, however, these strategies turned out to be less promising than was initially hoped. Linkers were able to rescue variants performing inadequately, but only led to slight improvements for the best-working variants. On the other hand, removing native competition only slightly improves the performance. This is probably due to the high level of redundancies (CotY/CotZ and CotV/CotX) as well as the high level of interdependencies in the crust. When considering that the crust is glycosylated, some enzymes might be less active or even inactive in this micro-environment. With this rationale, changing the surface properties using a mutant with an impaired crust polysaccharide structure (*cgeA*) slightly improved the activity of the *Bacillus pumilus* laccase. This might, in future, enable the performance of some enzymes requiring a more hydrophobic environment, such as lipases.

Nonetheless, a peculiarity discovered during this study could lead to a novel field of application: spore-derived self-assembled non-GMO including particles (SporoSNIPs). In some mutants, the assembly of the crust proteins onto the spore was highly disturbed, but the crust proteins themselves self-assembled in the mother cell instead (in the *cotZ* mutant the complete crust structure, or CotX and CotV in any mutant lacking CotY or CotZ). These fragments (SporoSNIPs) could potentially be enriched or completely separated from the spores for further utilization as biologically active particles, but without the disadvantage of containing living GMOs.

Zusammenfassung

Unter Hungerbedingungen durchläuft das Bodenbakterium *Bacillus subtilis* ein komplexes Überlebensprogramm, das schlussendlich zur Bildung von hoch widerstandsfähigen, jedoch inaktiven Endosporen führt. Diese Sporen beinhalten verpackte DNA, die von einem Peptidoglycan-Cortex und konzentrischen Proteinschichten umgeben ist, welche die Spore vor Umwelteinflüssen schützen. Diese Doktorarbeit konzentriert sich vorrangig auf die Kruste, da sie die äußerste Schicht des Mantels bildet.

Ziel dieser Doktorarbeit ist es, ausgewählte Proteine auf der Oberfläche der Spore zu präsentieren und dadurch biologisch aktive Partikel zu produzieren, sogenannte Sporobeads. Daher erscheint es nur logisch, hierfür auch die äußerste Schicht der Spore zu verwenden: die Kruste. Des Weiteren wird auch ein methodisch leicht zugängliches Vektorsystem (Sporovectors) erstellt. Da unser Wissen um die Beschaffenheit der Kruste bislang noch große Lücken aufweist, soll ein tieferes Verständnis dieser speziellen Schicht erreicht werden. Daher erläutert diese Studie zusätzlich das Interaktionsnetzwerk der sechs Krustenproteine (CotV,W,X,Y,Z und CgeA), ihre Rolle innerhalb der Kruste, sowie die Zusammensetzung und die aktiven Akteure, die in der vorgeschlagenen Modifizierung des Glykosylierungsprozesses beteiligt sind. Diese beiden Projekte profitieren voneinander. Die vorhandene Sporobead-Methodik sowie die ersten Erkenntnisse über die Krustenproteine erleichtern die Grundlagenforschung, die sich mit der Beschaffenheit der Kruste befasst. Im Gegenzug hilft uns jenes Wissen um die Beschaffenheit der Kruste die Leistung der Anker zu erklären und bietet eine Basis für weitere Verbesserungsstrategien.

Diese Studie beweist die Machbarkeit der Sporobeads. Die besten Anker waren CotY und CotZ, gefolgt von CotX und CotV. CotW und CgeA waren am wenigsten vielversprechend. Die Enzyme wurden während der Lagerung stabilisiert, und auch die Wiederverwendung gelang. Die relative Leistung der verschiedenen Anker lässt sich schon teilweise durch das Protein-Interaktionsnetzwerk und durch die Rolle der Proteine innerhalb der Kruste erklären. CotY und CotZ bilden den Hauptpfeiler der gesamten Struktur, während CotX und CotV eher eine unterstützende Funktion in der Ausbreitung der Struktur innehalten. CotW unterstützt den Aufbau des CotX/CotV-Pfeilers. CgeA, das am seltensten vorkommt, spielt scheinbar eine Rolle bei der Glykosylierung. CotZ verankert die Struktur der Kruste an den Mittelteil der Spore, wobei CotX und CotY sie möglicherweise schon lose an die Pole der Spore verankern.

CotX und CotV könnten Kandidaten für die Glykosylierung sein, aufgrund des konservierten Glykosylierungsmotivs in der CotX Superfamiliendomäne. Der Glykosylierungsmodus scheint ziemlich komplex zu sein. Es sind viele Akteure und vermutlich mindestens sechs verschiedene Zucker beteiligt, die in zwei unabhängige, jedoch wahrscheinlich quervernetzte Polysaccharid-Spezies unterteilt werden können: eine, die mit Rhamnose verwandt ist, und eine mit Galaktose. Die mutmaßlichen Funktionen der Akteure deutet darauf hin, dass die mit Rhamnose-verwandte Spezies möglicherweise den seltenen Zucker Viosamin oder VioNAc enthält. Des Weiteren ist das mit Rhamnose verwandte Polysaccharid potenziell mit einem einzigartigen, auf Viosamin oder VioNAc basierenden Zucker gekappt ist der mit einer Lysin-basierenden Seitenkette modifiziert sein könnte, ähnlich wie bei dem nahen Verwandten *Bacillus anthracis*. Das deutet darauf hin, dass diese Modifizierung nicht nur der Wasserverteilung der Spore dient, sondern aufgrund ihrer außergewöhnlichen Beschaffenheit auch zusätzlichen Schutz vor biologischer Plünderung bieten könnte.

Lehren, die aus der Beschaffenheit der Kruste gezogen werden, können im Gegenzug die Entwicklung potenzieller Verbesserungsstrategien vorantreiben. Allerdings gelang es zwar durch die Linker unterdurchschnittlich abschneidende Varianten zu retten, doch führten sie bei den am besten funktionieren Varianten nur zu einer geringen Verbesserung. Gleichermaßen führte die Entfernung des nativen Wettbewerbs nur zu geringen Leistungssteigerungen. Das ist wahrscheinlich auf die hohen Redundanzen (CotY/CotZ und CotV/CotX) zurückzuführen. Durch die Glykosylierung, könnten manche Enzyme in dieser Mikroumwelt weniger aktiv oder gar inaktiv sein. Vor diesem Hintergrund führte die Veränderung der Polysaccharidstruktur mit einer Mutante (*cgeA*) zu einer geringen Verbesserung der Aktivität der *Bacillus pumilus*-Laccase. Dies könnte in Zukunft die Leistung mancher Enzyme ermöglichen, wie beispielsweise Lipasen.

Trotz der minimalen Verbesserungen, könnte eine hierbei gemachte Entdeckung zu einer neuartigen Anwendungsgebiet führen: spore-derived self-assembled non-GMO including particles (SporoSNIPs). Manchmal war der Einbau der Krustenproteine stark beeinträchtigt, aber die Krustenproteine assemblierten stattdessen in der Mutterzelle (in *cotZ* Mutanten die gesamte Krustenstruktur, oder CotX und CotV in jeglichen Mutanten, die nicht CotY oder CotZ aufwiesen). Diese Fragmente (SporoSNIPs) könnten möglicherweise angereichert oder komplett von der Spore getrennt werden, zur Weiterverwendung als biologisch aktive Partikel, jedoch ohne den Nachteil, lebende, genetisch veränderte Organismen zu beinhalten.

Chapter 1: Introduction

1.1 Survival artist: the endospore of *Bacillus subtilis*

The Gram-positive bacterium *Bacillus subtilis* lives in the soil were it has to endure a high spectrum of changing environmental stressors such as dryness, nutrient scarcity, UV radiation and competition with other soil habitants[1]. The competition over specific nutrients can be very intense, and the availability of all nutrients is not reliable[1]. Therefore, many soil inhabitants are highly antagonistic and have evolved survival strategies in order to cope with life in this demanding ecological niche[1]. *B. subtilis* is no exception, and has consequently developed into a true survival artist. When nutrients are scarce, it can adopt a wide range of strategies to help it survive, its last resort being the production of a dormant endospore[2]. These strategies include the production of degradation enzymes (miner), the production of a matrix (biofilm), the production of toxins (cannibals), motility to find new niches and competency to scavenge nutrients from nucleic acids or to potentially adapt a strategy of survival (antibiotic resistance, degradation enzyme to utilize new nutrient sources, or others). As most of these strategies require only little energy to bring about a beneficial outcome (new nutrient sources, protection against competitors in the soil), they are the first lines of survival.

The production of the endospore itself, however, is very costly, and the aim is purely to survive until either the environment becomes more life-supporting or the spore is passively transported to a different niche where it can germinate again. If this change is initiated too early or too late, it results in the ecological disadvantage of either not multiplying and further competing with other soil inhabitants for the available resources and space, or in death, respectively. Therefore, there is an elaborate sensing and timing cascade dependent on the phosphorylation of the regulator Spo0A which leads to the initiation of the sporulation. This sensing cascade even involves cannibalism as a strategy to avoid the energy-consuming production of the spore. Nevertheless, the initiation of sporulation represents a point of no return[3, 4]. After this comes a fine-tuned series of sigma factor activations, also termed ping-pong cascade, which times and monitors the successive steps in mother cell and forespore, and synchronizes these events. These steps also include check-points for DNA damage, correct DNA packaging and correct engulfment to ensure that the spores that are produced are complete and viable after germination[4]. For the mother cell, sigma factor E and sigma factor

K and for the forespore, sigma factor F and sigma factor G coordinate the early and late processes of sporulation, respectively[4].

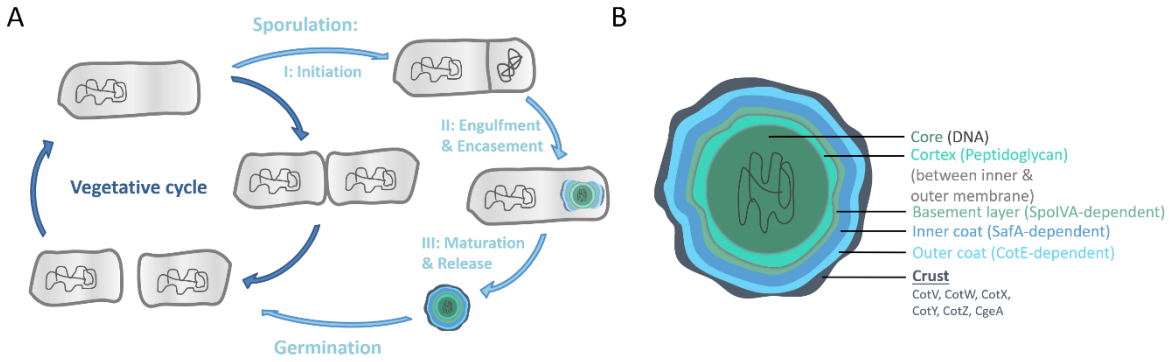


Figure 1: Production and architecture of the Bacillus subtilis *endospore*
***A**: The life cycle of* Bacillus subtilis *is shown here. When sufficient nutrients are available, the cell divides symmetrically in the vegetative cycle to produce biomass, just like in any bacterium. At the point in time when nutrients become scarce,* B. subtilis *differentiates into different cell types (which are explained in the main text), of which the differentiation into the dormant endospore (sporulation) is depicted here: Initiation (I), engulfement (II) by the mother cell (hence the term "endospore"), maturation and release of the spore (III). The spore can reenter the vegetative cycle via germination **B**: The architecture of the spore is depicted here, showing the core (containing the DNA packaged by DPA and SASPs), the cortex sandwiched by two membranes, and the proteinaceous coat: the basement layer, the inner coat, the outer coat and, outermost, the crust. The latter consists of at least six proteins, CotV, CotW, CotX, CotY, CotZ and CgeA. The formation of the basement layer and the inner and outer coats depends on the morphogenic proteins SpoIVA, SafA and CotE, respectively.*

After sporulation is initiated, the cell division site shifts to the poles by displacing the FtsZ division ring from the middle of the cell[5]. This leads to asymmetric cell division[5]. The smaller part of the cell is engulfed by the mother cell, resulting in the forespore (see Figure 1A). This process is driven by a membrane-bound machinery that degrades the cell wall. Only a thinned peptidoglycan layer, known as a germ cell wall[6], remains between the two membranes of the forespore. On top of this germ cell wall, a sporulation-specific peptidoglycan layer called a cortex is incorporated. The production of the cortex depends on the initiation of the basement layer, which is the first proteinaceous layer of the spore (see Figure 1B). This initiation is marked by the assembly of its morphogenetic protein SpoIVA as a cable-like mesh onto the forespore membrane[7]. SpoIVA is recruited by SpoVM[8], an amphipathic helix that integrates into the membrane. After recruitment, SpoIVA polymerizes, driven by ATP cleavage[7]. This mesh of SpoIVA coordinates the cortex production as well as the assembly of the spore coat[4]. The sporulation-specific lipid-II flippase is recruited to this mesh[8] and flips the peptidoglycan precursors into the intermembrane space, where they are then cross-linked[4]. The cortex protects the two membranes and therefore the core of the spore and is biochemically related to the prior thinned vegetative cell wall, but thicker and subsequently modified[9]. Simultaneously to cortex production, four distinct proteinaceous layers – the basement layer,

inner coat, outer coat and crust – assemble on the surface of the spore. Their formation is thought to be mostly driven by self-assembly[10]. The recruitment depends on core morphogenetic proteins (SpoIVA, SpoVM and SpoIVD for the basement layer, SafA for the inner coat and CotE for the outer coat) that seem to coordinate this highly structured process[11-13]. The assembly of each layer seems to also depend on the presence of the prior layer, an exception being the independence of the outer coat and the inner coat[4, 13]. The proteins for the coat are produced by the mother cell and assembly mostly initiates at the poles[14]. Even though each layer depends on the prior layer, their completion does not necessarily follow the same temporal chronology[14]. The outermost layer, the crust, was only discovered quite recently[15, 16], contains the six proteins CotV,W,X,Y,Z and CgeA, and is thought to depend on CotXYZ (Figure 1B).

1.2 Architecture of the spore coat: highly structured layers driven by self-assembly

How the proteinaceous layers of the spore are built up was shown quite nicely in an atomic force microscopy (AFM) study in diverse mutants, mostly missing the morphogenetic proteins or major proteins of each layer[13], meaning these mutants lacked parts of these structured layers. Additionally, (parts of) these layers were chemically removed to be able to see further into the structure. The exact types of mutants used and the details on the differences provide no benefit for the study presented in this thesis. Nevertheless, the general findings help understand the context of the spore coat and its recurring modes of highly structured self-organization, which could provide information that would help to understand the crust as the outermost layer of this structure. The AFM study found that the individual layers are subdivided into diverse structures: the outer pitted surface of the cortex, the basement layer, a multilayer structure on which nanodots are assembled, a fibrous/granular layer, a honeycomb layer, a rodlet layer and the outermost amorphous layer. The multilayer structure on which nanodots assembled was attributed to the inner coat, as it was substantially thinned and not tightly linked to the basement layer or cortex in the *safA* mutant (SafA being the morphogenetic protein of the inner coat). Additionally, it was missing in the misassembled spore coat sacculus in the *spoIVD* mutant (SpoIVD being the morphogenetic protein of the basement layer and a supporting morphogenetic protein for the inner coat). The nanodots were missing in the *cotE* mutant, meaning the latter might therefore contribute to their formation. As the *cotE* mutant (CotE being the morphogenetic protein of the outer coat)

additionally lacked the fibrous/granular structure, this structure was attributed to the outer coat. Its assembly was speculated to be facilitated by the nanodot structure, therefore representing the interface of these two layers (the multilayer, attributed to the inner coat, and the fibrous/granular layer, attributed to the outer coat). This is in accordance with CotE being the morphogenetic protein of the outer coat layer. The honeycomb and rodlet layers could not be attributed to any known structure of the coat, but were noted as new features of the outer structure (rodlet and amorphous/crust layer) and the respective interface (honeycomb layer). In this AFM study, they discussed two different modes of self-assembly for the different layers: 2D nucleation (for the multilayer structure) and a porous matrix (honeycomb layer) facilitating the self-assembly of amyloid-like structures (rodlet layer). 2D nucleation requires a high concentration of the coat proteins and 2D nuclei from which these layers spread. These nuclei could either be multi-molecular assembled coat proteins targeted at the spore surface, or impurities. The honeycomb structure, which creates the porous matrix on which the rodlets to polymerize, was shown to be able to self-assemble independently of the prior structures. The subsequent rodlet layer can therefore also be guided to assemble independently onto this mesh. These structures and modes of assembly show that the coat structure is highly organized, yet capable of assembling merely due to the intrinsic properties of the proteins and their interactions. An exception to this observation is the creation of the basement layer, which requires prior activation but self-assembles afterwards. This might mark a transition between a highly integrated sensing cascade (ping-pong of the sporulation[4]) and the self-organization in late stages of a survival process, where the exact energy status of the mother cell is not guaranteed. Therefore, the crust is highly likely to be a self-assembling structure as well. This is in accordance with the self-assembled supramolecular structures seen for some of the crust proteins when overexpressed in *Escherichia coli*[10].

1.3 The crust: the knowns of the architecture and protein interaction network

The crust was long termed an amorphous layer due to it only being visible when stained with ruthenium red under transmission electron microscopy (TEM), where it appeared to not be very highly structured[17]. Unlike most of the layers, which showed a highly regular structure under atomic force microscopy (AFM), this layer appeared amorphous[13]. How this correlates with the highly structured sheets (CotY) and fibers (CotV and CotW) that can be produced in overexpression experiments in *E. coli*[10] still remains unclear. The structure produced by the high-cysteine protein CotY in these sheets closely resembled the structure found for its

orthologue ExsY in the exosporium of the *B. cereus* group[18], which creates disulphide bonds even intracellularly, probably contributing to the spores' high level of chemical resistance. Natively, however, no structure could be determined for the crust. This raises the question whether these structures are natively relevant or just an artefact of overexpression. A likely reason for the amorphous character of the crust could also be possible modifications of the crust proteins masking the structure in TEM and AFM imaging. Additionally, the overexpression experiments only included one (CotY) or two (CotV and CotW) of the crust proteins. How these structures might relate with the other crust proteins remains unclear. For a better understanding of this relationship, it is of pivotal importance to look at the interaction network of these proteins, as the interaction/dependence might provide clues as to how this structure could be implemented natively.

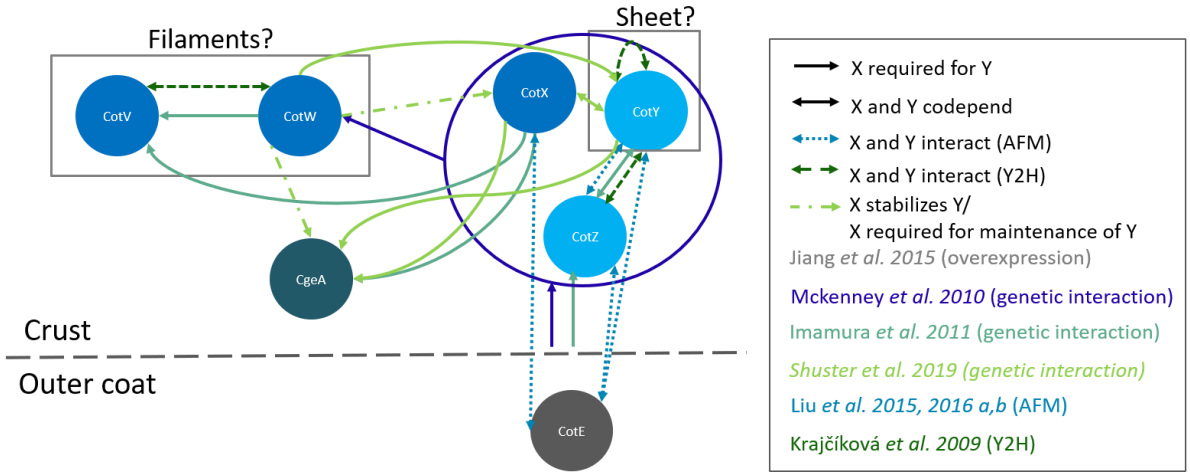


Figure 2: Known protein interactions of the six crust proteins
The knowns of the protein interaction network (excluding the most recent bacterial 2 hybrid study[19] for the sake of clarity) and structural information of the crust proteins are shown. The structural information comes solely from cryo-TEM imaging after overexpression in E. coli*, because natively, the crust always appears amorphous[13, 17]: CotV and CotW together produced filaments, and CotY produced highly structured sheets[10] (shown in grey boxes). Information on protein interaction derives either from genetic interaction[15, 16], a yeast 2 hybrid screen[20] or atomic force microscopy (AFM) studies[21-23]. The source of the information is color-coded and shown in the box on the right, as is the nature of the derived interaction (color-coded arrows).*

The crust is composed of the insoluble fraction (the five different proteins CotV,W,X,Y,Z)[24] and CgeA[15, 16] (see Figure 1B). How these proteins interconnect to form the crust as well as the native architecture is mostly unknown. What is known, however, is how some of these proteins interact or interdepend based on atomic force microscopy (AFM) or a row of two-hybrid assays. These methods, either being *in vitro* (AFM) or in a different organism (yeast or *E. coli*) raise the question if the findings are natively relevant. Three studies [15, 16, 25] give insight into the genetic interactions of the crust proteins. However, these studies either have gaps in the mutants they study or omit one or more of the crust proteins. These studies and the

current knowledge derived from them is shown in Figure 2. The morphogenetic protein(s) of the crust are concluded to be CotXYZ[16] or CotZ[15]. Imamura *et al.* showed that on the on hand CgeA and CotV depend on CotX and CotV additionally on CotW and on the other hand that CotY and CotZ codepend[15]. Shuster *et al*. 2019 additionally showed, that CotY and CotX codepend, which both depend on CotW either slightly (CotY) or for stabilization (CotX). They additionally showed, that CgeA depends on CotX and CotY. Nevertheless, additionally to giving an incomplete picture, there are also some discrepancies between the single studies. In contrast to Imamura et al. 2010, Shuster et al. 2019 concluded that CgeA maintenance rather than localization depended on CotW, and that CotZ does not depend on CotY.

AFM experiments as well as the two-hybrid assays confirm some of these findings: the interactions between CotY and CotZ, as well as between CotV and CotW[19, 20]. A very thorough bacterial two-hybrid assay reveals a whole interaction network of strong to weak interactions of the crust proteins as well as to the coat proteins (not depicted in Figure 2, as this study showed the interaction of almost all crust proteins with each other. Exceptions are CotX, which only interacted with CotW, CotW, which does not interact with CotY, and CgeA, which does not interact with CotV). This study gives a good basis for the interaction network, but lacks information that can only be collected natively: Are interactions natively relevant and to what extent (stabilizing, recruiting, codependent)? Does the respective crust protein play a role (and if so, what kind) in the structure as well as the location of the respective proteins? Nevertheless, this study also gives some insight into the link between the crust and the outer coat: CotY and CotZ[19] both interact with CotE, as well as with many other coat proteins. Accordingly, the AFM studies showed the interaction of CotY, CotZ and CotX with CotE[22, 23] as well. However, the overall picture of all interdependencies as well as the location and role of the respective crust protein in the structure of the crust remains incomplete.

1.4 The maturation of the endospore gives rise to ecological advantages such as its high level of resistance

Producing the dormant and highly resistant endospore requires some maturation steps, which can, other than depicted in Figure 1, occur at different points in time, and not necessarily only after spore release. These steps can occur during endospore formation. These include dipicolinic acid synthesis and uptake which dehydrates the endospore[26], protection of the

DNA with small acid soluble proteins (SASPs)[27], cortex production and modification[9], as well as proposed disulphide-bridge formation in the crust[18]. Some modification steps, such as protein cross-linking[28, 29], can even continue for a few days after spore release due to mother cell lysis[30]. These maturation steps grants the spore properties that ensure its survival in the harshest environments and even help in the relocation to new niches. The dehydrated core and the SASPs lead, among other things, to greater resistance against wet and dry heat, UV radiation and radiation. The cortex protects the membrane, and therefore also the core, against some chemicals and organic solvents[31, 32]. Its highly cross-linked and structured coat protects the spore against UV and oxidative stress[33] as well as biological degradation by enzymes[29] or even biological scavenging by competing soil organisms[34, 35]. The presumed glycosylation of the crust[17, 36-39] (which occurs at an unknown point in time) gives rise to the spore's hydrophilic nature. This property leads to the proposed function of the crust: Due to its hydrophilic surface, the spore is readily dispersed in the environment through (rain) water[15, 16, 36] and can therefore passively find new niches that could potentially be more friendly to bacterial growth. This gives it an ecological advantage over survival stages of other competitors which are not able to (passively) relocate to new niches.

1.5 The crust: sugar-coating helps move on to new pastures, or so it seems

The proteinaceous coat of the spore is modified in various ways, involving protein cross-linking[14, 15], disulphide bridge formation in the high cysteine crust proteins CotY and CotZ[18], and presumably also the sugar-coating or glycosylation of the crust[17, 36-39], as discussed above. The glycosylation of the crust, as already mentioned, gives rise to the proposed function of the crust as mostly stated in scientific literature: the water dispersal of the spores[15, 16, 36]. This function is proposed due to the phenotype of the mutants, which are more hydrophobic and tend to clump extensively. The readiness of the spores to be dispersed to new niches (as a soil bacterium, for example, from an abandoned pasture to another one full of nutrients) by (rain) water give them a peak ecological advantage over a survival strategy that rests solely on waiting for better times.

Even though the crust has often been termed a glycoprotein layer due to the fact that it stains with ruthenium red[17, 40], there is no hard evidence to suggest that the sugar is actually attached over protein glycosylation. Furthermore, an in-depth picture of the nature of the

sugar coat is lacking. There are a few indicators regarding which sugars might participate[36, 38] as well as some of the players, due to phenotypical evidence and gene product prediction[36, 37, 39]. Two of these studies led to the involved genes being renamed spore polysaccharide synthesis (*sps*)[36, 37] due to the phenotype of the mutants (more hydrophobic and clumping). Some work, as well as the predicted protein domains, also point to the involvement of *ytcA* and the *yfnHGFED* cluster in this spore maturation process[39]. The sugars involved in spore polysaccharides were examined by two of these studies, which might include rhamnose, galactose[36, 38], ribose, glucose, muramic acid, GlcNAc and quinovose (6-desoxy-glucose)[38], a rare sugar that is unique to *B. subtilis*. Additionally, one study showed a probable link between a sugar involved in the crust polysaccharide and the action of the candidate genes: *spsI-L* has been predicted, as well as partially experimentally validated, to produce rhamnose[41]. To date, however, the exact link between the sugars and the involved genes has not been established. Moreover, the possible structure of the polysaccharide and the attachment points remain unknown. Likewise, it is unclear if all the genes involved in the modification have been identified.

Nevertheless, these preliminary findings might already shift the viewpoint of what is the general state of affairs regarding the glycosylation of the crust as the determinant for water dispersal. What seems odd is the sheer number of genes potentially involved in this modification step, which outnumbers the crust genes. In addition, the preliminary findings on the sugars involved, one of which is also unique to *B. subtilis* (quinovose), indicates that the sugar-coating process might be more complex than required for water dispersal. This property alone could be accomplished in an easier and more energy-efficient manner. This suggests that water dispersal is not the sole purpose of crust glycosylation. In relatives of *B. subtilis*, which are pathogens, such as *B. anthracis*, the sugars attached to the exosporium have been proposed to be important for host cell interaction and internalization[42]. A complex polysaccharide structure including rare or even unique sugars is only logical in such a context. But *B. subtilis* is not a pathogen, which rules out this explanation. For this reason, the question arises as to what the true purpose of the crust glycosylation might be. To this end, a more detailed picture of the players involved in glycosylation as well as of the nature of the polysaccharide could provide more insight.

1.6 Sporobeads: utilization of the spore crust to produce self-displaying biological beads

Despite the above-mentioned complexity of the spore, one of the goals of this study is to utilize this outermost layer, the crust, as a platform for protein display. This protein self-immobilization leads to numerous advantages. For most real world applications, protein immobilization is of pivotal importance[43]. The exploitation of the diverse enzymes or protein functions benefit from their stabilization on the matrix surface during storage or application. Stabilization includes protection against denaturation by solvents and heat[43]. Additionally, handling such as end product separation, recovery or reusability is facilitated, making the immobilized protein the cheaper and more convenient option[43]. On top of all these advantages, immobilization opens up possibilities for some specific applications, such as continuous fixed-bed operations, multi-enzyme or cascade processes[43].

There are two different modes of immobilization: the immobilization of purified proteins on synthetic surfaces (such as silica beads), and the self-immobilization on diverse biological surfaces (such as phage display, cell wall targeting, and others[44]). Compared to each other, self-immobilization on biological surfaces is the more cost- and time-efficient alternative[44], as it does not require costly and time-consuming purification and subsequent fixation onto the desired surface of the protein of interest.

To this end, the endospore of *B. subtilis* can also be utilized as a biological surface for protein immobilization[44]. Spores have some unique features that make them superior to other systems, one being their high level of resistance to heat and organic solvents of these biological particles[31]. On top of that, self-immobilization onto the surface of the spores does not require membrane transportation due to the intracellular mode of spore production[4]. Therefore, proteins not readily secreted will benefit from this unique feature. Studies to date have mostly utilized the spore coat as an anchor for diverse proteins of interest[44, 45]. In the new light of the crust as the outermost layer, this is to some extent unexpected, as the crust should mask these underlying layers. The layer on top of the coat should exclude these underlying coat layers as a surface structure for immobilization. Nevertheless, it still proved feasible, as the spore coat is not as densely packed, making it a plausible matrix for protein immobilization: Germinants can permeate the coat to reach the receptors in the spore

membrane[45] and even some proteins can reach underlying layers until quite late in endospore maturation[14]. However, bigger proteins such as antibodies cannot reach these coat layers, as shown by immunostaining[15].

For this reason, the crust as a platform for protein immobilization seems the more promising and obvious choice. An additional benefit of the crust is that even though the surface properties change in crust mutants[15, 25], the resistance of the spore appears to remain unchanged. Accordingly, any disturbances in this layer due to the anchoring of a protein of interest is unlikely to change the resistance to heat and organic solvents. This makes some applications that require high temperatures or harsh organic solvents possible at all. Even though some crust proteins have been utilized for surface display[46], no conclusive study has compared the crust proteins as anchors for spore display. As such, this study aims to provide an easy and convenient method to test diverse possibilities of protein immobilization onto the crust. This is accomplished by designing so-called Sporovectors, where the gene of interest can easily be cloned and subsequently integrated into the genome of *B. subtilis*. This design facilitates the aimed conclusive study of the crust as a platform for protein display. The created protein-displaying spores were termed Sporobeads.

1.7 The objectives of this study: The connecting link between the knowledge of the crust's nature and the applicability of the Sporobeads

"What I cannot create, I do not understand." – This quote left to posterity on his black board by Richard Feynman after he passed away in 1988 has become the mantra of synthetic biology. It also means that foundational advances and application always go hand in hand. Foundational advances might help to reveal and implement applications, and conversely, the implementation of this knowledge as an application could help further understand the system. This relationship can lead to a design-create-test-learn(-redesign) cycle borrowed by synthetic biology from the more technical field of engineering.

This study benefits from that very same design-create-test-learn cycle. It all starts with the basic understanding of the crust: the proteins involved in this layer, and the fact that it is the outermost layer[15, 16]. The design of application (Sporovector system) is such that it is easy access to create and test a wide variety of possible Sporobeads. This easy design will help create a comprehensive collection of GFP fusions in crust and glycosylation gene mutants,

which will in turn lead to a better understanding of the interaction network and the nature of the crust, and address the open questions discussed above.

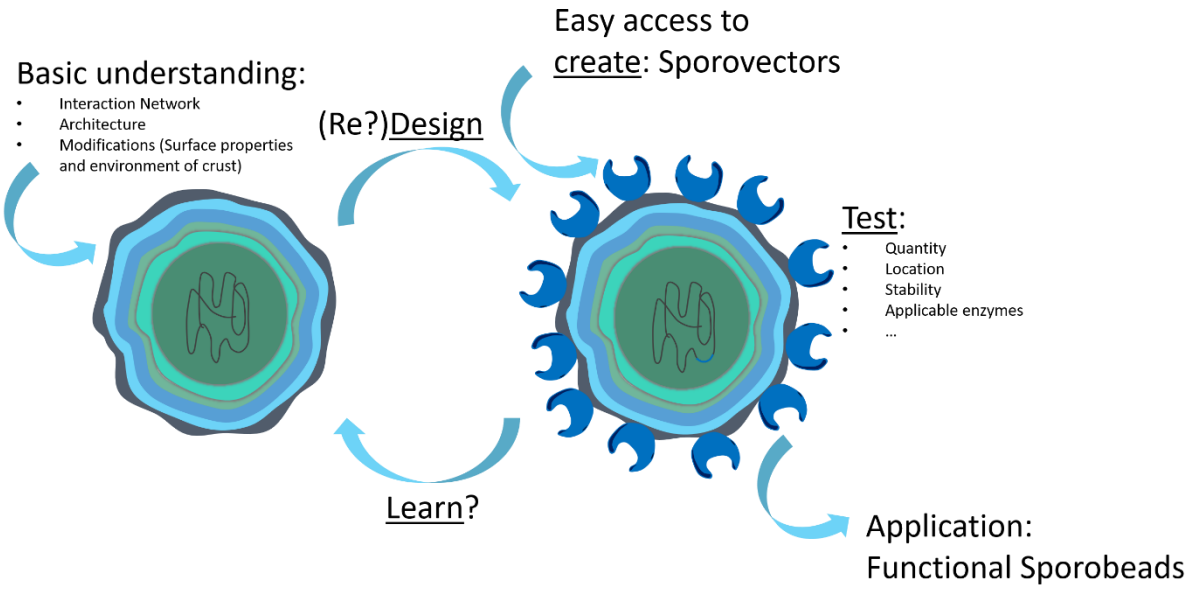


Figure 3: Design-Create-Test-Learn cycle: basic understanding of the spore crust and its application as Sporobeads
This study aims to benefit from the Design-Create-Test-Learn cycle, a tried-and-tested methodology borrowed from the field of engineering. The starting point is always the basic understanding of a system: the foundation for designing and creating a system to produce functional Sporobeads. In order to create a short-cut in this cycle, the "create" step is designed in such a manner that it is easy to create a vast amount of different Sporobeads through the Sporovectors. These Sporovectors not only help to test the application (the functional Sporobeads), but in turn help us understand the crust in more detail, as they can also be used to create a vast amount of GFP fusions in crust gene mutants or glycosyltransferase mutants. But also, the performance (quantity, location, stability, what types of enzymes might be applicable…) helps understand the nature of the crust and vice versa: This cross-talk between foundational research and application is a main theme of this thesis.

The connecting link between these two pillars of the study (nature of the crust and application) is what these two studies can teach each other. Accordingly, the quantity and location of the proteins of interest as well as the performance of the Sporobeads might give insight into the potential roles of the respective crust proteins. Conversely, insights into protein interactions as well as the modifications of the crust (and therefore the direct environment for the proteins/enzymes displayed) may help to explain possible effects in stability and applicable enzymes and might pave the way for redesigning and improving the working system of the Sporobeads. To improve the system, the information on the quantity of the proteins is quite helpful, as only proteins found natively in high numbers are a suitable anchor and represent an appropriate candidate for further optimization. The information regarding the interaction might also be interesting, as it might be possible to increase the capacity of the spores for the ectopic anchors by removing one or more of the native crust genes. Without the information on how these proteins relate to each other, this would be a

quite time-consuming approach. But by eliminating all variants where the protein is negatively influenced, this number might be decreased to a quite feasible amount. Also, the information on the environment of the spore's surface might be important with regard to selecting the possible enzymes for display: Enzymes that require a more hydrophobic environment might not operate as well in the hydrophilic nature of the glycosylated crust. The information on the key players and the structure and redundancy of this modification might give insight into how to change the property of the surface and therefore broaden the range of potentially displayable target enzymes. The information on the possible function of the crust and its modification might give insight into what could be impaired when utilizing the crust proteins as an anchor and what properties of the spore, for example resistance to chemicals, are still most likely in order.

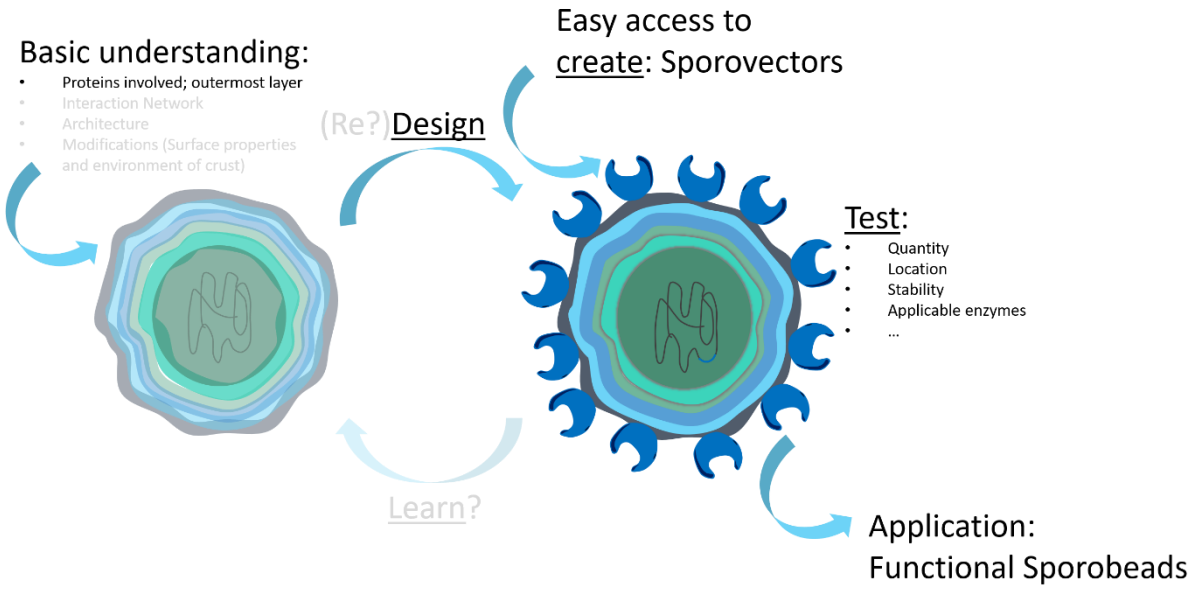
Taken together, the study of the nature of the crust not only helps to improve the application of the spore as a Sporobead; in turn, the performance and easy access of the application also helps us understand the crust to a better degree, in keeping with the spirit of synthetic biology.

Chapter 2: Sporobeads: The Utilization of the *Bacillus subtilis* Endospore Crust as a Protein Display Platform

This chapter presents the published work (Publication I):

Sporobeads: The Utilization of the *Bacillus subtilis* Endospore Crust as a Protein Display Platform. **Bartels J**, López Castellanos S, Radeck J, Mascher T. ACS Synth Biol. 2018 Feb 16; 7(2):452-461. doi: 10.1021/acssynbio.7b00285. Epub 2018 Jan 19

Background:



This study aimed to create self-immobilizing biological particles by translationally anchoring a protein of interest to the crust. This included designing and implementing Sporovectors and testing the application of the Sporobead system based on the basic understanding of the crust as the outermost layer and the proteins involved: CotV,W,X,Y,Z and CgeA. It provides first insights into the roles of these six proteins in the crust as well as the tools needed to learn more about the nature of the crust.

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Sporobeads: The Utilization of the *Bacillus subtilis* Endospore Crust as a Protein Display Platform

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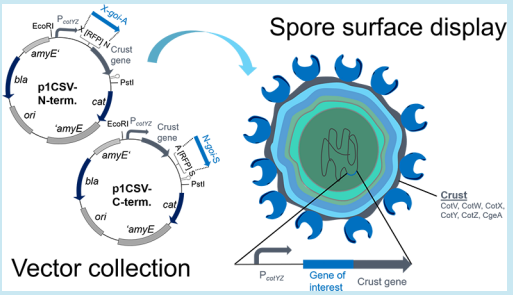
Supporting Information

ABSTRACT: Upon starvation, the soil bacterium *Bacillus subtilis* forms an intracellular, metabolically inactive endospore. Its core contains the DNA, encased by three protein layers protecting it against a multitude of environmental threats. The outermost layer, the crust, harbors great potential as a protein-displaying platform: a gene of interest can be translationally fused to a crust protein gene, resulting in endospores displaying the desired protein on their surface. To unlock this potential in a standardized fashion, we designed a suite of 12 vectors (Sporovectors), based on the BioBrick cloning standard. With these vectors, proteins can easily be fused N- or C-terminally to the six crust proteins CotV, CotW, CotX, CotY, CotZ, and CgeA under the control of the strongest crust gene promoter P_{cotYZ}. All Sporovectors were evaluated with GFP and two different laccases. On the basis of our data, CotY and CotZ represent the best anchor proteins. But there are significant differences in activity and functional stability between the two tested laccases. Our vector suite is a powerful tool to generate and evaluate a vast variety of functionalized endospores. It allows quickly identifying the best anchor and fusion site for the protein of interest. Our findings demonstrate that the crust of *B. subtilis* endospores is an inexpensive and easy platform for displaying different proteins of interest.



KEYWORDS: *sporulation, surface display, protein immobilization, BioBrick standard, Bacillus vectors*

The soil bacterium *B. subtilis* produces endospores under starvation conditions. This differentiation into a dormant stage is a highly relevant ability in its niche, the soil environment, where nutrients are not always readily available. The endospore is produced intracellularly by the mother cell, following an asymmetric cell division and the engulfment of the forespore, which is thereby encapsulated by two membranes. It is protected by a specialized and spore-specific peptidoglycan layer, termed the cortex, which is thicker than and differentially modified compared to the vegetative cell wall. The peptidoglycan precursors for the cortex are flipped into the intermembrane space by a sporulation-specific lipid-II flippase and then cross-linked.[1] Simultaneously, four distinct proteinaceous layers—the basement layer, inner coat, outer coat, and crust—are assembled on the surface of the spore by the mother cell. This process initiates at the poles and the individual layers are completed at different time points.[2] Their formation is thought to be mostly driven by self-assembly,[3] depending on core morphogenetic proteins that serve as hubs to coordinate this highly structured process: SpoIVA for the basement layer, SafA for the inner coat, and CotE for the outer coat[4,5] (Figure 1). The assembly of each layer depends on the prior layer, with the exception of the inner and outer coat, which are independent from each other. The unique mechanism of intracellular assembly of this surface structure by the mother cell abolishes the need for transporting these proteins across the inner and outer membrane, the cell wall, and the cortex. The outermost layer, the crust, was only discovered quite recently,[6,7] and is thought to depend on CotXYZ (Figure 1). It further contains the proteins encoded by the *cotVWXYZ*-cluster and CgeA from the *cgeAB* operon (Figure 2A).

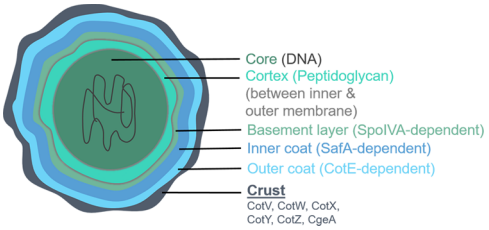


Figure 1. Architecture of the *B. subtilis* endospore: The core contains the DNA; the peptidoglycan cortex is sandwiched between inner and outer membrane; the basement layer, the inner coat, the outer coat, and outermost the crust. The latter consists of at least six proteins, CotV, CotW, CotX, CotY, CotZ, and CgeA. Formation of the basement layer, inner, and outer coat depends on the morphogenic proteins SpoIVA, SafA, and CotE, respectively.

***B. subtilis* Endospores as a Platform for Protein Display.** Protein immobilization on different surfaces, synthetic or biological, is important for the biotechnological or medical exploitation of diverse protein functions.[8] The main benefits of immobilization lie in the stabilization of and convenience in handling the enzymes. The product can easily be separated

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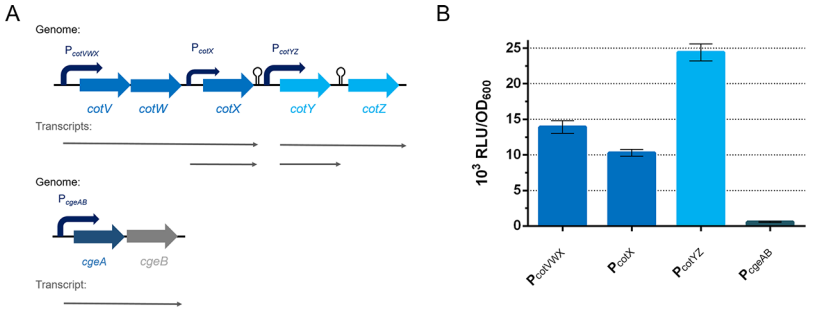

Figure 2. Genomic organization and promoter strengths of crust gene operons. (A) Operon structure of the *cotVWXYZ* cluster[30] and the *cgeAB* operon and their resulting transcripts (gray arrows). The *cotVWX* operon is transcribed from the P_{cotVWX} promoter. Additionally, *cotX* is transcribed from the internal promoter P_{cotX}. The transcript *cotYZ* is under transcriptional control of the P_{cotYZ} promoter. An internal hairpin-loop after *cotY* leads to an incomplete termination. The *cgeAB* operon is transcribed by the P_{cgeAB} promoter. Genomic regions and transcripts are not drawn to scale. (B) Crust gene promoters (P_{cotYZ}, P_{cotVWX}, P_{cotX} and P_{cgeAB}) were transcriptionally fused to the *lux* operon and luminescence was measured in a microtiter plate reader to determine luciferase activity (Supplemental Figure S1). Mean peak activity is plotted as relative luminescence units (RLU) per OD_{600} as a measure of maximal promoter activity per cell. The error bars show the standard deviation calculated from biological triplicates.

from the enzyme, thereby reducing protein contaminations and facilitating recovery and reusability of the enzyme, which can be an important economic factor.[8] Immobilization also enhances the stability during storage, as well as during operational conditions, by protecting the enzyme from denaturation caused by heat, solvent, or autolysis.[8] Additionally, it enables distinct applications such as continuous fixed-bed operations, multienzyme or cascade processes, or the optimal performance in nonaqueous media.[8] The accessibility to the media and the substrate is enhanced while the enzyme is stabilized toward denaturation by the organic medium.[8]

In comparison to the immobilization of proteins on synthetic surfaces, such as silica beads, which is a highly laborious and expensive procedure that requires purified proteins, the self-immobilization on biological surfaces has become a cost- and time-efficient alternative.[9] These range from phage display, to targeting of the cell surface, cell wall, or other diverse surface structures of cells.[9] In recent years, the endospore of *B. subtilis* has been studied as a protein display platform, due to its high resistance, its dormancy, and its unique mechanism of producing a proteinaceous coat intracellularly.[9] This provides the potential for self-immobilization by genetically anchoring proteins of interest to proteins forming these layers. Hence, the respective fusion proteins are incorporated into the highly structured coat of the spore.[9]

Since no membrane transport is required, this surface display can also be applied to proteins not readily secreted. The high resistance and dormancy of the endospore facilitate long-term storage and even the utilization of organic solvents or other conditions not tolerated by other biological surface display systems during storage or operational procedures. Several studies have already demonstrated the capability and advantages of spores for protein display (as summarized in refs 9 and 10). These studies have mostly focused on inner and outer coat proteins as anchors using antigens or specific enzymes as proof-of-principle. Despite some progress, no comprehensive and comparative evaluation has so far been conducted. The crust in particular, has hardly been addressed so far.[11−17]

The Advantages of the Crust as a Platform for Protein Display. As the outermost layer, the crust—which has only been discovered quite recently[6,7]—seems to be the obvious choice for surface display.

Previous studies have demonstrated that proteins found in the coat can be utilized as anchors for functional display.[9,10] This is possible, as the spore coat is not densely packed and is permeable for some molecules, as seen with germinants. In fact, even proteins can pass through these layers, at least until late in the endospore maturation.[2] Still, some applications may not tolerate a layer on top of the proteins displayed, which might block the access of substrates or protein interaction partners in mature spores, as seen with immunostaining.[6] In these cases, the crust will be the preferable target.

Additionally, most crust gene mutants do not show any sensitivity phenotype. This observation indicates that the utilization of crust proteins as anchors will not affect the beneficial resistance of the endospore, for example against organic solvents. The only phenotype observed in crust mutants seems to be an aggregation of the spores.[6,18] Hence, the proposed function of the crust is the prevention of spore aggregation and a dispersal of the spores in aqueous fluids. Therefore, utilizing the crust as an anchor for surface display, has the potential advantage that the derived spore maintains most of its advantages.

The Goal of this Study. Our study aims to unlock the potential of the crust as a surface for protein display. It provides a comprehensive evaluation of all known crust proteins, CotV, CotW, CotX, CotY, CotZ, and CgeA as anchors, utilizing green fluorescent protein (GFP) and two different laccases as test cases.

We provide a collection of 12 standardized ectopic vectors derived from the backbones of the *Bacillus* BioBrick Box,[19] which all integrate at the *amyE* locus. Our vector suite has been comprehensively evaluated under standardized conditions, in order to demonstrate their potential for protein surface display on the spore crust.

RESULTS AND DISCUSSION

P_{cotYZ} Is the Strongest Crust Promoter and Therefore Most Suitable for Surface Display. For an efficient spore display, the desired fusion proteins need to be expressed under the control of a strong promoter that is intrinsically activated to ensure the incorporation of large amounts of fusion proteins with the right timing during differentiation. We therefore chose to evaluate the four native crust gene promoters P_{cotVWX}, P_{cotX}, P_{cotYZ}, and P_{cgeAB} in order to identify the best candidate for our purpose (see Figure 2A).

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The promoters were cloned into the pBS3C*lux* reporter vector of the *Bacillus* BioBrick Box[19] to generate transcriptional promoter-*lux* fusions. The resulting reporter strains were grown in DSM to induce sporulation, and the resulting promoter activity was measured throughout the growth cycle in a microtiter plate reader (Figure S1). As shown in Figure 2B, P_{cotYZ} is the strongest of the four promoters tested. It was therefore used for the construction of all Sporovectors (see Figure 3 and Table 1) to achieve the highest possible efficiency in expression and therefore protein display.

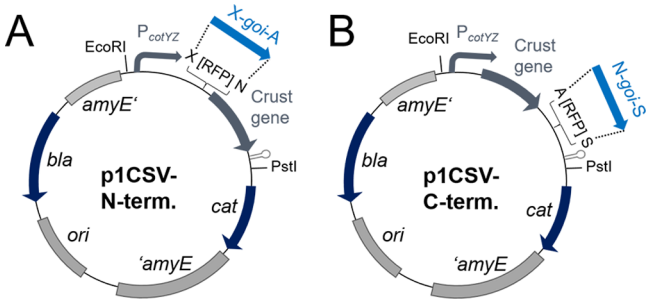

Figure 3. Vector maps of the Sporovectors. Vector maps of the pBS1C-Sporovectors, for N-terminal (A) and C-terminal (B) fusions to the crust gene. Both utilize the pBS1C-backbone,[19] which carries a *cat* gene mediating chloramphenicol resistance in *B. subtilis* flanked by homologous integration sites for the *amyE* locus in the genome of *B. subtilis*, as well as the *bla* gene for an ampicillin resistance and an origin of replication (ori) for vector propagation in *Escherichia coli*. The Sporovector-specific part is located between the restriction sites *Eco*RI and *Pst*I. It contains the strongest crust operon promoter P_{cotYZ}, one of the crust genes (*cotV*, *cotW*, *cotX*, *cotY*, *cotZ*, or *cgeA*), an *rfp*-cassette, for red-white screening, and a transcriptional terminator. The *rfp*-cassette is located either on the future N- (A) or the C-terminal side (B) of the resulting crust protein fusion and will be exchanged for the gene of interest. The enzymes of use are *Xba*I (X), *Ngo*MIV (N), *Age*I (A), and *Spe*I (S), where N and A produce compatible overhangs, following the BioBrick RFC25[20] cloning standard for fusion proteins, but including a ribosome binding site (RBS) for *B. subtilis*.

Twelve Sporovectors Allow N- and C-Terminal Fusions to All Crust Protein Genes. All Sporovectors (see Figure 3 and Table 1) are based on the backbone pBS1C from the *Bacillus* BioBrick Box[19] and integrate into the chromosome at the *amyE* locus, thereby enabling the verification of positive integration events by a simple starch test. The Sporovector-specific part is located between the restriction sites *Eco*RI and *Pst*I. It contains the strongest crust operon promoter P_{cotYZ}, one of the crust genes (*cotV*, *cotW*, *cotX*, *cotY*, *cotZ* or *cgeA*), an *rfp*-cassette, for red-white screening, and a *rf* transcriptional terminator. The *rfp*-cassette is located either on the future N- or the C-terminal end of the resulting crust protein fusion and will be exchanged for the gene of interest. For the N-terminal variants, the *rfp*-cassette is excised with *Xba*I and *Ngo*MIV restriction, while the corresponding gene of interest is cut with *Xba*I and *Age*I. For the C-terminal variants, the *rtf*-cassette is removed by *Age*I and *Spe*I digestion, while the corresponding gene of interest is cut with *Ngo*MIV and *Spe*I. *Ngo*MIV and *Age*I produce compatible overhangs, following the BioBrick RFC25[20] cloning standard for translational fusion proteins, thereby leaving a two amino acid scar. A detailed description of Sporovector construction is provided in the Methods section. In addition, a guide describing how to utilize the Sporovectors can be found in the Supporting Information.

GFP Fusions to Crust Proteins Reveal a General Preference for the N-Terminal Fusion Site. Initially, we evaluated the spore display efficiency of all 12 Sporovectors with translational GFP fusions (Figure 4). This reporter easily allows quantification of protein display both at bulk and single spore level, using fluorescence microscopy. The latter will provide information on the distribution and heterogeneity of the protein display at the single spore level. Additionally, the signal can be quantified by direct comparison to standardized beads emitting a defined fluorescence signal.

Wildtype spores (W168) show the known autofluorescence signal. In the N-terminal versions, the proteins CotX, CotY, and CotZ show comparably high GFP signals, but differ in the distribution and heterogeneity of GFP display (Figure 4). CotV and CotW show a significantly reduced GFP signal, while CgeA seems to be the weakest anchor with only very little fluorescence observed in the N-terminal version (Figure 4, see asterisk). C-terminal fusions are much weaker throughout.

Table 1. List of Sporovectors

Sporovector[a]	backbone[b]	integration site[c]	antibiotic resistance[d]		BGSC-Nr.[e]	Jbeir public[f]
			E. coli	*B. subtilis*		
p1CSV-CgeA-N	pBS1C	*amyE*	Amp	*Cm*	ECE363	JPUB_009501
p1CSV-CgeA-C	pBS1C	*amyE*	Amp	*Cm*	ECE364	JPUB_009502
p1CSV-CotV-N	pBS1C	*amyE*	Amp	*Cm*	ECE365	JPUB_009503
p1CSV-CotV-C	pBS1C	*amyE*	Amp	*Cm*	ECE366	JPUB_009504
p1CSV-CotW-N	pBS1C	*amyE*	Amp	*Cm*	ECE367	JPUB_009505
p1CSV-CotW-C	pBS1C	*amyE*	Amp	*Cm*	ECE368	JPUB_009506
p1CSV-CotX-N	pBS1C	*amyE*	Amp	*Cm*	ECE369	JPUB_009507
p1CSV-CotX-C	pBS1C	*amyE*	Amp	*Cm*	ECE370	JPUB_009508
p1CSV-CotY-N	pBS1C	*amyE*	Amp	*Cm*	ECE371	JPUB_009509
p1CSV-CotY-C	pBS1C	*amyE*	Amp	*Cm*	ECE372	JPUB_009510
p1CSV-CotZ-N	pBS1C	*amyE*	Amp	*Cm*	ECE373	JPUB_009511
p1CSV-CotZ-C	pBS1C	*amyE*	Amp	*Cm*	ECE374	JPUB_009512

[a]The denomination derives from a shortened code for the pBS1C (p1C) as the backbone, SV for Sporovector, followed by the crust protein coded in the Sporovector and the translational fusion site (N=N-terminal, C=C-terminal). [b]The backbone is the *B. subtilis* backbone pBS1C[19] [c]In *B. subtilis* they integrate into the genome at the amyE-locus, which can be verified by the inability to degrade starch on starch minimal plates. [d]They all confer ampicillin resistance in *E. coli* and chloramphenicol resistance in *B. subtilis*. [e]The Sporovectors are readily available from the BGSC.[24] [f]The sequences can be found in the JBEI ICE Registry.[29]

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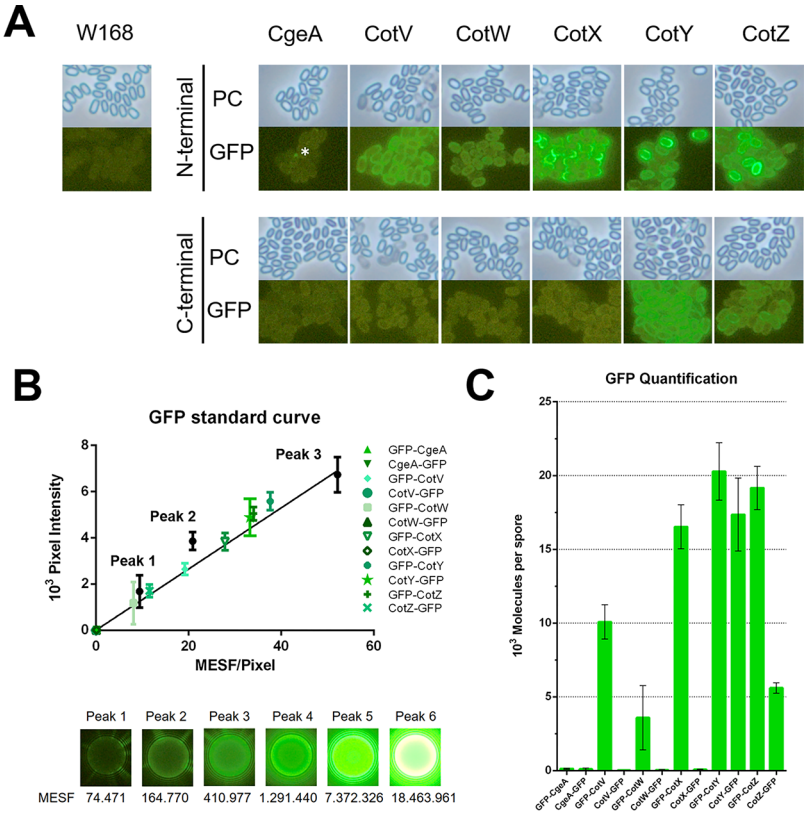

Figure 4. Evaluation and quantification of the six crust anchors utilizing GFP. Microscopic analysis of *B. subtilis* endospores displaying GFP either C- or N-terminally fused to one of the six different anchor proteins CgeA, CotV, CotW, CotX, CotY, or CotZ. (A) Phase contrast (top) and fluorescence microscopy pictures (bottom) of the spores displaying GFP. On the left side, the wildtype (W168) is depicted as a reference. The asterisk indicates the occasionally observed GFP-signal for the N-terminal CgeA variant. It should be noted that the color space of the autofluorescence is quite different than that of GFP, which can be numerically measured with a spectrum (data not shown) and is shifted slightly into the red. Therefore, it is possible to separate this signal from the GFP signal, not only qualitatively, but also quantitatively by utilizing a color threshold (for details refer to Methods). (B) Quantification of the GFP signal using a standard curve (black) created with the help of standardized GFP beads. These include six different peaks of intensity (depicted on the bottom) that carry different quantities of GFP molecules, given as molecular equivalent of the solvent fluorophore (MESF). The curve depicts the peaks 1−3 of the GFP-beads and relates the Pixel Intensity (relative unit) with GFP-density (MESF normalized by the pixel size of the GFP-beads (MESF/Pixel)). For each measurement, a separate standard curve (black) was created; a representative data set is shown here. The data points in green (different shades and symbols, refer to the legend) show the pixel intensities obtained for the measurement of the respective GFP-displaying spores and the corresponding GFP-density (MESF/Pixel) obtained utilizing the respective standard curve. The error bars show the standard deviation of biological triplicates. (C) For quantification of GFP-displaying spores, the GFP density obtained from the standard curve (B, MESF/Pixel) was multiplied by the average pixel size of the spore, to obtain the MESF, which is equivalent to functional GFP molecules per spore. The error bars show the standard error of the mean calculated from biological triplicates. For further details on the measurement and calculation refer to the Methods section.

The only C-terminal versions with a significant GFP signal are those to CotY and CotZ. For CotY, the signal of the C-terminal fusion is similar in strength to the N-terminal version, but more homogeneous. For CotZ the C-terminal signal seems to be weaker. Only a negligible GFP signal can be observed for spores expressing C-terminal GFP fusions to CgeA, CotV, CotW, and CotX. Overall, the N-terminus therefore seems to be more suitable as a fusion site for anchoring proteins to the surface of the spore.

To quantify the amount of GFP fusion proteins on the spore surface, GFP calibration beads were utilized. They are provided as a mixture of beads showing six different peaks of fluorescence intensity that correspond to known amounts of GFP molecules (molecular equivalent of solvent fluorophore, MESF). A comparison of the fluorescence intensity of these GFP-displaying spores with the fluorescence intensity of the GFP beads therefore allows estimating the number of GFP molecules per spore (Figure 4B and Figure 4C). For the N-terminal GFP fusions to CotX, CotY, and CotZ approximately 16 000 to 20 000 molecules per spore were determined, approximately 10 000 for CotV, and 4000 for CotW. Again, the numbers for the CgeA-dependent GFP display are much lower, with approximately 130 molecules per spore, demonstrating that this is the least favorable anchor protein. C-terminal GFP fusions to CotY resulted in approximately 17 000 molecules per spore, while CotZ displays about 5000 GFP molecules. The remaining GFP signals for C-terminal fusions are negligible.

Proof of Concept: Laccase Fusion Spores Exhibit Enzymatic Activity. The disadvantage of using GFP as a reporter for protein display on the spore surface is the low detection sensitivity. Moreover, it provides no information about protein stability and activity, as GFP is very stable on its own and performs no enzymatic activity. But for applied purposes, such information is crucial, since one of the primary motivations of immobilizing enzymes on surfaces is to increase their stability, But often at the price of a loss of enzymatic

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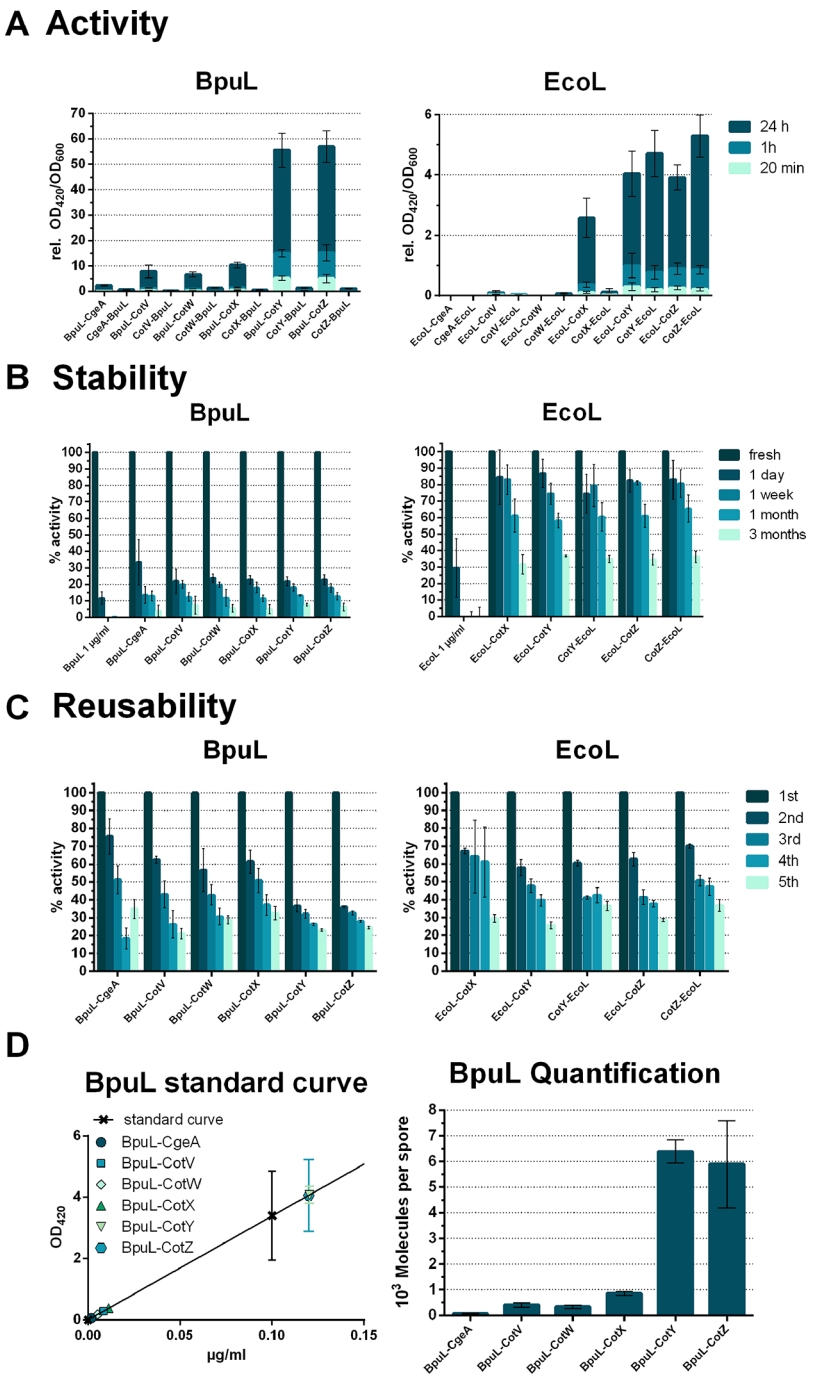

Figure 5. Evaluation and quantification of the six crust anchors utilizing the laccases from *E. coli* and *B. pumilis*. Activity of the two different laccases from *E. coli* (EcoL) and *B. pumilis* (BpuL) fused to the six different crust anchors CgeA, CotV, CotW, CotX, CotY, and CotZ (and thereby displayed on the spore surface) was determined by ABTS activity assays. For panels A–C, BpuL is shown on the left and EcoL is on the right. (A) Laccase activity from the reaction measured after 20 min, 1 h, and 24 h is expressed in the unit OD_{420}/OD_{600} as an indirect measure for the activity per spore. (B–D) Only those variants with notable activity are depicted. (B) Stability of the spores in comparison to the soluble purified enzyme: the activity (assayed after 1 h of reaction time) of the spore-displayed enzyme in comparison to the soluble purified enzyme (relative activity in %) from freshly prepared spores or enzyme and after 1 day, 1 week, 1 month, and 3 months of storage at room temperature in the appropriate buffer. (C) Remaining activity after reusing the same spores for multiple reactions, by recycling and washing these after each reaction. The relative activity (in %) after 1 h is depicted after each successive reaction over four recycling steps. (D) Quantification of active enzymes displayed on the spore with all six crust anchors. The quantification was only performed for BpuL, since EcoL could not be enriched to sufficient purity. On the left, a zoom-in of the standard curve is depicted (in black), including only two (0 and 0.1 μg/mL) of the four points utilized for the standard curve. The latter relates the activity of purified BpuL to the respective concentration of the enzyme (0, 0.1, 0.5, and 1 μg/mL). The activity of the BpuL-displaying spores (different shades of blue and symbols, refer to the legend) is shown with the corresponding calculated concentration of the enzyme with help of the standard curve. On the right, the results of the subsequent quantification are shown in active laccase molecules per spore for the respective anchor proteins. For this, the calculated enzyme concentration was normalized by the number of spores in each reaction. All error bars represent the standard deviation calculated from biological triplicates. For further details on the measurement refer to the Methods section.

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activity after immobilization.[8] We therefore decided to evaluate these parameters using laccases as model enzymes.

The anchor proteins were evaluated with two different laccases: a laccase from the spore coat of *B. pumilus* (BpuL) and a periplasmic laccase from *E. coli* (EcoL). Spores displaying the crust protein fusions were prepared to study the (re)usability and stability of the enzymatic activity on the resulting laccase-displaying Sporobeads. For these experiments, the native laccase gene of *B. subtilis*, *cotA*, was exchanged with a resistance marker to avoid background laccase activity.

A clear enzymatic activity could be demonstrated for about half of all strains in the course of 24 h, but the product yield varied significantly between the different constructs (Figure 5A). For BpuL, all versions were active, although the N-terminal variants were considerably stronger. CotY and CotZ were the best anchors (~55 au), followed by CotV, CotW, and CotX, which had approximately the same activity (~8−10 au). The lowest activity was exhibited by the CgeA-anchored fusion. For EcoL, the overall activity was lower, reaching a maximum of 5 au after 24 h (CotZ-EcoL). Here again, CotZ and CotY worked best, with both N- and C-terminal fusion constructs reaching the highest activity (4−5 au), followed by EcoL-CotX (2.5 au). The activity of the remaining variants was negligible. The enzymatic activities obtained for the fusion protein variants of both laccases nicely reflect the results obtained with the GFP fusion proteins, which also indicated that CotY and CotZ are the best-suited anchor proteins, particularly when using N-terminal fusions (Figure 4). These differences in activity between the two laccases depending on the different anchors may reflect the different properties of the two fusion proteins: BpuL is natively active in the hydrophobic context of the highly structured spore coat of *B. pumilus*, while EcoL is a soluble periplasmic enzyme and therefore rather hydrophilic.

Some of the anchors analyzed above have already been utilized in previous studies for displaying antigens or enzymes. CotX, CotY, and CotZ were utilized for displaying an L-arabinose isomerase (only CotX)[11] and a β-Galactosidase.[13,14] CotZ was additionally utilized for different antigens: FliD from *Clostridium difficile*[15] and UreA from *Helicobacter acinonychis*,[15] which was also displayed with CgeA.[12] All studies used C-terminal fusions, which turned out to be less favorable in our study. Even though CotX and CgeA in the C-terminal version showed very little activity with the two laccases, these studies showed the feasibility of these variants. While the activity of a desired fusion protein displayed on the spore crust will always depend on the individual combination of proteins used, our data strongly suggest that N-terminal fusions to CotY and CotZ seem to be the most promising constructs.

Spore Display Increases the Stability and Allows Reuse of Laccases. One of the major benefits of immobilizing enzymes is their increased stability and potential recycling for future use.[8] We therefore analyzed the stability and reusability of the laccase-displaying spores (Figure 5B/C). The purified soluble laccase BpuL lost its activity quite rapidly, with only 10% and 1% of the initial activity remaining after 24 h and 1 week, respectively. The stability of spore-displayed BpuL is significantly increased: the activity drops to approximately 20−25% in the first 24 h, and then stabilizes at about 10% in three months' period tested. Immobilization of EcoL on the *B. subtilis* spore led to an even more pronounced increase in stability: while the purified enzyme lost around 70% of its activity in 24 h, the immobilized variants displayed activity around 80% up until 1 week after preparation, 60% after one month, and 40% after 3 months. This translates to a half-life just short of three months, compared to less than 24 h for the purified soluble enzyme. Overall, the immobilization of laccases on the spore surface dramatically stabilizes the enzyme during storage at room temperature thereby demonstrating a major advantage of spore display.

Another reason to immobilize enzymes is the possibility to recover expensive proteins and reuse them for further reactions. This possibility was analyzed by recovering the laccase-displaying spores for five subsequent reactions and determining the retained activity: for both laccases, the remaining enzymatic activity was determined to be 30−70% for the first recycling step and still 20−30% after the fourth recycling step (see Figure 5C). These results demonstrate a second advantage of our spore display, since our Sporobeads can easily be recovered from reactions by simple centrifugation and reused multiple times.

Finally, we estimated how much of the anchors' capacity was linked to an active enzyme, by quantifying the N-terminal fusions of BpuL to crust proteins. The number of active molecules per spore was calculated by comparing the activity of the purified enzyme to the spores with a standard curve (see Figure 5D, left side), normalized by the number of spores in the reaction (see Figure5D, right side). Both BpuL-CotY and BpuL-CotZ spores carry the equivalent of ~6000 active laccase proteins. CotX is the third-best anchor (858), followed by CotW, CotV, and CgeA (335, 410, and 67, respectively). In comparison, GFP-quantification revealed 135 (GFP-CgeA) to 20276 (GFP-CotY) GFP-molecules per spore. Assuming that the capacity of displayed laccases is comparable to that determined with GFP, the subset of active enzyme immobilized on the spore crust can therefore be estimated. For CgeA it is approximately 50%, for CotY and CotZ it is approximately 30%, for CotW it is approximately 11% and for CotX 5%. Even though the percentage is highest for CgeA, this anchor is still the least favorable one due to its overall low amount on the spore crust. For the best anchor proteins, CotZ and CotY, a fraction of 30% active enzyme is comparable to other immobilization techniques.[8]

CONCLUSION

We have demonstrated that the *B. subtilis* endospore crust is well suited for immobilizing proteins and enzymes. The benefits of our spore display are cost and labor efficient self-immobilization, high enzyme stability, and the possibility of easily recovering the immobilized enzymes for multiple rounds of use. Immobilization also improves protein handling and simplifies the fast separation from the product after the reaction. Currently, the anchors' capacity is not fully exhausted when compared to GFP, but with around 30% activity retained, Sporobeads can compete with other immobilization techniques, while having clear economic benefits, a higher shelf life and integrity against solvents. N-terminal fusions to CotY and CotZ seem to be the most promising candidates for achieving the best anchoring capacities on the spore crust. Nevertheless, the performance of the anchors will most likely vary significantly between different target proteins, based on their individual properties. We therefore provide a collection of 12 Sporovectors to enable constructing both N- and C-terminal fusions to all six anchors sites in the spore crust. Our toolbox provides a comprehensive and easy-to-apply system for convenient protein surface display on the outermost endospore layer. As shown, it offers major advantages for protein

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immobilization and (re)usability and allows a fast creation and testing of an array of protein-displaying spores in a standardized manner.

METHODS

Bacterial Growth Conditions. *B. subtilis* and *E. coli* were routinely grown in lysogeny broth (LB medium) (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) at 37 °C with agitation (220 rpm). All strains used in this study are listed in the Supporting Information, Table S2. Selective media for *B. subtilis* contained chloramphenicol (5 μg mL^{-1}), or erythromycin in combination with lincomycin (1 μg mL^{-1}; 25 μg mL^{-1} for mls^{r}). Selective media for *E. coli* contained ampicillin (100 μg mL^{-1}), kanamycin (50 μg mL^{-1}), or chloramphenicol (35 μg mL^{-1}). Solid media additionally contained 1.5% (w/v) agar.

Transformation. *E. coli* (XL1 blue, Stratagene, DH10B or DH5α, Thermo Fisher Scientific) competent cells were prepared and transformed according to the protocol provided on OpenWetWare.[21] Transformations of *B. subtilis* were carried out as described previously.[22] The integration of plasmids into the *B. subtilis* genome was verified on starch plates (*amyE*).

DNA Manipulation. General cloning procedures, such as endonuclease restriction digest, ligation, and PCR, were performed with enzymes and buffers from New England Biolabs (NEB; Ipswich, MA, USA) according to the respective protocols. For cloning purposes, Phusion polymerase was used for PCR amplifications, otherwise, OneTaq was the polymerase of choice. PCR-purification was performed with the HiYield PCR Gel Extraction/PCR Clean-up Kit (Süd-Laborbedarf GmbH (SLG), Gauting, Germany). Plasmid preparation was performed with alkaline lysis plasmid preparation. Allelic replacement mutagenesis of the *cotA* gene using long flanking homology (LFH)-PCR was performed as described previously.[23] All plasmids generated during this study are listed in Table S1, all primer sequences are given in Table S3.

Cloning of the Sporovectors. Table 1 gives an overview of all the Sporovectors generated and evaluated during this study. They are all available from the BGSC.[24]

General Cloning. To adhere to the RFC25 standard,[20] certain forbidden restriction sites had to be removed from the vectors. The gene *cotV* contained the forbidden restriction site *Eco*RI, the gene *cgeA* the forbidden restriction site *Age*I and the *rfp*-cassette (from pSB1C3) contained two forbidden *Age*I sites, which had to be removed utilizing the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The gene *cotY* contained a forbidden *Ngo*MIV restriction site at the 5′-end of the gene, which was removed by including the mutated base pair in the forward primer. For the Sporovectors, the required genes (*cgeA*, *cotV*, *cotW*, *cotX*, *cotY*, and *cotZ*) were amplified with primers fitting the RFC25 standard (refer to the primer list in Table S3), and subcloned into the pSB1C3[25] using the restriction sites *Eco*RI and *Pst*I.

N-Terminal Sporovectors. For these Sporovector versions, the respective crust gene and the double terminator BBa_B0014 (Registry of Standard Biological Parts[26]) were cloned together, by digesting the respective crust gene with *Eco*RI and *Spe*I and the double terminator with *Xba*I and *Pst*I and ligating them into pSB1K3,[25] cut with *Eco*RI and *Pst*I. The promoter P_{cotYZ} was amplified with primers containing the restriction sites *Eco*RI at the 5′-end and *Xba*I at the 3′-end. The *rfp*-cassette was amplified with primers introducing the RFC10[27] region containing the *Xba*I site at the 5′-end and containing the *Ngo*MIV site at the 3′-end. These parts were cloned together in a one pot reaction to generate the Sporovector by cutting the promoter P_{cotYZ} with *Eco*RI and *Xba*I, the *rfp*-cassette with *Xba*I and *Ngo*MIV, the crust gene/terminator fragment with *Ngo*MIV and *Pst*I, and ligating these fragments with the backbone pBS1C cut with *Eco*RI and *Pst*I.

C-Terminal Sporovectors. For these Sporovector versions, the promoter P_{cotYZ} was subcloned in RFC10 standard into pSB1C3. The respective crust gene and the promoter P_{cotYZ} were cloned together by digesting the promoter with *Eco*RI and *Spe*I and the respective crust gene with *Xba*I and *Pst*I and ligating them into the pSB1K3, cut with *Eco*RI and *Pst*I. The double terminator was amplified from the respective BioBrick plasmid (BBa_B0014) with the restriction sites *Spe*I at the 5′-end and *Pst*I at the 3′-end. The *rfp*-cassette was amplified from pBS1C with the restriction sites *Age*I at the 5′-end and *Spe*I at the 3′-end. These parts were cloned together in a one pot reaction to harbor the Sporovector by cutting the respective promoter/crust gene fragment with *Eco*RI and *Age*I, the *rfp*-cassette with *Age*I and *Spe*I, the double terminator with *Spe*I and *Pst*I, and ligating these fragments with the backbone pBS1C cut with *Eco*RI and *Pst*I.

Cloning of the Gene of Interest. To clone the genes of interest (encoding GFP, or the two laccases, BpuL and EcoL), they need to adhere to the RFC25 standard, including an RBS for N-terminal fusions. The *gfp* gene was already available in this standard from the parts registry.[25] The genes for the two laccases BpuL and EcoL (parts registry) were available but not yet adjusted to RFC25. The gene encoding BpuL contained too many forbidden restriction sites so that the RFC25 compatible restriction sites *Ava*I instead of *Ngo*MIV and *Bsp*EI instead of *Age*I were chosen and added by PCR. For the gene encoding EcoL, the *Bacillus* RFC25 standard was added by PCR. These PCR products were subcloned into pSB1C3 using the restriction sites *Eco*RI and *Pst*I. For N-terminal variants, the gene of interest was cloned by digesting with *Xba*I and *Age*I (or *Bsp*EI in case of BpuL) and cloned into the respective Sporovector cut with *Xba*I and *Ngo*MIV. For the C-terminal variants the gene of interest was cloned by digesting with *Ngo*MIV (or *Ava*I in the case of BpuL) and *Spe*I and cloned into the respective Sporovector cut with *Age*I and *Spe*I. For a detailed explanation on how to clone and use the Sporovectors readers are referred to in the Supporting Information.

Bioluminescence Assays for Determing Promoter Strengths. Luciferase activities of the strains harboring pBS3C*lux*-derivates[19] with the respective promoters P_{cotVWX}, P_{cotX}, P_{cotYZ}, and P_{cgeA} were assayed using a Synergy2 multimode microplate reader from BioTek (Winooski, VT, USA). The reader was controlled using the software Gen5.

Cultures were inoculated as triplicates 1:100 from an overnight culture grown in LB to 100 μL of Difco Sporulation Medium (DSM, 0.8% w/v Tryptone, 0.1% w/v KCl, 1 mM $MgSO_4$, 10 μM $MnCl_2$, 1 μM $FeSO_4$, 0.5 mM $CaCl_2$) per well in 96-well plates (black walls, clear bottom; Greiner Bio-One, Frickenhausen, Germany), and incubation occurred at 37 °C with agitation (medium intensity).

Cell growth was monitored by optical density at a wavelength of 600 nm (OD_{600}). Raw luminescence output (relative luminescence units, RLU) was first corrected by the RLU of a strain harboring an empty pBS3C-*lux* and then normalized to cell density by dividing each data point by its corresponding corrected OD_{600} value. The maximum of the resulting graph (Figure S1), ignoring the initial fluctuation due

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to the low OD_{600}, was utilized as a measure of promoter strength.

Spore Preparation. The *B. subtilis* strains were inoculated in 50 mL of Difco Sporulation Medium (DSM, 0.8% w/v Tryptone, 0.1% w/v KCl, 1 mM $MgSO_4$, 10 μM $MnCl_2$, 1 μM $FeSO_4$, 0.5 mM $CaCl_2$) and grown at 37 °C with agitation (220 rpm) for 48 h to ensure sporulation. These spore cultures were harvested by centrifugation at 10 000*g* for 8 min, resuspended in 10 mL of dH_2O and treated for 1 h at 37 °C with 75 μg/mL lysozyme to lyse remaining cells. Afterward, the prepared spores were washed with 10 mL of dH_2O, 10 mL of 0.05% SDS, and then three times with 10 mL of dH_2O. The spores were then resuspended in either 2 mL of dH_2O or the appropriate buffer (adapted from ref 22).

Fluorescence Microscopy and GFP Quantification. The GFP-displaying spores were viewed on an agarose pad (1% Agarose in dH_2O, cast in press-to-seal silicone isolators with 20 mm diameter and 1 mm depth from life technologies) under the Olympus AX70 microscope utilizing the 100× objective (UPlanFl 100*x*/1,30 Oil). The spores were documented with phase contrast and with the GFP fluorescence channel utilizing the filter cube U-MNIB (IF blue long pass; Ex., 470−490 nm; Em., 515−∞). The pictures were taken with a XC10 color camera from Olympus with 500 and 1000 ms exposure times and the same microscope settings, and saved as a tagged image file (tif).

For Image analysis, the java based program Fiji (Fiji is just ImageJ) was used. The setting for opening the image was changed from automatic to 16-bit (0−65535). The image type was then converted to RGB, to enable color thresholding.

For the quantification, AcGFP Flow Cytometer Calibration Beads (Takara Clontech) were utilized. The same procedure as above was followed. The mean pixel intensity above background was measured for each of the six GFP-beads individually. This allowed separating the six different intensity peaks with the diameter of the circle fitting the size of the GFP-beads. Moreover, this procedure also provided a scale for the area of the beads in pixel. The mean pixel intensity was sorted by the six intensity peaks and a standard curve was created by plotting them against the molecular equivalence of solvent fluorophore (MESF) divided by pixel size of the GFP-beads as a measure of fluorophore density (MESF/Pixel).

The spores were measured by utilizing a color threshold with the color space HSB (Color Threshold setting). First, the threshold for brightness was adjusted until all spores were marked (approximately 4−6 to 255, pass). The number of pixels obtained during this measurement was used for calculating the number of all spores by dividing this by the pixel number of one spore. Then the threshold for hue was adjusted to 64−255, pass. This excludes the autofluorescence of the spores as seen with the wildtype (W168). The intensity above the background was measured, also measuring the pixel number. The pixel number obtained from this measurement was used for calculating the number of spores with GFP signals, by dividing this by the pixel number of one spore. Four fluorescence pictures per sample were analyzed (>100 spores). The mean pixel intensity was calculated by adding all mean pixel intensities multiplied by the number of spores with the GFP signal and then divided by the total number of spores.

To quantify the molecules of GFP per spore, the MESF/Pixel was calculated by dividing the mean pixel intensity by the slope of the standard curve obtained with the GFP-beads. Then the MESF, which is equivalent to the number of GFP molecules, was calculated by multiplying the MESF/Pixel by the size of one spore in the pixel to get the number of GFP per spore.

Protein Purification of the Laccases. The plasmids containing the genes for the *B. pumilis* laccase (BpuL) and the gene for the *E. coli* laccase (EcoL) driven by the T7-promoter and containing a C-terminal 6xhis-tag were transformed into KRX and BL21 protein expression strains (Promega), respectively. Protein expression was induced utilizing 500 mL of self-induction media ZYM-5052[28] in chicane flasks, in which the glucose was substituted with 2 g/L rhamnose for the KRX strain. The cells were harvested by centrifugation (10 000*g*, 8 min), frozen at −80 °C, and then resuspended in 20 mL of loading buffer (20 mM KP-Buffer, pH = 7.5, 500 mM NaCl, 5 mM imidazole, pH = 8.0, 0.1 mM PMSF, 10% glycerol, 5 mM DTT, 5 mM β-mercaptoethanol) on ice. The cells were disrupted utilizing a french press (Thermo IEC French Press, Thermo Fisher Scientific, 20K Pressure cell with 1100 PSI). Then DNase was added to degrade the genomic DNA, and the mixture was incubated for 10 min on ice. The sample was filtered (Filtropur S 0.45 μm, Sarstedt) and then loaded onto a 1 mL Ni-NTA-Agarose column (Ni-NTA Agarose and Polypropylene column (1 mL), Qiagen), which had been equilibrated by washing it with 5 column volumes (CV) dH_2O followed by 5 CV loading buffer. The loaded column was then washed with 10 CV loading buffer and 10 CV washing buffer (same as loading buffer, but with 50 mM imidazole). Elution was performed with each 2 mL of elution buffers with increasing amounts of imidazole (same as loading buffer, but with 100, 200, and 500 mM imidazole) and collected in 500 μL fractions on ice.

The appropriate fractions were determined utilizing SDS-PAGE. These fractions were desalted utilizing a desalting column (PD-10 desalting column, GE Healthcare life science) following the respective protocol and exchanging the buffer to the appropriate buffer for the ABTS-laccase activity assay (100 mM Sodium-acetate buffer pH = 5.0, 0.4 mM $CuCl_2$). The protein concentration of the sample was determined with a 1:10 dilution in technical triplicates utilizing the Bradford reagent (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad) and a standard curve determined with BSA (Bovine Serum Albumin, albumin fraction V, Sigma-Aldrich) of 1, 2.5, 5, 10, 25, 50, 75, and 100 μg/mL in the Synergy2 multimode microplate reader from BioTek (Winooski, VT, USA). The OD_{590}/OD_{450} ratio was utilized to determine the standard curve and the concentration of the protein sample was calculated.

Laccase Activity Assay with ABTS and Subsequent Quantification. The assay measures the activity of laccases by their ability to oxidize the chromogenic substrate ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) (green) to $ABTS^+$ (blue), which leads to a shift of peak absorbance of light from the wavelength of 405 to 420 nm. The reaction is set up in the appropriate buffer (100 mM sodium acetate buffer pH = 5.0, 0.4 mM $CuCl_2$, the latter ensuring the integrity of the copper-reactive center), with the final concentration of 5 mM of ABTS (ABTS BioChemica, Applichem).

For the spores displaying the laccases, a host strain lacking the laccase CotA (TMB2131) was utilized to avoid background activity. This strain was also used as a reference for the laccase background activity. For the reaction, the optical density of the spore suspensions is adjusted to an OD_{600} of approximately 0.5 to ensure similar amounts of spores. For the purified enzyme,

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the reaction is set up with the concentrations of 0, 0.1, 0.5 1, 2, 5, and 10 μg/mL in the Synergy2 multimode microplate reader from BioTek (Winooski, VT, USA). The reaction with BpuL was incubated at room temperature, the reaction with EcoL at 50 °C. For determining the enzymatic activities on the spores, the OD_{420} was measured after 20 min, 1 h, and 24 h, discontinuously by centrifugation of the reaction, to measure the supernatant. The OD_{420} of the reference strain was subtracted. For the purified enzymes, the same data points were measured, but continuously. For determining the stability of the enzymes, reactions were set up with 1 day old, 1 week old, 1 month old, and 3 months old spores and purified enzyme, performing the same measurement as above.

To measure the reusability of the spores displaying the laccase, the reaction was set up with one-day old spores (to ensure no significant loss of activity) and measured after 1 h for five subsequent times with the same spores, with washing in-between the reactions.

The quantification was only performed with BpuL, for which a purity of >95% was achieved for the overexpressed enzyme. In contrast, the purification of the laccase EcoL was less successful and therefore not considered further. With the data from the ABTS assay, the standard curve was obtained by relating the activity of purified BpuL in the unit OD_{420} with the respective concentration of the enzyme (0, 0.1, 0.5, and 1 μg/mL). Higher concentrations were excluded, as only the linear part of the curve was utilized. The activity of the respective BpuL-displaying spores was used to calculate the concentration of enzyme with the help of the standard curve. The molecules per spore of the respective anchors were calculated by normalizing the concentration by the number of spores in each reaction, as counted with a Thoma chamber (depth 0.01 mm, 0.0025 mm^2, Poly-Optik GmbH).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00285.

Plasmid, strain and primer lists; promoter strength measurement; how-to-use guide to the Sporovectors (PDF)

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Author Contributions

J.B., J.R., and S.L. constructed the vectors. S.L. additionally evaluated the crust promoter strength. All other experiments were performed by J.B. J.B. and T.M. designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), substrate of laccases; BpuL, *Bacillus pumilus* laccase (*cotA*); EcoL, *Escherichia coli* laccase (*cueO*); MESF, molecular equivalent of solvent fluorophore, number of GFP molecules per GFP-bead; RFC10, request for comments 10, BioBrick cloning standard; RFC25, request for comments 25, BioBrick Freiburg standard for translational fusion

■ REFERENCES

(1) Higgins, D., and Dworkin, J. (2012) Recent Progress in *Bacillus subtilis* Sporulation. *FEMS Microbiol. Rev. 36*, 131−148.

(2) McKenney, P. T., and Eichenberger, P. (2012) Dynamics of Spore Coat Morphogenesis in *Bacillus subtilis*. *Mol. Microbiol. 83*, 245−260.

(3) Jiang, S., Wan, Q., Krajcikova, D., Tang, J., Tzokov, S. B., Barak, I., and Bullough, P. A. (2015) Diverse Supramolecular Structures Formed by Self-assembling Proteins of the *Bacillus subtilis* Spore Coat. *Mol. Microbiol. 97*, 347−359.

(4) McKenney, P. T., Driks, A., and Eichenberger, P. (2013) The *Bacillus subtilis* Endospore: Assembly and Functions of the Multilayered Coat. *Nat. Rev. Microbiol. 11*, 33−44.

(5) Driks, A., and Eichenberger, P. (2016) The Spore Coat. *Microbiol. Spectrum 4*, na.

(6) Imamura, D., Kuwana, R., Takamatsu, H., and Watabe, K. (2011) Proteins Involved in Formation of the Outermost Layer of *Bacillus subtilis* Spores. *J. Bacteriol. 193*, 4075−4080.

(7) McKenney, P. T., Driks, A., Eskandarian, H. A., Grabowski, P., Guberman, J., Wang, K. H., Gitai, Z., and Eichenberger, P. (2010) A Distance-Weighted Interaction Map Reveals a Previously Uncharacterized Layer of the *Bacillus subtilis* Spore Coat. *Curr. Biol. 20*, 934−938.

(8) Sheldon, R. A. (2007) Enzyme Immobilization: The Quest for Optimum Performance. *Adv. Synth. Catal. 349*, 1289−1307.

(9) Isticato, R., and Ricca, E. (2014) Spore Surface Display. *Microbiol. Spectrum 2*, na.

(10) Wang, H., Wang, Y., and Yang, R. (2017) Recent Progress in *Bacillus subtilis* Spore-surface Display: Concept, Progress, and Future. *Appl. Microbiol. Biotechnol. 101*, 933−949.

(11) Liu, Y., Li, S., Xu, H., Wu, L., Xu, Z., Liu, J., and Feng, X. (2014) Efficient Production of D-Tagatose Using a Food-Grade Surface Display System. *J. Agric. Food Chem. 62*, 6756−6762.

(12) Iwanicki, A., Piątek, I., Stasiłojć, M., Grela, A., Łęga, T., Obuchowski, M., and Hinc, K. (2014) A System of Vectors for *Bacillus subtilis* Spore Surface Display. *Microb. Cell Fact. 13*, 1−9.

(13) Wang, H., Yang, R., Hua, X., Zhang, W., and Zhao, W. (2016) An Approach for Lactulose Production Using the CotX-Mediated Spore-Displayed β-Galactosidase as a Biocatalyst. *J. Microbiol. Biotechnol. 26*, 1267−1277.

(14) Wang, H., Yang, R., Hua, X., Zhao, W., and Zhang, W. (2015) Functional Display of Active β-galactosidase on *Bacillus subtilis* Spores Using Crust Proteins as Carriers. *Food Sci. Biotechnol. 24*, 1755−1759.

(15) Negri, A., Potocki, A., Obuchowski, M., Hinc, K., and Iwanicki, A. (2013) Expression and Display of *Clostridium difficile* Protein FliD on the Surface of *Bacillus subtilis* Spores. *J. Med. Microbiol. 62*, 1379−1385.

(16) Hinc, K., Iwanicki, A., and Obuchowski, M. (2013) New Stable Anchor Protein and Peptide Linker Suitable for Successful Spore Surface Display in *B. subtilis*. *Microb. Cell Fact. 12*, 22.

DOI: 10.1021/acssynbio.7b00285
ACS Synth. Biol. 2018, 7, 452−461

(17) Qian, L., Ning, D., and Wu, C. (2010) Surface Display of GFP Using CotX as a Molecular Vector on *Bacillus subtilis* Spores. *Shengwu Gongcheng Xuebao 26*, 264−269.
(18) Roels, S., and Losick, R. (1995) Adjacent and Divergently Oriented Operons under the Control of the Sporulation Regulatory Protein GerE in *Bacillus subtilis*. *J. Bacteriol. 177*, 6263−6275.
(19) Radeck, J., Kraft, K., Bartels, J., Cikovic, T., Dürr, F., Emenegger, J., Kelterborn, S., Sauer, C., Fritz, G., Gebhard, S., and Mascher, T. (2013) The *Bacillus* BioBrick Box: Generation and Evaluation of Essential Genetic Building Blocks for Standardized Work with *Bacillus subtilis*. *J. Biol. Eng. 7*, 1−17.
(20) Grünberg, R., Arndt, K., and Müller, K. (2009) Fusion Protein (Freiburg) BioBrick assembly standard, http://hdl.handle.net/1721.1/45140.
(21) Competent E. coli cells.. http://openwetware.org/wiki/TOP10_chemically_competent_cells (accessed 2017).
(22) Harwood, C., and Cutting, S. M. (1990) *Molecular Biological Methods for Bacillus*, John Wiley & Sons Ltd., Chichester, England.
(23) Mascher, T., Margulis, N. G., Wang, T., Ye, R. W., and Helmann, J. D. (2003) Cell Wall Stress Responses in *Bacillus subtilis*: the Regulatory Network of the Bacitracin Stimulon. *Mol. Microbiol. 50*, 1591−1604.
(24) Bacillus Genetic Stock Center. http://www.bgsc.org/ (accessed 2017).
(25) Vilanova, C., and Porcar, M. (2014) iGEM 2.0 - Refoundations for Engineering Biology. *Nat. Biotechnol. 32*, 420−424.
(26) Registry of Standard Biological Parts. http://parts.igem.org/Main_Page (accessed 2017).
(27) Knight, T. (2007) Draft Standard for Biobrick Biological Parts, http://hdl.handle.net/1721.1/45138.
(28) Studier, F. W. (2014) Stable Expression Clones and Auto-induction for Protein Production in *E. coli*. *Methods Mol. Biol. (N. Y., NY, U. S.) 1091*, 17−32.
(29) JBEI ICE Registry, https://public-registry.jbei.org/folders/330 (accessed 2017).
(30) Zhang, J., Ichikawa, H., Halberg, R., Kroos, L., and Aronson, A. I. (1994) Regulation of the Transcription of a Cluster of *Bacillus subtilis* Spore Coat Genes. *J. Mol. Biol. 240*, 405−415.

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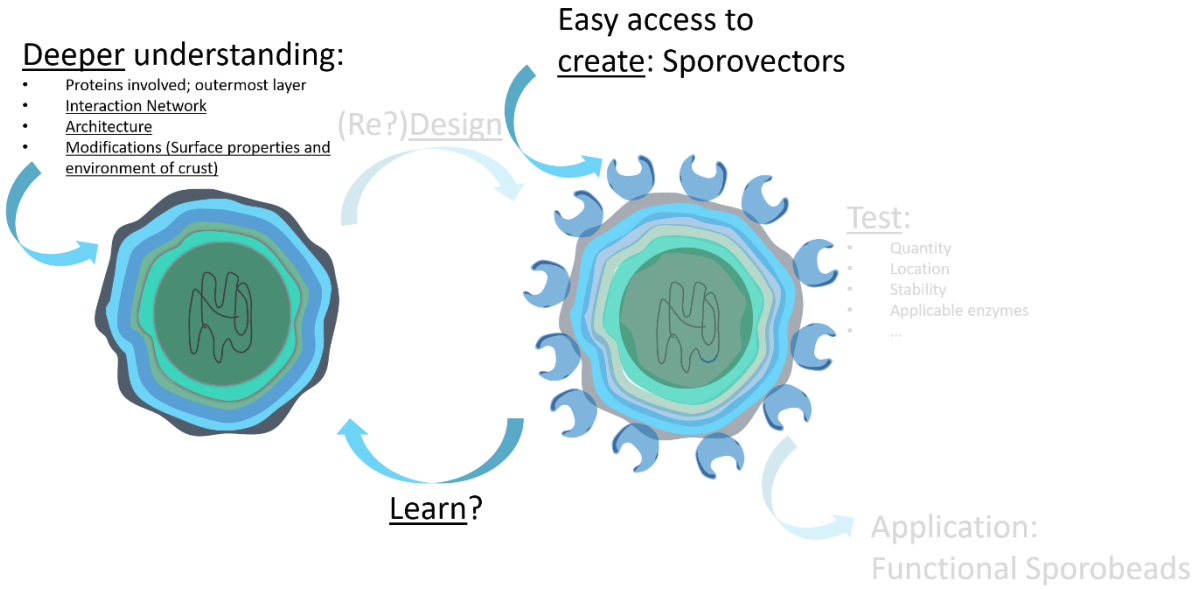
Chapter 3: The *Bacillus subtilis* endospore crust: protein interaction network, architecture and glycosylation state of a potential glycoprotein layer

This chapter presents the published work (Publication II):

The *Bacillus subtilis* endospore crust: protein interaction network, architecture and glycosylation state of a potential glycoprotein layer. **Bartels J**, Blüher A, López Castellanos S, Günther M, Richter M, Mascher T, Mol Microbiol. doi:10.1111/mmi.14381

Background:



This study aims to understand the nature of the crust in a deeper and conclusive manner. To achieve this objective, the Sporovector system is utilized to create a vast number of GFP fusions in a complete set of crust gene mutants as well as in glycosyltransferase mutants. Additional methods including electron microscopy and HPLC with these mutants give further insights on the nature of the crust. This leads to a deeper understanding of the interaction network, the architecture as well as the modifications of the crust. This can, in turn, be of use to redesign and further improve the application of the Sporobeads.

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The *Bacillus subtilis* endospore crust: protein interaction network, architecture and glycosylation state of a potential glycoprotein layer

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Summary

The endospore of *Bacillus subtilis* is formed intracellularly upon nutrient starvation and is encased by proteinaceous shells. The outermost layer, the crust, is a postulated glycoprotein layer that is composed of six proteins: CotV, W, X, Y, Z and CgeA. Despite some insight into protein interactions and the identification of players in glycosylation, a clear picture of its architecture is still missing. Here, we report a comprehensive mutational analysis that confirms CotZ as the anchor of the crust, while the crust structure is provided by CotV, CotX and CotY. CotY seems to be the major structural component, while CotV and CotX are polar and co-depend on each other and partially on CotW. CotW is independent of other crust proteins, instead depending on outer coat proteins, indicating a role at the interface of crust and coat. CgeA is co-expressed with putative glycosyltransferases (CgeB and CgeD) and implicated in crust glycosylation. In accordance, we provide evidence that CgeB, CgeCDE, SpsA-L, SpsM and SpsNOPQR (formerly YfnHGFED) contribute to the glycosylation state of the spore. The crust polysaccharide layer consists of functionally linked rhamnose- and galactose-related variants and could contain rare sugars. It may therefore protect the crust against biological degradation and scavenging.

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Introduction

The soil bacterium *Bacillus subtilis* can form endospores upon nutrient starvation; a dormant cell capable of enduring harsh conditions for long periods of time, while still being able to respond to nutrient upshifts to initiate spore germination and resuming a vegetative life (Higgins and Dworkin, 2012). The endospore is produced inside the mother cell after an asymmetric cell division and the consecutive engulfment of the forespore compartment by the mother cell. The core, containing the dehydrated DNA, is surrounded by two membranes, which are protected by a spore-specific peptidoglycan layer termed cortex, and then encased in four concentric layers of proteins: the basement layer, the inner coat, the outer coat and the crust. These layers are produced by the mother cell and added to the forespore (mostly from the poles inward). This process occurs in a self-organizing fashion and is coordinated by morphogenetic coordination proteins: SpoIVA for the basement layer, SafA for the inner coat, CotE for the outer coat and CotXYZ for the crust have been suggested for this role (McKenney *et al.*, 2010; 2013; Imamura *et al.*, 2011; McKenney and Eichenberger, 2012; Plomp *et al.*, 2014). After or during this process, many of the proteins are cross-linked (Abhyankar *et al.*, 2015) to produce a mature spore capable of enduring harsh conditions such as heat, dryness, pressure (Nicholson *et al.*, 2000) and biological scavenging (Klobutcher *et al.*, 2006; Laaberki and Dworkin, 2008) to a certain degree. For most of the layers, the protein interaction network as well as the structure are known, at least to some extent (McKenney and Eichenberger, 2012; McKenney *et al.*, 2013; Plomp *et al.*, 2014). For the outermost layer, the crust, this knowledge is still incomplete.

Protein interaction network and structure of the crust

The crust contains at least six different proteins: CotVWXYZ, which were long known as part of the insoluble fraction of the spore coat (Zhang *et al.*, 1993), and CgeA (McKenney *et al.*, 2010; Imamura *et al.*, 2011). Most of the data on the protein interaction network are

based on atomic force microscopy (AFM) or two-hybrid assays. Genetic interaction studies based on GFP fusions propose CotXYZ (McKenney *et al.*, 2010; Shuster *et al.*, 2019a) or CotZ (Imamura *et al.*, 2011) as key morphogenetic proteins of the crust on which all others depend. Furthermore, Imamura *et al.* showed that CotY and CotZ co-depend, that CotV and CgeA depend on CotW, while CotV additionally depends on CotX (Imamura *et al.*, 2011). Shuster et al. 2019 additionally showed, that localization of CotY depends on CotW to some extent, CotY and CotX codepend, CotX is stabilized by CotW and that the localization of CgeA depends on CotX and CotY. In contrast to Imamura et al. 2010 they showed, that CgeA maintenance rather than localization depended on CotW and that CotZ does not depend on CotY (Shuster *et al.*, 2019a). The interaction between CotY and CotZ was confirmed by AFM experi-ments, while the interactions between CotY and CotZ as well as between CotV and CotW were demonstrated by two-hybrid assays (Krajčíková *et al.*, 2009; 2017; Liu *et al.*, 2015). The link between the crust and the outer coat seems to depend on the interaction of CotX, CotY and CotZ with CotE (as well as many other coat proteins) as demonstrated by two-hydrid assays and AFM (Liu *et al.*, 2016a; 2016b; Krajčíková *et al.*, 2017).

According to AFM data, the crust is proposed to be an amorphous layer (Plomp *et al.*, 2014) that can only be visualized by transmission electron microscopy (TEM) when stained with ruthenium red (Waller *et al.*, 2004). This assumption of the crust being an amorphous layer seems to contradict the highly structured hexagonal sheets (CotY) and fibres (CotV and CotW) that are pro-duced when overexpressed in *E. coli* (Jiang *et al.*, 2015). The structure produced by the cysteine-rich protein CotY (and presumably its paralogue CotZ) in these sheets (Jiang *et al.*, 2015) resembles that of its orthologue ExsY in the exosporium of the *B. cereus* group (Terry *et al.*, 2017), which forms intracellular disulphide bonds and contributes to the chemical resistance of these spores.

Protein modification and maturation of the crust

The need for ruthenium red staining in TEM imaging (Waller *et al.*, 2004) indicates that the crust might carry gly-cosylated moieties (Luft, 1971). Glycosylation of the crust, which is supported by additional evidence (Wunschel *et al.*, 1995; Abe *et al.*, 2014; Cangiano *et al.*, 2014; Arrieta-Ortiz *et al.*, 2015), leads to proposing that dispersal of the oth-erwise more hydrophobic spores in the aqueous phase is the main function of this outermost layer (McKenney *et al.*, 2010; Imamura *et al.*, 2011; Abe *et al.*, 2014). Glycosylation was linked to the *sps* (spore envelope polysaccharide syn-thesis) genes: the cluster *spsA-L* (Cangiano *et al.*, 2014) and the split gene *spsM* (Abe *et al.*, 2014). Mutant spores defective in these genes are more hydrophobic, clump and do not easily disperse in water. Moreover, the gene products of *ytcA* and the *yfnHGFED* cluster seem to be involved in this spore maturation process (Arrieta-Ortiz *et al.*, 2015). The spore polysaccharides seem to include rhamnose, galactose, ribose, glucose, muramic acid, GlcNAc and the rare sugar quinovose (6-desoxy-glucose), which is unique to *B. subtilis* (Wunschel *et al.*, 1995; Abe *et al.*, 2014). Initial data indicated that the gene products of *spsI-L* seem to be involved in producing rhamnose (Plata *et al.*, 2012). Beyond this, no further information exists on the proposed spore crust glycosylation.

The aim of this study

This study aims at filling the existing gaps on native protein interactions, co-dependencies and the relationship to the structure and localization of crust proteins by a comprehensive mutational study. Our results indicate that CotZ anchors the crust structure, which is provided by CotY, CotV and CotX, at the middle part of the spore. The affinity of CotY and CotX to the poles of the spore might provide the primary attachment. CotV and CotX co-depend on each other and the conjoined structure might be stabilized by CotW at the interface of the crust to the outer coat. CgeA might play a role in crust glycosylation. This study additionally aimed at identifying the players involved in the addition of crust polysaccharides. Our results indicate that CgeB, CgeCDE, SpsA-L, SpsM and SpsNOPQR (formerly YfnHGFED) contribute to the glycosylation state of the spore. The spore polysaccharide probably contains two different polysaccharide variants, one rhamnose-related and one galactose-related type. Moreover, HPLC data together with the putative protein functions suggest that it might contain the rare sugar viosamine and also a related capping sugar.

Results

Functional or structural roles of spore crust proteins

Initially, the gene sequences and operon structure (Zhang *et al.*, 1994; Roels and Losick, 1995) of the crust genes were evaluated (Fig. 1A). Remarkably, CotV and CotX as well as CotY and CotZ not only share common protein domains (CotX and CotZ superfamily respectively), but are also co-transcribed, indicating a strong functional interdependency between them. Moreover, *cotW* is transcribed with *cotV* and *cotX* (Zhang *et al.*, 1994), indicating that the corresponding proteins might also be functionally linked. In contrast, *cgeA* is located in a separate gene cluster (Roels and Losick, 1995), which might suggest a distinct physiological role of its gene product.

The crust was proposed to be a glycoprotein layer, based on its staining with the promiscuous sugar-staining

dye ruthenium red (Waller *et al.*, 2004). Therefore, the crust protein sequences were screened for bacterial N-glycosylation motifs (taken from [Kowarik *et al.*, 2006]). CotX is the only protein that possesses a stringent glycosylation motif (E/D-X-N-X-S/T, where X is not proline, see Fig. 1A, black star), which is located inside the first of the two CotX superfamily domains. A less stringent motif (N-X-S/T, see Fig. 1A, grey star) can also be found in the second CotX domain at the identical position. The crust protein CotV has a less stringent glycosylation motif at the same position of the CotX superfamily domain. This positional conservation of the glycosylation motifs between all three domains of CotX and CotV suggests that these two proteins could be glycosylation targets. CotY and CotZ also contain less stringent glycosylation motifs (N-X-S/T, see Fig. 1A, grey star) at a different position in the shared

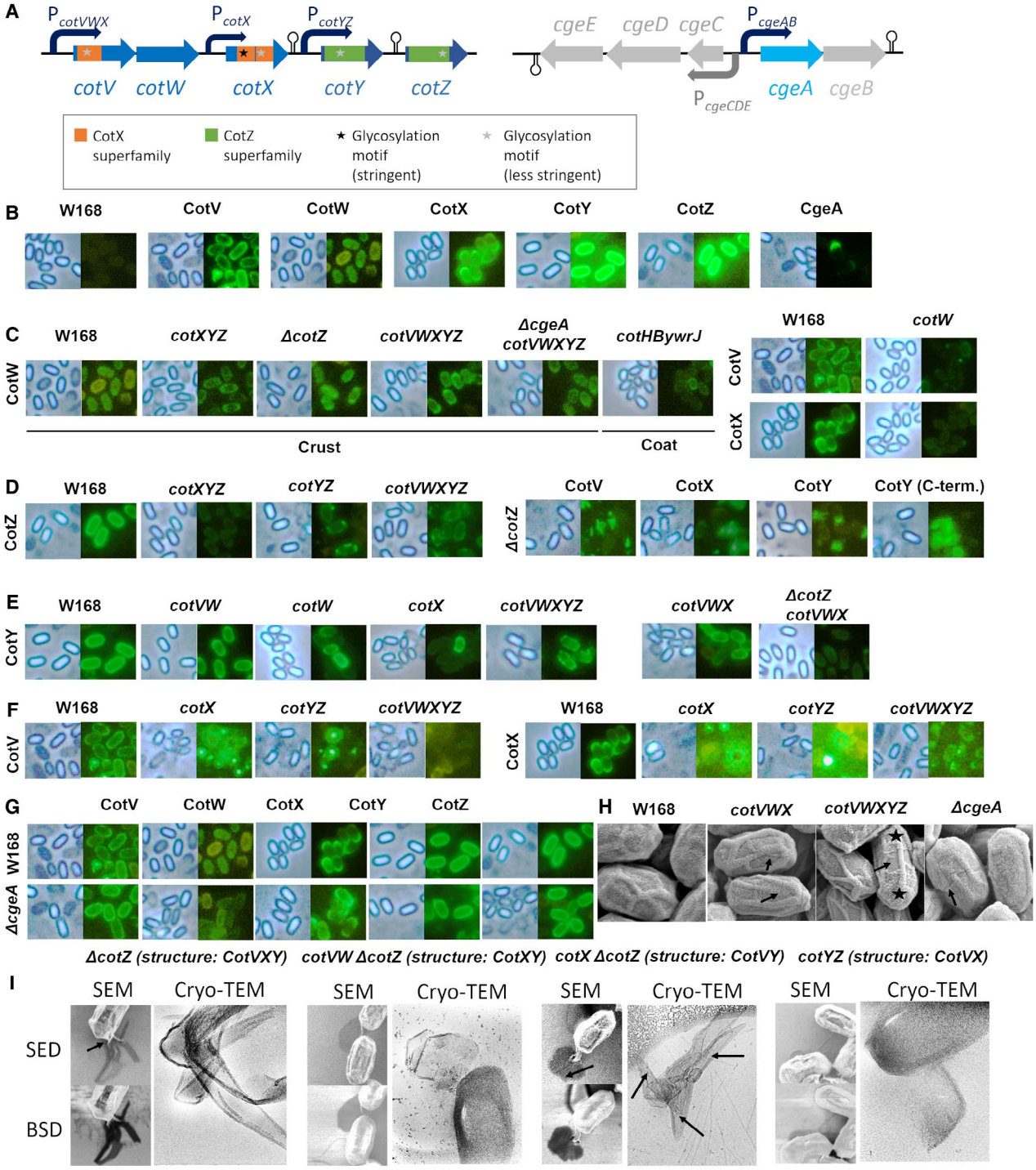

Fig. 1. Operon structure, distribution, genetic interaction and architecture of the crust proteins. The pictures are adapted for optimal visibility and do not represent the actual quantities (except for Fig. 1B), but the pictures within each subpanel were always adapted with the same parameters to ensure comparability. For a quantitative representation of the complete data set, see Fig. S1.
A. Operon structure of the crust genes (Zhang *et al.*, 1994; Roels and Losick, 1995): While *cotVWXYZ* are clustered together, *cgeA* is encoded in a different chromosomal location. CotX and CotZ superfamily domains are indicated in orange or green respectively. Glycosylation motifs are highlighted by black (stringent) and gray (less stringent) stars (motifs taken from [Kowarik *et al.*, 2006]).
B. Distribution of crust proteins on the spores of the *B. subtilis* W168. Wild type spores (weak autofluorescence) are shown as negative control. All microscopic pictures were adapted with the same parameters to ensure comparability. For this and the following panels, phase contrast (left picture) and the corresponding fluorescence channel (right picture) are shown.
C–G. Spores displaying N-terminal GFP-fusions (if not indicated otherwise) to the respective crust protein (indicated by the protein names) in different mutant backgrounds (indicated by is, Δ indicating a clean deletion). Each corresponding series of panels was adapted using the same parameters to ensure comparability, but brightness/fluorescence intensities cannot be compared between different panel series, since different settings applied to optimize presentation of each series.
H. SEM data with gold labeling, showing the wild type (W168) and isogenic mutants of *cotVWX*, *cotVWXYZ* and *cgeA*. Ridge structures and rough cap structures are indicated by black arrows and black stars respectively.
I. SEM data (without gold labeling) and cryo-TEM data from the cotZ mutant. For the complete SEM data set, see Fig. S2. The SEM detection method SED (secondary electron detection) is suitable to show surface structures, while the BSD (backscattered electron detection) shows differences in height and composition.

CotZ superfamily domain. Together, the role of this proposed glycosylation could be counteracting the hydrophobicity of the CotX superfamily domain, thereby adjusting these proteins for the hydrophilic nature of the crust, which is important to allow the dispersal of the spores in the aqueous environment (McKenney *et al.*, 2010; Imamura *et al.*, 2011; Abe *et al.*, 2014).

Next, we investigated the distribution and quantity of the spore proteins to gain further insight into the spore crust composition and architecture. The distribution of the crust proteins was evaluated with GFP-fusions. Even though the assessment of native abundance and distribution has some limitations with ectopic GFP-fusions (for a detailed discussion refer to the supplemental material), it nevertheless gives first insights into the potential roles and dependencies of the respective proteins, CotY seems to be the most abundant crust protein, closely followed by CotZ, then CotX and lastly CotV (Fig. 1B). CotY and CotZ share the same domain (CotZ superfamily) and both distribute evenly on the spore. This common location together with the high quantity suggests that these two proteins might provide the core structure of the crust. In contrast, CotX and CotV, which also share a common domain (CotX superfamily), locate preferentially to the poles of the spore. While this distribution is more pronounced for CotX, it might also indicate a functional link between the two proteins. Both polar localization and lower abundance of CotX and CotV might indicate that these proteins are probably non-essential for the crust structure (since they are absent from the middle parts of the spore), but instead rather structurally support the initiation or propagation of the crust structure from the spore poles.

In contrast, CotW is poorly and quantitively unequally distributed on the spores, particularly lower or missing on premature spores (phase gray), indicating that this protein might be added late during spore maturation. This predominantly α-helical protein lacks a recognizable functional domain. It can produce thin sheet layers at the water–air interface and can replace its ortholog in the exosporium of *Bacillus megaterium* (Manetsberger *et al.*, 2018). Taken together, this indicates that CotW could produce a supportive, thin layered structure that is incorporated at the hydrophilic crust to hydrophobic coat interface in a later stage of spore maturation.

CgeA seems to be the least abundant of all crust proteins and not detectable on all spores. In fact, the mature spores (phase white) seem to harbor little to no CgeA protein, while its abundance is slightly higher on premature spores (phase gray), pointing toward a role early in crust development. Since the CgeA signal also localizes to the spore poles, its function might be linked to CotX and CotV. As mentioned above, these two proteins are the most likely glycosylation targets of the crust. Since CgeA was implied in spore maturation and is co-transcribed or co-regulated with putative glycosyltransferases (CgeB and CgeD respectively) (Zhu and Stülke, 2018), it is attractive to suggest a role in coordinating crust glycosylation.

CotW: a linker protein at the interface of outer coat and crust

To assess the dependencies of the spore crust proteins to each other and decipher the protein–protein interaction network of the crust, GFP-crust protein fusions were visualized in a collection of crust gene mutants. The complete data set can be found in supplemental Fig. S1.

Localization of CotW is virtually independent of any other crust protein, as there were no significant differences in both quantity and distribution of GFP-CotW in any of the crust gene mutants (Fig. 1C). The mutants *cotXYZ* and *cotZ* are described or shown in the literature to misassemble crust-like structures (McKenney *et al.*, 2010; Imamura *et al.*, 2011), and therefore will be further termed as ‘crust displacement mutants’. But localization of ectopically expressed GFP-CotW was unaffected in both of these crust displacement mutants and even in *cotVWXYZ* and *cgeA-cotVWXYZ* crust mutant strains, in which the crust is completely missing.

In contrast, CotW was slightly perturbed when the outer coat proteins CotH and CotB were missing (Fig. 1C). This observation indicates that CotW might play a role at the interface between outer coat and crust. Such a linker role is further underpinned by the (partial) dependency of the crust proteins CotV and CotX on CotW (Fig. 1C). CotW therefore seems to be important for stabilizing parts of the crust (CotV/CotX) without playing an important structural role, as indicated by the crust displacement in the main structural mutants *cotXYZ* and *cotZ* (McKenney *et al.*, 2010; Imamura *et al.*, 2011), where CotW remains on the spore surface.

CotZ presumably anchors the crust structure to the middle of the spore

Our data on CotZ localization confirm previous observations that this protein is a main determinant of the crust (McKenney *et al.*, 2010; Imamura *et al.*, 2011) (Fig. 1D). Its distribution partially depends on CotY, but seems to be mostly independent of any other crust protein. The co-dependency of CotY and CotZ is reflected in the fact that they both share homologue domains (Finn *et al.*, 2016), are transcribed together (Zhang *et al.*, 1994) (Fig. 1A and above), and that tight interactions could be demonstrated by yeast/bacterial two-hybrid screens, as well as by atomic force microscopy (Krajčíková *et al.*, 2009; 2017; Liu *et al.*, 2015). CotZ seems to hold an important anchoring function for the crust, since the main structural components – CotV, CotX and CotY – are displaced in a *cotZ* mutant (Fig. 1D and I). The SEM and cryo-TEM data of the *cotZ* mutant demonstrate that the crust structure is displaced from the spore, but is still attached to the poles of the spores (see black arrow). This observation indicates that CotZ anchors the structure at the center of the spore, but that additional, CotZ-independent anchoring structures exist at the poles. The latter most likely precedes the CotZ-dependent anchoring, since the spore crust, like other spore layers, initiates at the poles (McKenney and Eichenberger, 2012).

CotY seems to be the main structural component of the crust

CotY is a cysteine-rich protein that forms layered structures when overexpressed in *E. coli* (Jiang *et al.*, 2015). It seems to be a major part of the crust structure, together with CotV and CotX. CotY is slightly perturbed in the absence of CotV, CotX or CotW (Fig. 1E). The latter could be an indirect effect, due to CotV and CotX depending on CotW. Nevertheless, CotY is still able to assemble around the spore, albeit in lower quantities and preferentially at the poles, even in the complete crust mutant *cotVWXYZ*, indicating that it has interaction partners in the outer coat that localizes to the spore poles. This interpretation is supported by described interactions of CotY with CotE and other coat proteins, based on AFM (Liu *et al.*, 2016b) or a bacterial two-hybrid screen (Krajčíková *et al.*, 2017). The importance of CotY as a main structural component of the crust is illustrated in mutants lacking CotV CotW, and CotX, in which CotY still nicely assembles around the spore (Fig. 1E). Additional deletion of *cotZ* in this background then leads to a complete loss of CotY-incorporation, demonstrating that the structural integrity of CotY is CotZ-dependent.

The SEM data of the spore surface demonstrate the structural importance of CotY: The wild type and the *cotVWX* mutant (in which the two other structural components, CotV and CotX are missing) appear almost indistinguishable with only minor perturbations of spore crust integrity, that is, an additional loss of CotY leads to major perturbations (Fig. 1H).

To further investigate the role of CotV, CotX and CotY, SEM and cryo-TEM analyses were performed for mutants each lacking one of these structural components (Fig. 1I). In the absence of CotY, there were only few, and small displaced crust fragments that seem to form small cap-like structures. In the presence of CotY, the structures were larger and more abundant: in the additional presence of CotX, these structures appeared as extended cap-like, while the presence of CotV resulted in branched structures (see black arrows). Taken together, CotY seems to provide the major part of the crust structure (together with its homologue CotZ), CotX seems to be part of the polar cap-like structure, while CotV seems to be involved in the propagation of the crust structure from the polar cap-like structure to the middle part of the spore (where the structure is anchored by CotZ).

CotV and CotX are co-dependent structural components of the spore crust

CotV and CotX are minor parts of the crust structure. They share homologue domains (Finn *et al.*, 2016) and their structural genes are transcribed together with *cotW*, while *cotX* is additionally also transcribed on its own (Zhang *et al.*, 1994). Figure 1F shows data of CotV (on the left) and CotX (on the right), which behave quite similar. CotV is CotX-dependent, but can still self-assemble in a *cotYZ* mutant to a structure distinct from the crust fragments. In contrast, no GFP-CotV can be detected in the complete crust mutant *cotVWXYZ*. CotX produces similar structures as CotV in the *cotX* and *cotYZ* mutants (Fig. 1F), indicating a joint structure of these two proteins. But in contrast to CotV, CotX still visibly assembles on the spore in the complete crust mutant *cotVWXYZ*. This observation fits AFM data demonstrating an interaction

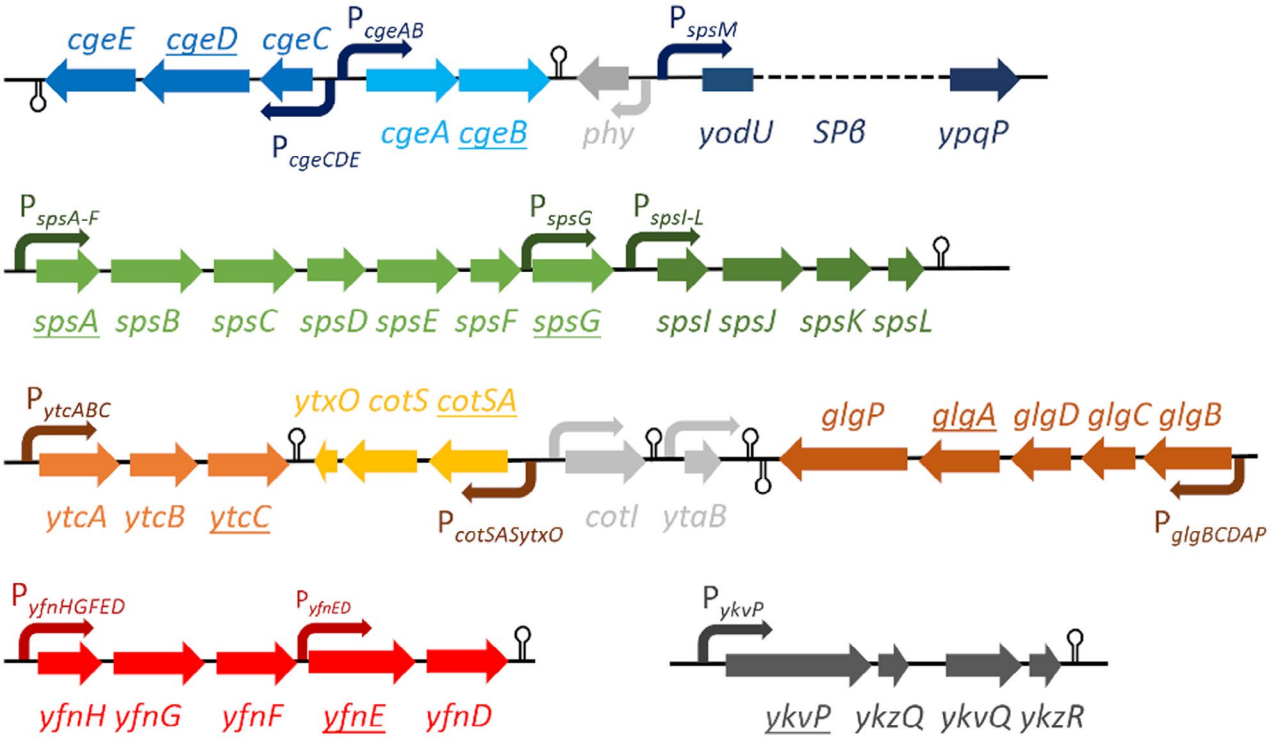

Fig. 2. SigE- and SigK-dependent operons encoding putative glycosyltransferases. See the methods sections for details on the identification of these clusters. Detailed information on all genes of these clusters can be found in Table S4. Thick arrows indicate promoters (taken from subtiwiki), the stem-loop structures terminators.

between the morphogenetic outer coat protein CotE and CotX (Liu *et al.*, 2016b).

CgeA might be a glycosylation hub and not a structural component of the spore crust

CgeA seems to be a minor part of the spore crust that might not play a structural role. The quantity and distribution of the N-terminal GFP-fusion to CgeA is not significantly affected in the different mutants (Fig. S1). CgeA also does not seem to be involved in the recruitment or initiation of the crust structure, since the localization of the other crust proteins is not perturbed in the *cgeA* mutant (Fig. 1G). Moreover, the spore surface of a *cgeA* mutant appears comparable to the wild type in SEM images, with few additional ridges appearing in the structure (Fig. 1H). Like other crust gene mutants described before (Imamura *et al.*, 2011), the *cgeA* mutant showed a clumping phenotype. In addition, it strongly adhered to polypropylene and developed matt colonies after sporulation (data not shown).

CgeA is co-transcribed or co-regulated with two putative glycosyltransferases (CgeB and CgeD, respectively). Additionally, it is located next to a split gene (*spsM*), which is associated with the spore envelope polysaccharide synthesis, hence the name *sps* (Abe *et al.*, 2014) (see Fig. 2). As CgeA itself is not predicted to be involved in glycosylation, but itself is part of the spore crust (Imamura *et al.*, 2011), it is attractive to interpret CgeA as the glycosylation hub of the crust, which plays a role in coordinating the glycosylation. This would also explain the phenotype of the mutant.

The SigK and SigE regulons contain putative glycosyltransferases and additional sugar-modifying enzymes

If CgeA indeed acts as the glycosylation hub of the spore crust, CgeB, CgeD and SpsM might be modifying enzymes for spore envelope polysaccharides. To gain a comprehensive picture of the crust glycosylation, we next identified all possible glycosyltransferases that could be involved in transferring the sugars for the spore envelope polysaccharides. Toward this end, the SigK and SigE regulons were screened for all genes encoding putative glycosyltransferase domain. This search revealed 10 genes, of which one was excluded due to its known function in the formation of lipid II (*murG*). The remaining nine genes are found in five genetic clusters that contain additional operons potentially involved in spore crust glycosylation (Fig. 2 and Table S4). We also included the split gene *spsM*, as it was already described to take part in spore

polysaccharide synthesis (Abe *et al.*, 2014) and is located next to *cgeA*.

The *spsA-L* cluster plays a role in spore envelope polysaccharide biosynthesis (Cangiano *et al.*, 2014) and is also predicted to be involved in the production of rhamnose-nucleotides (*spsI-L*) (Plata *et al.*, 2012). One of its gene products, SpsC, binds the outer surface of the spore (Knurr *et al.*, 2003) and has an aminotransferase domain, which is potentially involved in modifying sugar moieties. The *glgBCDAP* operon was implicated in glycogen synthesis during sporulation (Kiel *et al.*, 1994). The first and third genes from the *ykvP-ykzQ-ykvQR* encode proteins with LysM domains (Buist *et al.*, 2008), which could bind sugar moieties of peptidoglycan either in the cell wall or the spore cortex and might potentially also bind the spore crust polysaccharides. Additionally, these clusters also encode other predicted modifying enzymes, like the already mentioned putative aminotransferase SpsC, the putative acetyltransferases CgeD/SpsD, a putative CDP-glycerophosphotransferase (SpsB), some nucleotide transferases (SpsG, SpsI, YfnH, YfnF, YfnD, GlgC, GlgD), which are necessary to produce the substrate of the glycosyltransferases, as well as putative sugar epimerases (SpsJ, SpsK, SpsL, SpsM, YtcA, YtcB, YfnG), which could prime the sugar moieties for further modifications. The abundance and diversity of glycosyltransferases and sugar modifying enzymes encoded in the SigK and SigE regulons indicate that the spore envelope polysaccharide could be quite complex. We therefore aimed at gaining a first insight if these genes and corresponding operons were involved in the glycosylation of the crust.

Identification of glycosyltransferases involved in determining the glycosylation state of the spore envelope

Next, we phenotypically characterized a collection of glycosyltransferase mutants from the SigK and SigE regulons with regard to the glycosylation state of the spore crust envelope (Fig. 3, and supplemental Fig. S3). The most obvious phenotype observed was the loss of hydrophilicity, which results in the clumping of the spores (data not shown) as well as the adhesion to polypropylene (such as falcon tubes, data not shown) and silica (used as carrier for SEM) as seen in Fig. 3A. This behavior is displayed by all mutants lacking *cgeB*, *spsM* and *spsA-L* and mostly correlates with the loss of the mucus-like shine of the colony after sporulation (supplemental Fig. S3), which indicates at least a partial loss of the polysaccharide layer. But some mutants deviated from this correlation: The *cgeD* mutant did not adhere to silica or polypropylene (even sedimenting poorly during centrifugation), but still seemed to have lost the polysaccharide layer, as it produces matte colonies after sporulation. The mutant lacking *yfnHGFED* also sedimented poorly during centrifugation, but still produced colonies with a mucus-like shine. The Indian ink staining revealed that this mutant produces an extended polysaccharide layer compared to the wild type (shown in Fig. 3B). Abundance and localization of GFP-fusion to crust genes in these mutants appeared comparable to the wild type (supplemental Fig. S3) and SEM-analyses revealed that the spore surface appeared unaffected compared to the wild type, except for occasional ridges (shown in Fig. 3C, white arrows, representative with the triple mutant *cgeB spsM spsA-L*). This indicates that the glycosylation of the crust is not essential for the structural integrity of the spore crust.

The mutants were also visualized in the genetic background of the crust displacement mutant *cotZ*, to see if the structure of the displaced crust differed from the wild type. While this was not the case (data shown in supplemental Fig. S3), this investigation revealed an unexpected outer coat phenotype for the mutants *ytcABC*, *ytcC* and *cotSASytxO* (Fig. 3D): This phenotype is characterized by the displacement and structural disruption of the two structured caps of the outer spore coat (shown with black asterisks) and therefore a loss of the smooth center part (shown with a black arrow).

Lectin staining reveals no significant changes in quantity of the sugar moieties, but a change in the polymerization state

To further evaluate the nature of the polysaccharide layer, lectins were utilized to stain the sugar moieties. We chose rhamnose- and galactose-specific lectins, since previous studies on *spsM* indicated the presence of these sugars in the polysaccharide layer (Abe *et al.*, 2014). Lectin blots of the spore surface extracts did not reveal any significant differences in the presence and quantity of the sugar moieties in the glycosyltransferase mutants (Fig. S3D). Next, lectin stains of the mutant spores were prepared to study the sugar distribution and quantity at the single spore level (Fig. 3E, left). Surprisingly, the wild type shows no signal for the lectin stain, while the glycosyltransferase mutants lacking *cgeB*, *cgeD*, *spsM*, *spsA-L* and *yfnHGFED* show an increased signal for rhamnose and galactose (the *cgeD* mutant only showed wild-type signals for galactose).

We next applied boiling to break down and remove the polysaccharide layer prior to lectin staining (Fig. 3E right). A comparison of the wild type with the triple mutant *cgeB spsM spsA-L* (as a representative of the glycosyltransferase mutants) now revealed comparable signals of the boiled samples between the two strains. Hence, the lectins seem to be incapable of binding the undisturbed polysaccharides of the wild type. This observation indicates that the glycosyltransferase mutants seem to harbor perturbed and truncated polysaccharides, maybe even down to monosaccharides.

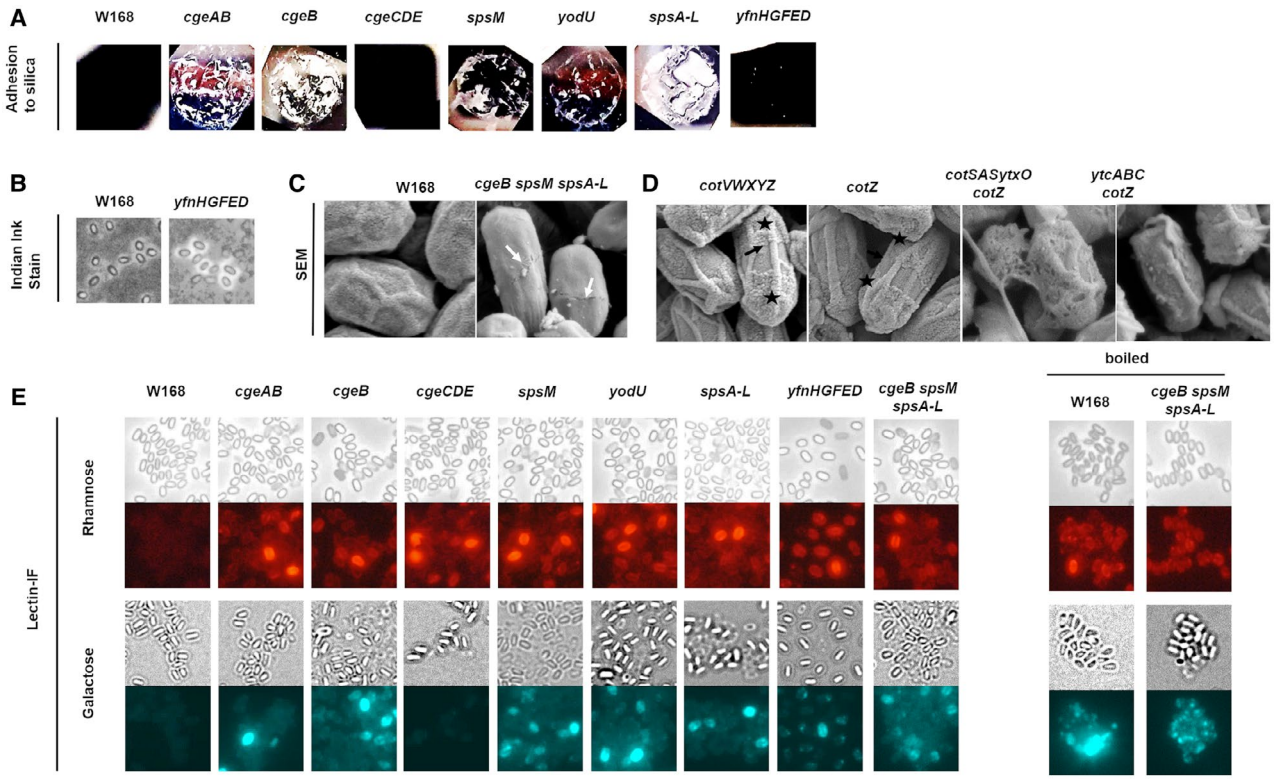

Fig. 3. Phenotype and lectin staining of the glycosyltransferase mutants.
A. Adhesion to silica indicates that the spores are more hydrophobic, presumably due to the missing polysaccharide modification of the crust, which is proposed to be responsible for water dispersal of the spores (McKenney *et al.*, 2010; Imamura *et al.*, 2011; Abe *et al.*, 2014).
B. Indian Ink stain of the *yfnHGFED* mutant in comparison to the wildtype indicates an extension of the polysaccharide layer of the crust on a subset of the spores. All other mutants did not show a significant difference to the wild type (data not shown).
C. SEM pictures of the triple mutant (*cgeB spsM spsA-L*) in comparison to the wild type show some ridges in the surface structure of the crust (see white arrows). This is not visible for all spores, indicating that the structure of the spore crust is not severely disturbed.
D. SEM pictures (with gold labeling) of the three mutants *cotSASytxO*, *ytcABC* and *ytcC* in the *cotZ* mutant background in comparison to the wild type and crust mutants: *cotZ* and *cotVWXYZ* (which show the outer coat: two structured caps shown with black stars and a smooth center shown with black arrows) reveal a disturbed outer coat.
E. On the left: Lectin stain of the spores from the glycosyltransferase mutants with lectins against rhamnose on the top in red (CSL, Oncorhynchus keta L-rhamnose binding lectin) and galactose on the bottom in turquoise (AIA, lectin from Artocarpus integrifolia (Jacalin)) with the phase contrast picture and the bright phase picture of the spores respectively. On the right: The triple mutant lacking *cgeB*, *spsM* and *spsA-L* as well as the wild type stained with lectins against rhamnose and galactose boiled. The complete data of the phenotypes can be found in supplemental Fig. S3.

Our results also demonstrate that there seems to be at least two independent (although most likely closely interlinked) polysaccharide variants, one based on rhamnose and one based on galactose (or comparable sugars): Only the rhamnose variant seems to be perturbed in the *cgeCDE* mutant spores which are additionally as hydrophilic as wild-type spores (Fig. 3A). The *yfnHGFED* mutant not only has an extended polysaccharide layer but also shows a higher signal for rhamnose and a slightly higher signal for galactose, indicating that the sugars are accessible to the lectins in this layer (see Fig. 3B and E).

Taken together, the presence and quantity of the sugar moieties are not affected by the glycosyltransferases tested. Instead, the structural integrity of the polysaccharides seems to be impaired, which argues for a high redundancy of these enzymatic functions, possibly though at different sites of action, but all influencing the intact production of the PS-layer.

The monosaccharide composition in the mutant spores suggests a rhamnose interrelated pathway leading to a capping sugar

Finally, we analyzed the monosaccharide composition of spore surface extracts of the wild type and glycosyltransferase mutants by HPLC (Fig. 4). While galactose and rhamnose could not be identified for wild-type spores based on sugar standards, the six peaks observed could potentially mask small amounts of these sugars,

A

Sugar	Glucose	Mannose	Galactose	Xylose	Rhamnose	Ribose
Retention time [min]	12,45	13,13	13,15	13,28	13,98	14,63

B

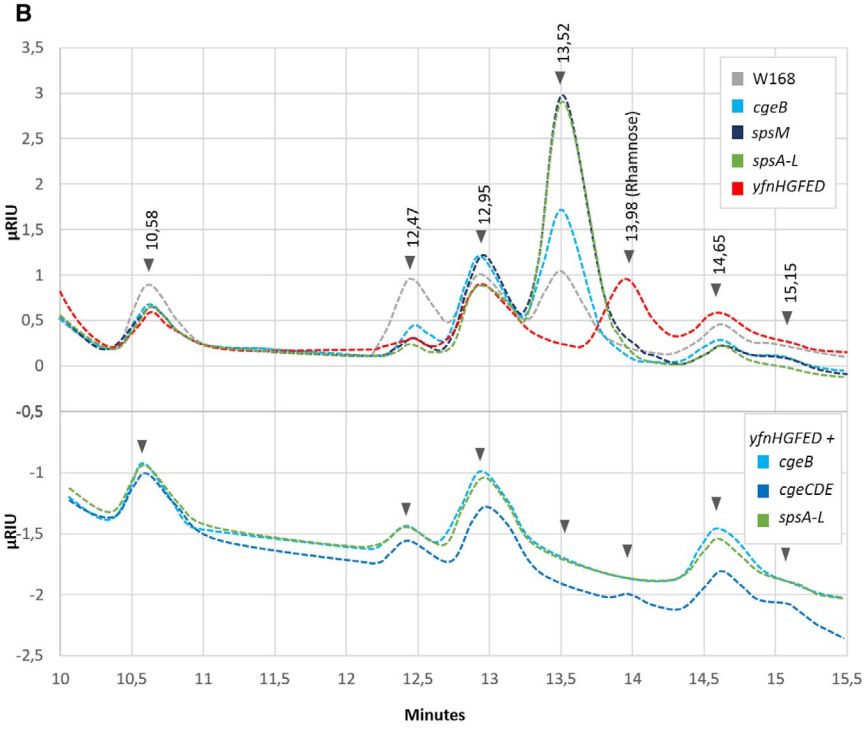

Fig. 4. Monosaccharide composition of the glycosyltransferase mutants investigated with HPLC. Spore surface extracts of the glycosyltransferase mutants were separated by HPLC.
A. Retention times of the sugar standards (glucose, mannose, galactose, xylose, rhamnose and ribose).
B. Top: HPLC data of the spore surface extracts of glycosyltransferase mutants; y-axis, refractory index; x-axis retention time in minutes. The peak at 12,47 minutes could be glucose and the peak at 14,65 minutes could be ribose. The peak at 13,98 relates to rhamnose. The other sugars could not be identified.

(Fig. 4B, top, gray curve, compared to the peaks of the sugar standards of Fig. 4A). Of the six peaks, two might correlate with the standards: the peak at 12,47 minutes could be glucose (standard at 12,45 minutes), while the peak at 14,65 minutes could represent ribose (standard at 14,63 minutes). It is impossible to tell if the peak that elutes at 12,95 minutes (shortly before galactose at 13,15 minutes) and the peak thatis detected at 13,52 minutes (shortly before rhamnose at 13,98 minutes) might be derivatives of rhamnose or galactose, which would explain the lectin results (Fig. 3C).

All glycosyltransferase mutants have a reduced peak at 12,47 minutes (presumably glucose), indicating that glucose might be the universal precursor, which is transferred by at least one of the spore-specific glycosyltransferases. The mutants *cgeB*, *spsM* and *spsA-L* additionally accumulate an unknown sugar, eluting at 13,52 minutes. This peak is missing in the *yfnHGFED* mutant, indicating that it is either transferred or produced by the gene products of this operon. The accumulation of this peak could suggest that this sugar might act as a pool for the production of other sugar moieties produced by the gene products of *cgeB*, *spsM* and *spsA-L*.

In the *yfnHGFED* mutant, rhamnose (detected at 13,98 minutes) accumulates. This might be the reason for the extended polysaccharide layer, which was observed by Indian ink staining (Fig. 3B) Based on the lectin signal for rhamnose (see Fig. 3B), this extended polysaccharide layer could therefore be composed of a rhamnose-containing polymer. As the peak at 13,52 minutes is missing in this mutant, it is compelling to think of this sugar moiety or a resulting sugar as a capping or junction moiety, which would normally prevent the respective glycosyltransferase from adding or producing rhamnose moieties uncontrollably. To test this assumption, double mutants combining *yfnHGFED* with either *cgeB*, *cgeCDE* and *spsA-L* were generated and the spore surface extracts were again analyzed by HPLC (Fig. 4B, bottom). The double mutants with *cgeB* and *spsA-L* do not accumulate rhamnose anymore and also produce matt colonies

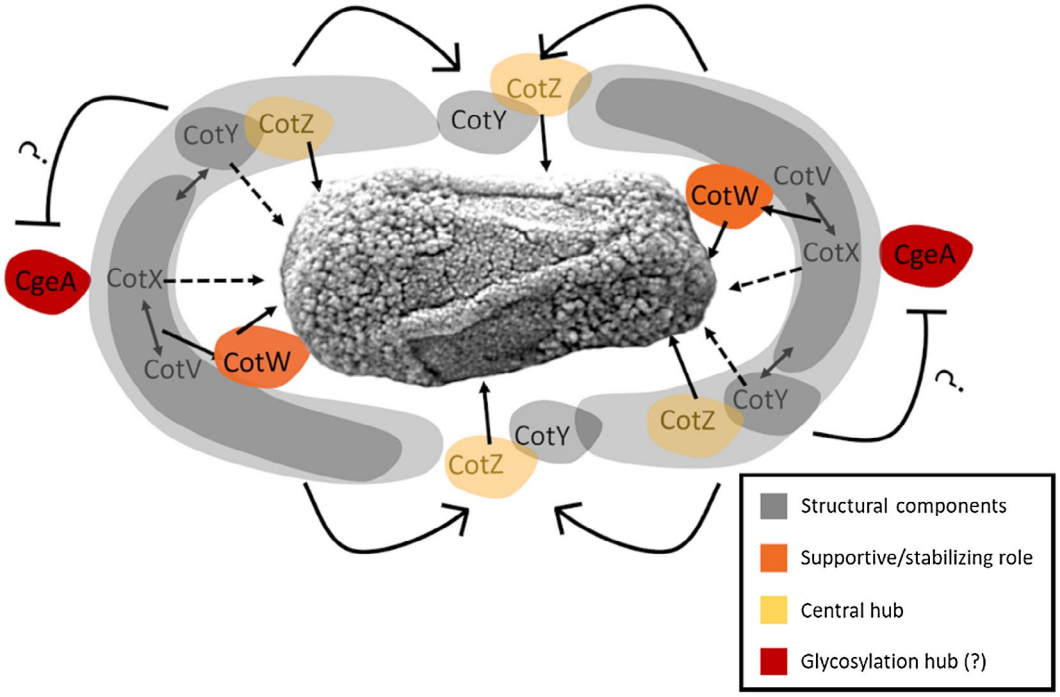

Fig. 5. Model of the crust interaction network. The interaction model of the crust deduced from the data of crust gene mutant spores of crust expressing GFP-crust protein fusions. The colors indicate the postulated roles: gray: structural components, orange: supportive/stabilizing role, yellow: central hub/anchor of the crust and red: glycosylation hub. The outer coat, as seen on the spore, is divided in two rough cap-like structures and a more or less smooth middle part as already described (Plomp *et al.*, 2014). See discussion for details.

(data not shown). In the *cgeCDE* mutant, this peak is still present, indicating that the gene products of this *cgeCDE* are not involved in producing or transferring rhamnose. But since this method is only semi-quantitative and no peak could be conclusively identified, these studies only provide a first insight and will require more detailed and quantitative follow-up studies.

Discussion

This study aimed at understanding the interactions of crust proteins and the nature of the crust. Based on the data obtained, an interaction model was deducted that illustrates the hierarchy and physiological role for the proteins of the crust (Fig. 5).

An interaction network of the spore crust

Like many parts of the spore coat, the crust initiates at the poles (McKenney and Eichenberger, 2012). Therefore, it is tempting to suggest that the polar nature of some of the crust proteins and the initiation at the pole is based on a cap-like structure that forms on top of the outer coat pole.

CotV and CotX. The GFP data suggest that this cap could be a joint structure of the polarly located proteins CotV and CotX, which probably support the initiation (CotX) and propagation (CotV) from the spore poles. While neither yeast nor bacterial two-hybrid assays could demonstrate an interaction between CotV and CotX, this could be due to the very hydrophobic nature of CotX, which likely lead to misfolding and therefore false negatives in these screens (Krajčíková *et al.*, 2009; 2017). Nevertheless, our GFP data suggest a strong link between these two proteins, which show mostly similar behavior in the mutant screen, and both depend on CotW (Fig. 1 and Fig. S1), even though CotW alone is not sufficient to bind these proteins to the spore. This co-dependency is supported by data showing that CotX degrades in a *cotVW* and *cotV* mutant (Shuster *et al.*, 2019a).

CotW itself is distributed across the whole spore surface and is independent of any other crust protein. Instead, it partially depends on the outer coat proteins CotH, CotB and YwrJ (not shown in Fig. 5), suggesting a role in the interface of the outer coat and the crust. It seems to be incorporated into the crust late during maturation, since it is still absent in immature, that is, phase-gray spores. Such a 'late incorporation' has also been previously observed for the coat protein CotD, demonstrating that small proteins can transcend an already formed spore coat (McKenney and Eichenberger, 2012). This suggests that CotW might not be part of the structure, since it remains

on the spore in a *cotZ* (crust displacement) mutant, but rather plays a supportive or stabilizing role for CotV and/or CotX. Accordingly, an interaction of all three proteins has been demonstrated in yeast (CotW and CotV) (Krajčíková *et al*., 2009) or bacterial two-hybrid assay (CotW and CotX) (Krajčíková *et al*., 2017). Additionally, CotV and CotW together produce a filament-like structure when overexpressed in *E. coli*, whereas CotV produces none alone (Jiang *et al*., 2015), suggesting that these two proteins interact and that the structure produced by CotV is stabilized by CotW. This stabilizing role was also proposed recently, as CotX is degraded in *cotVW* mutants, but less in *cotV* mutants (Shuster *et al*., 2019a).

CotY. The GFP-data suggests that the core structure of the crust is mainly formed by CotY (together with its homologue CotZ), a cysteine-rich protein that produces a highly stable sheet structure when overexpressed in *E. coli* (Jiang *et al*., 2015). It was shown to interact in the yeast two-hybrid assay with itself and CotZ (Krajčíková *et al*., 2009), while bacterial two-hybrid assays suggested an interaction of CotY with all crust proteins except CotX and many coat proteins (Krajčíková *et al*., 2017), underlining its importance for the crust structure. It is also to some extent dependent on CotV and CotX for propagation, as recently published (Shuster *et al*., 2019a). This study also demonstrated that the crust structure is unperturbed in a *cotY* mutant. Therefore, CotY is the main structural component, but seemingly has a redundant function with its homolog CotZ.

CotZ is required to anchor the crust structure to the spore, as described previously (McKenney *et al*., 2010; Imamura *et al*., 2011). Accordingly, this structure is displaced in a *cotZ* mutant, and only remains attached at the spore poles. This residual polar attachment of the crust structure is probably mediated by CotY and CotX (Liu *et al*., 2016b; Krajčíková *et al*., 2017). CotZ was shown to interact with CotE in AFM experiments (Liu *et al*., 2016a) and weakly with two additional coat proteins in a bacterial two-hybrid assay (Krajčíková *et al*., 2017). CotZ is mainly required to anchor the crust structure at the middle of the spore. It is distributed in high quantities across the whole spore surface but seems to represent a non-essential part of the crust structure, which is still assembled in a *cotZ* mutant. CotY and CotZ are paralogous proteins (CotZ superfamily) that interact strongly (Liu *et al*., 2015). It is therefore possible that the missing CotZ in the crust structure of the *cotZ* mutant could be replaced by CotY. The central role of CotZ as the main morphogenetic protein of the spore crust was recently confirmed in a study on genetic dependencies of crust proteins (Shuster *et al*., 2019a).

CgeA is a low-abundant spore crust protein that is mostly found as a polar spot or cap on premature phase-gray spores, but seems to be less abundant or absent on mature phase-white spores. Other crust proteins do not depend on CgeA and their incorporation is not affected by its absence in a *cgeA* mutant (Fig. S1), in line with recent findings (Imamura *et al*., 2011; Shuster *et al*., 2019a). While CgeA does not seem to play a structural role, it seems to share its binding location with some of the other crust proteins. Accordingly, CgeA was shown to interact weakly with many crust and coat proteins in a bacterial two-hybrid assay (Krajčíková *et al*., 2017). The *cgeA* gene is co-transcribed with two putative glycosyltransferases (*cgeB* and *cgeD*). It is therefore tempting to think of CgeA as a glycosylation hub, which plays a role in coordinating the crust glycosylation (see next section for details). As the polarly located crust proteins CotX and CotV both contain conserved glycosylation motifs (in the CotX superfamily domain), these might be the major targets of glycosylation. While a role of CgeA in crust glycosylation is also discussed in a recently published study, these authors suggest CgeA as a target of glycosylation (Shuster *et al*., 2019a) and not as glycosylation hub. If true, its glycosylation would differ from the typical bacterial N-glycosylation, as CgeA does not include a conserved glycosylation domain (Kowarik *et al*., 2006). The exact role of CgeA therefore still remains an open question for further research.

Involvement of glycosylation in the attachment of the crust–coat interface

Most of the glycosyltransferase mutants (shown to be involved in crust glycosylation) as well as the *cgeA* and *cotX* mutant showed ridges in the crust surface (Figs. 1 and 3) and in TEM the crust structure (beads-on-a-string) ballooned away for the *cgeA* and *cotX* mutant (Shuster *et al*., 2019a). These phenotypes are presumably identical and show a more loosely attached crust, which is detached by the harsher treatment during the TEM probe preparation. This together with the phenotype (see below for details) of the *cotSASytxO* and *ytc(AB)C* mutants and their putative functions might indicate a role for glycosylation in the attachment of the crust to the coat interface. These genes are predicted to encode glycosyltransferases or sugar epimerases. Additionally, the neighboring gene encodes a putative nucleotidyl transferase (*ytdA*), which was shown to be phenotypically involved with the spore polysaccharides (Arrieta-Ortiz *et al*., 2015). Accordingly, the spore still exhibits a thin polysaccharide layer in the *cotXYZ* mutant, which lost the crust, indicating another attachment point for the spore polysaccharides (Shuster *et al*., 2019a). But the exact role of glycosylation in crust-to-coat interface attachment remains an open question for future research.

The spore crust as a glycosylated protein layer

We identified and deleted all glycosyltransferase-encoding operons of the mother cell-specific SigE and SigK regu-lons, as well as the previously described split gene *spsM* (Abe *et al*., 2014). This mutant collection was then pheno-typically screened. We could demonstrate a role in spore glycosylation for *spsM*, *spsA-L* (which was also previously described [Cangiano *et al*., 2014]), *cgeAB*, *cgeCDE* and *yfnHGFED*. We therefore propose to rename the *yfnHG-FED* cluster to *spsNOPQR*, as this operon is involved in spore polysaccharide synthesis, just like *spsA-L* and *spsM* (Abe *et al*., 2014; Cangiano *et al*., 2014).

Despite clear indications that the five mutated operons are involved in crust glycosylation, since their deletion resulted in matt colonies and hydrophobic spores, the amount and composition of sugar moieties remained surprisingly unchanged for most of them. The *yfnHGFED* mutant was the only exception, since the HPLC profil-ing demonstrated the loss of one unknown sugar spe-cies, while it accumulated rhamnose instead. This lack of change in the sugar composition might indicate some degree of functional redundancy that needs to be further analyzed.

The combined data of the HPLC and lectin analysis indicate that the structure of the polysaccharide might be impaired (Figs. 3E and 4B). The sugar pathways involved are either intertwined or generate highly similar sugars. The phenotypes of the *yfnHGFED* and the *cgeCDE* mutant provided some insights into the possible mecha-nism of crust polysaccharide synthesis.

The ***cgeCDE* mutant** sedimented poorly and was the only mutant, for which the rhamnose lectin could readily bind, while the galactose lectin could not, indicating that the sugars bound by these lectins belong to two different polysaccharide species. Since lectin binding was coupled for all other strains, these two polysaccharide variants (potentially a rhamnose-related and a galac-tose-related variant) might be cross-linked and highly dependent on each other, with the function encoded in the *cgeCDE* operon playing a role in cross-linking them. A recent unpublished study on the glycosylation of the crust also saw a similar phenotype for sedimentation and an extended polysaccharide layer (Shuster *et al*., 2019b). This extended layer could be accounted for by a one-sided unattached linker molecule.

The ***yfnHGFED* mutant** also sedimented poorly and produced an extended polysaccharide layer, again in agreement with observations by Shuster *et al.* (2019b), This mutant accumulated rhamnose while lacking an unknown sugar species (eluting at 13,52 minutes) that was enriched in the *spsM, spsA-L* and *cgeAB* mutants (Fig. 4). This observation indicates that the gene products of these operons are involved in the same biosynthetic pathway as the gene products of *yfnHGFED*. Accordingly, this phenotype can be rescued by additionally mutating *spsA-L* and *cgeB* (Fig. 4), as well as *spsI* alone (Shuster *et al*., 2019b). As rhamnose accumulates in the *yfnHG-FED* mutant, the rhamnose synthesis pathway already known to be executed by *spsIJKL* (Plata *et al*., 2012) was the starting point for developing a model on spore polysaccharide biosynthesis in *B. subtilis* that is in agreement with our data.

Spore polysaccharide biosynthesis in B. subtilis*: a hypothesis*

We propose that the rhamnose pathway has a junction leading to a sugar moiety, which caps the polysaccharide and hinders the uncontrolled addition of rhamnose, a sugar only found at most in low levels in the wild type. The postulated functions of the genes involved in spore polysaccharide biosynthesis (See Fig. S4 and Table S4) provided hints on their role in this pathway, the nature of the sugar moieties, the junction between this and the other two pathways and the source of the observed redundancy. Fig. 6A shows the model in a schematic fashion, and Fig. 6B explains the phenotype of the two unique mutants which led to the model. For a more detailed discussion, please refer to supplemental Figs. S5–S7 as well as S4 for the detailed operon structure with the respective putative functions of all genes involved and the detailed explanation of the model.

Our model involves three independent, yet redundant pathways: one leading to rhamnose (executed mainly by *spsIJKL*, but redundant enzymes may exist), one leading to the proposed capping sugar, which is diverted from this pathway to form viosamine, and leading to a unique sugar derived from either viosamine or VioNAc. Biosynthesis of the latter would involve mainly *yfnHGFED* and the operon *yodTSRQPkamAyokU,* which is located directly next to *cgeCDE,* and could also involve *spsM*. A third, independent pathway, would produce the same sugar moieties, but as a cross-linkage site and not the precursor for the capping moiety. The latter would mostly involve the products of *cgeCDE* and presumably *spsC*. The model postulates that the sugar moieties and pathways involved are the same, based on the identical sugar profiles of the respective mutants, as observed by HPLC: if the individual pathways would produce different sugars, the peak profile would change, unless the quantity is too low for detection.

The cross-linking to the galactose-related variant is proposed to occur via polyglycerophosphate, as SpsB is a putative polyglycerophosphate transferase and this molecule would fit the function. Other than binding of the galactose-specific lectine and the putative functions of the genes involved, we have very little insight into the composition of

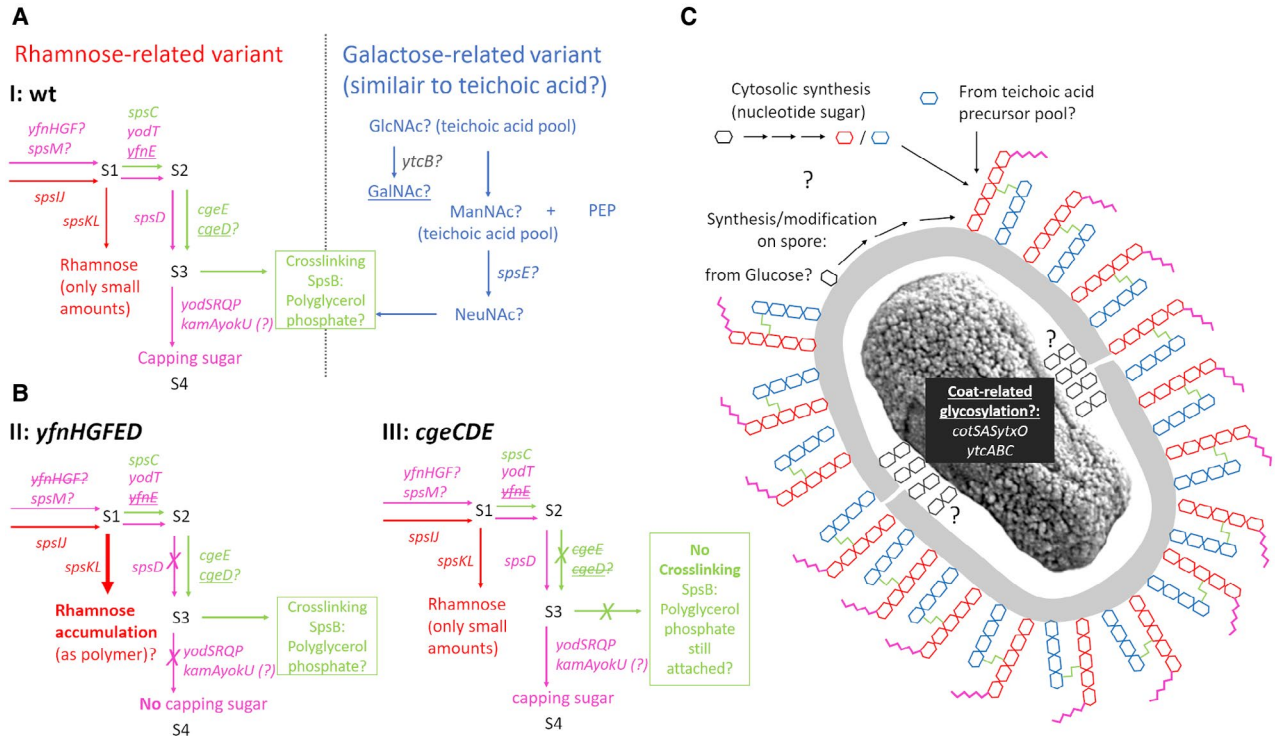

Fig. 6. Model of spore crust polysaccharides. The model suggests (at least) two different PS variants, one based on galactose or a similar sugar (blue) and one based on rhamnose (shown in red), presumably capped by a unique sugar (shown in pink). These two variants might be cross-linked (shown in green), e.g. via polyglycerolphosphate (based on the putative function of SpsB, see Table S4).
A. For the rhamnose-related variant, there are three independent, yet partially redundant pathways: one leading to rhamnose (red, involving *spsIJKL*), one leading to a capping sugar (pink, involving *yfnHGFED*, *spsM*, *spsD* maybe also *yodT* and presumbably *yodSRQPkamAyokU*) and one leading to the sugar that is the point of attachment for the cross-linking to the other PS-variant (in green, involving *cgeCDE* and maybe *spsC*, cross-linked via polyglycerolphosphate). For the proposed pathways, refer to supplemental Figs. S5–S7. The respective sugars are proposed to be as follows: S1: 4-keto-6-deoxy-Glucose; S2: Viosamine; S3: VioNAc and S4: VioNAc with a modified lysine side chain (see supplemental Fig. S6).
B. Proposed pathway alterations in the *yfnHGFED* mutant (II) and the *cgeCDE* mutant (III).
C. Summary of the model on the spore surface: rhamnose- (red), and galactose-related variant (blue), postulated outer coat glycosylation (black). These two variants might be by polyglycerolphosphate (green), similar to teichoic acid. The rhamnose-related variant might be capped by a unique sugar (pink). The precursors of the polysaccharides are presumably produced in the mother cell and at some point transferred to the spore crust (likely targets are either CotX or CotV), where additional modification steps might occur. Some crust polysaccharides might be produced from glucose (shown in black) directly onto the spore surface, as glucose seemed to be part of the spore polysaccharide composition (Fig. 4B). See discussion for details.

the galactose-related variant. One gene (*spsE*) encodes a putative NeuNAc synthetase, which suggests that this variant could be similar to teichoic acid, containing NeuNAc. We would like to propose that GalNAc and related sugars are bound to polyglycerophosphate, which then links to the rhamnose-related variant. YtcB is a postulated UDP-glucose-4-epimerase (see Table S4), which could produce GalNAc. But without biochemical evidence, the exact nature of the galactose-related variant remains unclear.

Taken together, we propose that the two different polysaccharides, one related to rhamnose and one derived from galactose, are cross-linked via polyglycerolphoshate (Fig. 6C). This cross-link could link NeuNAc (predicted to be produced by SpsE) from the galactose-related variant to VioNAc in the rhamnose-related variant (see supplemental Fig. S7 for details). The rhamnose-related variant might also be capped by a unique sugar similar to what was shown for *Bacillus anthracis*. In this close relative, a side chain derived from leucine is transferred to the amino group of the rare sugar viosamine (4-amino-4,6-dideoxy-glucose) (Dong *et al.*, 2008; Dong *et al.*, 2010). A comparable mechanism could be envisioned for *Bacillus subtilis* and might involve the gene products of the *yodTSRQPkamAyokU* operon, which is located next to *cgeCDE* (supplemental Fig. S6). This capping might prevent the uncontrollable polymerization of the rhamnose polysaccharide (see Fig. 6B (II) for the *yfnHGFED* mutant). But future research will be required to unravel the true nature of the spore polysaccharides.

Accordingly, the presumed unique nature of the crust polysaccharide might suggest an additional role next to

the dispersal in water: the protection to biological degradation and scavenging. The mere property of water dispersal would not require as many players as well as such a high amount of energy investment. Conclusively, many players and the complexity of the polysaccharide synthesis might indicate that the end result of these reactions, though being non-unique in parts, might lead to structures rare or unique in nature, therefore providing protection against commonly found degradation enzymes. We therefore propose an additional function of the spore polysaccharide layer (McKenney *et al.*, 2010; Imamura *et al.*, 2011; Abe *et al.*, 2014): protection against biological degradation and scavenging commonly found in biologically demanding conditions (Klobutcher *et al.*, 2006; Laaberki and Dworkin, 2008), where spores are normally formed under.

Experimental procedures

Bacterial growth conditions

B. subtilis and *E. coli* were routinely grown in lysogeny broth (LB medium) (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) at 37°C with agitation (220 rpm). All strains used in this study are listed in Additional file 1: Table S1. Selective media for *B. subtilis* contained chloramphenicol (5 μg ml^{-1}), erythromycin in combination with lincomycin (1 μg ml^{-1}; 25 μg ml^{-1} for mls^r), spectinomycin (100 μg ml^{-1}) or kanamycin (10 μg ml^{-1}). Selective media for *E. coli* contained ampicillin (100 μg ml^{-1}). Solid media additionally contained 1.5% (w/v) agar.

Transformation

Transformations of *B. subtilis* were carried out as described previously (Harwood and Cutting, 1990). The integration of the DNA into the *B. subtilis* genome was verified on starch plates (*amyE*), when the crust-GFP fusions based on the Sporovectors (Bartels *et al.*, 2018) were utilized or by colony-PCR after allelic replacement mutagenesis. All plasmids used in this study are listed in Additional file 1: Table S2.

Allelic replacement mutagenesis

Allelic replacement mutagenesis of the crust and glycosyltransferase genes/operons using long flanking homology (LFH)-PCR was performed as described previously (Mascher *et al.*, 2003). All primers used in this study are listed in Additional file 1: Table S3.

Spore colony morphology

About 10 μL of the overnight cultures of the strains was dotted onto 90 mm Difco Sporulation Medium-Plates (DSM, 0.8% w/v Tryptone, 0.1% w/v KCl, 1 mM $MgSO_4$, 10 μM $MnCl_2$, 1 μM $FeSO_4$, 0.5 mM $CaCl_2$, 1.5 % (w/v) Agar). These plates were incubated at room temperature for at least two weeks to enable complete sporulation of the colony.

Spore preparation

The *B. subtilis* strains were inoculated in 50 ml (for Electron microscopy, lectin blots/fluoreszenz microscopy, HPLC) or 2 ml (for GFP fluorescence microscopy) Difco Sporulation Medium (DSM, 0.8% w/v Tryptone, 0.1% w/v KCl, 1 mM $MgSO_4$, 10 μM $MnCl_2$, 1 μM $FeSO_4$, 0.5 mM $CaCl_2$) and grown at 37°C with agitation (220 rpm) for 48 hours to ensure sporulation. These spore cultures were harvested by centrifugation at 10,000× *g* for 8 minutes, resuspended in 10 ml or 2 ml of dH_2O and treated for 1 hour at 37°C with 75 μg ml^{-1} lysozyme to remove the remaining vegetative cells. Afterwards, the prepared spores were washed with 10/2 ml dH_2O, 10/2 ml 0.05% SDS and then one time with 10/2 ml dH_2O. The spores were then resuspended in either 2 ml or 200 μl dH_2O (adapted from [Harwood and Cutting, 1990]).

Fluorescence microscopy

The GFP-displaying spores were inspected on agarose pads (1% Agarose in H_2O, cast in Press-to-seal™ silicone isolators with 20 mm diameter and 1 mm depth from Life Technologies) using an Olympus AX70 upright fluorescence microscope utilizing the 100× objective (UPlanFl 100×/1,30 Oil). The spores were documented with phase contrast and the GFP fluorescence channel utilizing the filter cube U-MNIB (IF blue long pass, Ex. 470–490 nm, Em. 515-∞). The pictures were taken with a XC10 color camera from Olympus with 500 ms exposure times and the same microscope settings and saved as tagged image file (tif).

Electron microscopy

For the scanning electron microscopy (SEM), two different methods were utilized. First, the traditional CP-drying method, where the spores were dried by incubating the spore suspension in increasing concentrations of ethanol (70%, 80%, 90%, 96% and 100%) for 20 minutes each, to substitute the water with ethanol. Then the spores were washed with fluid carbon dioxide in a CPDryer (Baltec CPD030) to substitute the ethanol with the carbon dioxide. The carbon dioxide was eliminated by heating the chamber to 31°C and a pressure of 75 bar, to assure that the fluid carbon dioxide transitions to gas phase, creating a dry probe. The pressure was then released, the probe was brought onto a conductive probe carrier, sprayed with gold and measured with the secondary electron detector at 5 kV (Zeiss Supra 40VP).

Second, the probes were measured natively (without drying and gold labeling), by applying the probe directly on a silica wafer, letting it dry, applying it on a carrier and assuring conductivity with silver polish from the silica wafer to the metal part of the carrier. These probes were also measured with the secondary electron detector or the backscattered electron detector (Zeiss Gemini 982 with LaB6-cathode).

Cryogenic transmission electron microscopy (cryo-TEM) images were recorded using a Libra 120 microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Two microliter of specimen were placed onto a holey carbon TEM grid (Lacey type, 300 mesh), blotted with filter paper and vitrified in liquid ethane at –178°C using a Grid Plunger (Leica Microsystems GmbH, Wetzlar, Germany). Frozen grids

were transferred into a Gatan 626 (Gatan GmbH, München, Germany) cryo-TEM holder. Images were recorded at an accelerating voltage of 120 kV while keeping the specimen at –170°C.

Detection of galactose and rhamnose with lectins

For the detection of the sugar components of the polysaccharide layer, the lectins for galactose (*Artocarpus integrifolia* (Jacalin) Lectin (AIA) - Cy5 from bioworld) and Rhamnose (*Oncorhynchus keta* (Chum salmon) Lectin (CSL3) - Cy3 from bioworld) were chosen. Two methods were applied: lectin blots from spore surface extracts and immunofluorescence of the spores.

For the lectin blots, 10 µl of a spore surface extract (supernatant of a spore suspension incubated at 98°C with a SDS buffer (125 mM Tris-HCl (pH 6.8), 2% SDS and 4% β-mercaptoethanol)) was spotted onto a PVDF membrane (activated with 100% Methanol). The membrane was incubated with 1% BSA in PBS-T (137 mM NaCl, 12 mM Phosphate, 2.7 mM KCl, pH 7.4, 0.1% (v/v) Tween 20) overnight at 4°C. The next day, the membrane was washed three times with PBS-T for 10 minutes each and then incubated with 0.1 µg ml^{-1} of the respective lectin in PBS-T for one hour at room temperature. Afterwards, the membrane was washed three times with PBS-T for 10 minutes each. The secondary antibody (Anti-Cyanine antibody (HRP) from abcam) was diluted 1:5,000 in PBS-T and the membrane was incubated with this solution for one hour at room temperature. Finally, the membrane was washed three times with PBS and then developed with AceGlow™ (VWR).

For immunofluorescence, the spore suspension was blocked with 5% BSA in PBS for 30 minutes and then incubated with 1 µg ml^{-1} of the respective lectin in PBS for one hour, followed by three washing steps with PBS for 5 minutes. The probes were viewed on an agarose pad (1% agarose in H_2O, cast in Press-to-seal™ silicone isolators with 20 mm diameter and 1 mm depth from Life Technologies). For the rhamnose lectin, the samples were analyzed using an Olympus AX70 fluorescence microscope utilizing the 100× objective (UPlanFl 100×/1,30 Oil), the phase contrast channel, and the Cy3 channel (filter cube U-M41007A, Cy3, Ex. 530–560 nm, Em. 575–645 nm). The pictures were taken with a XC10 color camera from Olympus with 500 ms exposure times and identical microscope settings and saved as tagged image files (tif). For the galactose lectin, the samples were analyzed using a DeltaVision Elite microscope (GE Healthcare) utilizing the 100× objective (Planapochromat 100×; NA 1.4 Oil), the bright field channel and the Cy5 channel (single band pass emission filter Cy5 = 679/34 and an excitation wavelength for Cy5 = 632/22). The pictures were taken with a 'Scientific CMOS camera' with 500 ms exposure times, applying identical microscope settings and saved as tagged image file (tif).

The pictures of the rhamnose lectins were modified with Adobe Photoshop (2017) by shifting the magenta–green slider in the color balance option completely to the magenta side. This depletes the pictures (which derive from a color camera) of the autofluorescence of the spores, thereby clarifying the signal. The contrast and brightness were adapted for all images in the same manner. The pictures of the galactose lectins were analyzed utilizing Fiji, an image processing package for ImageJ (downloaded from https://fiji.sc/). Contrast and brightness were adapted for all images in the same manner and the color channel was changed to cyan (as these pictures derive from a black-and-white camera).

HPLC-based analysis of monosaccharide composition

To assess the monosaccharide composition of the spore surface, 200 µl of the spore suspension (which were adjusted to an optical density of 50) was dried in the DNA Speed Vac (Savant DNA 110) for one hour at drying rate high. The dried samples were incubated for one hour at 105°C in 200 µl of 4M trifluoroacetic acid (TFA) to break down the polysaccharides into monosaccharides. The samples were air dried, then dried in the DNA Speed Vac again and resuspended in 200 µl of dH_2O. The samples were then centrifuged for 10 minutes at 4°C and 15.000 g to separate the extract from the spores. From these extracts, 30 µl was injected into the HPLC (Smartline HPLC Series KNAUER, column: Eurokat H, 10 µm, 300 × 8 mm), with 0.05 N sulfuric acid as the mobile phase with a flow rate of 0.5 ml min^{-1} at 65°C. Sugar solutions (galactose, glucose, mannose, rhamnose, ribose and xylose) were injected as standards. The sugars were detected with an UV-detector (210 nm) and a RI detector. The data were evaluated with the ChromGate Client software.

Bioinformatic screening of the SigK and SigE regulons for glycosyltransferases

To determine all possible candidates for the glycosylation of the spore crust, the mother cell-specific sporulation regulons of the sporulation sigma factors SigK and SigE were screened for glycosyltransferase domains. For this, all relevant glycosyltransferase domains from Pfam (version 32.0, accessed 06/18/2019 excluding eukaryotic domains) were compiled. The SigK and SigE regulons were downloaded from SubtiWiki 2.0 (Zhu and Stülke, 2018). The resulting gene lists were submitted to UniProt (The UniProt Consortium, 2017) to extract the corresponding protein sequences in FASTA format. These sequences were then submitted to SeqDepot (Ulrich and Zhulin, 2014) to obtain pre-computed information (including Pfam domains [Finn *et al*., 2016]). The resulting output file was then screened for glycosyltransferase domains utilizing a python script. The list of domains and the python script can be found in supplemental material.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

JB and SL constructed the strains. MR ran the HPLC experiments. MG and AB assisted with the electron microscopy. All other experiments were performed by JB. JB evaluated the data. JB and TM designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials. Raw data were generated at TU Dresden. Derived data supporting the findings of this study are avail-able from the corresponding author JB on request.

References

Abe, K., Kawano, Y., Iwamoto, K., Arai, K., Maruyama, Y., Eichenberger, P., *et al.* (2014) Developmentally-excision of the SPβ prophage reconstitutes a gene required for spore envelope maturation in *Bacillus subtilis*. *PLoS Genetics*, **10**, e1004636.

Abhyankar, W., Pandey, R., Ter Beek, A., Brul, S., de Koning, L.J. and de Koster, C.G. (2015) Reinforcement of *Bacillus subtilis* spores by cross-linking of outer coat proteins during maturation. *Food Microbiology*, **45**, 54–62.

Arrieta-Ortiz, M.L., Hafemeister, C., Bate, A.R., Chu, T., Greenfield, A., Shuster, B., *et al.* (2015) An experimentally supported model of the *Bacillus subtilis* global transcriptional regulatory network. *Molecular Systems Biology*, **11**, 839.

Bartels, J., López Castellanos, S., Radeck, J. and Mascher, T. (2018) Sporobeads: the utilization of the *Bacillus subtilis* endospore crust as a protein display platform. *ACS Synthetic Biology*, **7**, 452–461.

Buist, G., Steen, A., Kok, J. and Kuipers, O.P. (2008) LysM, a widely distributed protein motif for binding to (peptido) glycans. *Molecular Microbiology*, **68**, 838–847.

Cangiano, G., Sirec, T., Panarella, C., Isticato, R., Baccigalupi, L., De Felice, M., *et al.* (2014) The sps gene products affect the germination, hydrophobicity, and protein adsorption of *Bacillus subtilis* spores. *Applied and Environment Microbiology*, **80**, 7293–7302.

Dong, S., McPherson, S.A., Tan, L., Chesnokova, O.N., Turnbough, C.L. and Pritchard, D.G. (2008) Anthrose biosynthetic operon of *Bacillus anthracis*. *Journal of Bacteriology*, **190**, 2350–2359.

Dong, S., McPherson, S.A., Wang, Y., Li, M., Wang, P., Turnbough, C.L., Jr., *et al.* (2010) Characterization of the enzymes encoded by the anthrose biosynthetic operon of *Bacillus anthracis*. *Journal of Bacteriology*, **192**, 5053–5062.

Finn, R.D., Coggill, P., Eberhardt, R.Y., Eddy, S.R., Mistry, J., Mitchell, A.L., *et al.* (2016) The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Research*, **44**, D279–D285.

Harwood, C.R. and Cutting, S.M. (1990) *Molecular Biological Methods for* Bacillus. Chichester: John Wiley & Sons Ltd.

Higgins, D. and Dworkin, J. (2012) Recent progress in *Bacillus subtilis* sporulation. *FEMS Microbiology Reviews*, **36**, 131–148.

Imamura, D., Kuwana, R., Takamatsu, H. and Watabe, K. (2011) Proteins involved in formation of the outermost layer of *Bacillus subtilis* spores. *Journal of Bacteriology*, **193**, 4075–4080.

Jiang, S., Wan, Q., Krajcikova, D., Tang, J., Tzokov, S.B., Barak, I., *et al.* (2015) Diverse supramolecular structures formed by self-assembling proteins of the *Bacillus subtilis* spore coat. *Molecular Microbiology*, **97**, 347–359.

Kiel, J.A., Boels, J.M., Beldman, G. and Venema, G. (1994) Glycogen in *Bacillus subtilis*: molecular characterization of an operon encoding enzymes involved in glycogen biosynthesis and degradation. *Molecular Microbiology*, **11**, 203–218.

Klobutcher, L.A., Ragkousi, K. and Setlow, P. (2006) The *Bacillus subtilis* spore coat provides "eat resistance" during phagocytic predation by the protozoan *Tetrahymena thermophila*. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 165–170.

Knurr, J., Benedek, O., Heslop, J., Vinson, R.B., Boydston, J.A., McAndrew, J., *et al.* (2003) Peptide ligands that bind selectively to spores of *Bacillus subtilis* and closely related species. *Applied and Environment Microbiology*, **69**, 6841–6847.

Kowarik, M., Young, N.M., Numao, S., Schulz, B.L., Hug, I., Callewaert, N., *et al.* (2006) Definition of the bacterial N-glycosylation site consensus sequence. *The EMBO Journal*, **25**, 1957–1966.

Krajčíková, D., Lukáčová, M., Müllerová, D., Cutting, S.M. and Barák, I. (2009) Searching for protein-protein interactions within the *Bacillus subtilis* spore coat. *Journal of Bacteriology*, **191**, 3212–3219.

Krajčíková, D., Forgáč, V., Szabo, A. and Barák, I. (2017) Exploring the interaction network of the *Bacillus subtilis* outer coat and crust proteins. *Microbiological Research*, **204**, 72–80.

Laaberki, M.-H. and Dworkin, J. (2008) Role of spore coat proteins in the resistance of *Bacillus subtilis* spores to *Caenorhabditis elegans* predation. *Journal of Bacteriology*, **190**, 6197–6203.

Liu, H., Krajcikova, D., Zhang, Z., Wang, H., Barak, I. and Tang, J. (2015) Investigating interactions of the *Bacillus subtilis* spore coat proteins CotY and CotZ using single

molecule force spectroscopy. *Journal of Structural Biology*, **192**, 14–20.
Liu, H., Qiao, H., Krajcikova, D., Zhang, Z., Wang, H., Barak, I., *et al.* (2016a) Physical interaction and assembly of *Bacillus subtilis* spore coat proteins CotE and CotZ studied by atomic force microscopy. *Journal of Structural Biology*;**195**:245–251.
Liu, H., Krajcikova, D., Wang, N., Zhang, Z., Wang, H., Barak, I., *et al.* (2016b) Forces and kinetics of the *Bacillus subtilis* spore coat proteins CotY and CotX binding to CotE inspected by single molecule force spectroscopy. *The Journal of Physical Chemistry B*, **120**, 1041–1047.
Luft, J.H. (1971) Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. *The Anatomical Record*, **171**, 347–368.
Manetsberger, J., Ghosh, A., Hall, E. and Christie, G. (2018) Orthologues of *Bacillus subtilis* spore crust proteins have a structural role in the *Bacillus megaterium* QM B1551 spore exosporium. *Applied and Environmental Microbiology*, **84**, e01734-18.
Mascher, T., Margulis, N.G., Wang, T., Ye, R.W. and Helmann, J.D. (2003) Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon. *Molecular Microbiology*, **50**, 1591–1604.
McKenney, P.T. and Eichenberger, P. (2012) Dynamics of spore coat morphogenesis in *Bacillus subtilis*. *Molecular Microbiology*, **83**, 245–260.
McKenney, P.T., Driks, A., Eskandarian, H.A., Grabowski, P., Guberman, J., Wang, K.H., *et al.* (2010) A distance-weighted interaction map reveals a previously uncharacterized layer of the *Bacillus subtilis* spore coat. *Current Biology*, **20**, 934–938.
McKenney, P.T., Driks, A. and Eichenberger, P. (2013) The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nature Reviews Microbiology*, **11**, 33–44.
Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J. and Setlow, P. (2000) Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and Molecular Biology Reviews*, **64**, 548–572.
Plata, G., Fuhrer, T., Hsiao, T.L., Sauer, U. and Vitkup, D. (2012) Global probabilistic annotation of metabolic networks enables enzyme discovery. *Nature Chemical Biology*, **8**, 848–854.
Plomp, M., Monroe Carroll, A., Setlow, P. and Malkin, A.J (2014) Architecture and assembly of the *Bacillus subtilis* spore coat. *PLoS ONE*, **9**, e108560.
Roels, S. and Losick, R. (1995) Adjacent and divergently oriented operons under the control of the sporulation regulatory protein GerE in *Bacillus subtilis*. *Journal of Bacteriology*, **177**, 6263–6275.
Shuster, B., Khemmani, M., Abe, K., Huang, X., Nakaya, Y., Maryn, N., *et al.* (2019a) Contributions of crust proteins to spore surface properties in *Bacillus subtilis*. *Molecular Microbiology*, **111**, 825–843.
Shuster, B., Khemmani, M., Nakaya, Y., Holland, G., Iwamoto, K., Abe, K., *et al.* (2019b) Expansion of the spore surface polysaccharide layer in *Bacillus subtilis* by deletion of genes encoding glycosyltransferases and glucose modification enzymes. *Journal of Bacteriology*, JB.00321-00319.
Terry, C., Jiang, S., Radford, D.S., Wan, Q., Tzokov, S., Moir, A., *et al.* (2017) Molecular tiling on the surface of a bacterial spore - the exosporium of the *Bacillus anthracis/cereus/thuringiensis* group. *Molecular Microbiology*, **104**, 539–552.
The UniProt Consortium. (2017) UniProt: the universal protein knowledgebase. *Nucleic Acids Research*, **45**, D158–D169.
Ulrich, L.E. and Zhulin, I.B. (2014) SeqDepot: streamlined database of biological sequences and precomputed features. *Bioinformatics*, **30**, 295–297.
Waller, L.N., Fox, N., Fox, K.F., Fox, A. and Price, R.L. (2004) Ruthenium red staining for ultrastructural visualization of a glycoprotein layer surrounding the spore of *Bacillus anthracis* and *Bacillus subtilis*. *Journal of Microbiological Methods*, **58**, 23–30.
Wunschel, D., Fox, K.F., Black, G.E. and Fox, A. (1995) Discrimination among the *B. cereus* Group, in Comparison to *B. subtilis*, by structural carbohydrate profiles and ribosomal RNA spacer region PCR. *Systematic and Applied Microbiology*, **17**, 625–635.
Zhang, J., Fitz-James, P.C. and Aronson, A.I. (1993) Cloning and characterization of a cluster of genes encoding polypeptides present in the insoluble fraction of the spore coat of *Bacillus subtilis*. *Journal of Bacteriology*, **175**, 3757–3766.
Zhang, J., Ichikawa, H., Halberg, R., Kroos, L. and Aronson, A.I. (1994) Regulation of the transcription of a cluster of *Bacillus subtilis* spore coat genes. *Journal of Molecular Biology*, **240**, 405–415.
Zhu, B. and Stülke, J. (2018) SubtiWiki in 2018: from genes and proteins to functional network annotation of the model organism *Bacillus subtilis*. *Nucleic Acids Research*, **46**, D743–D748.

Supporting Information

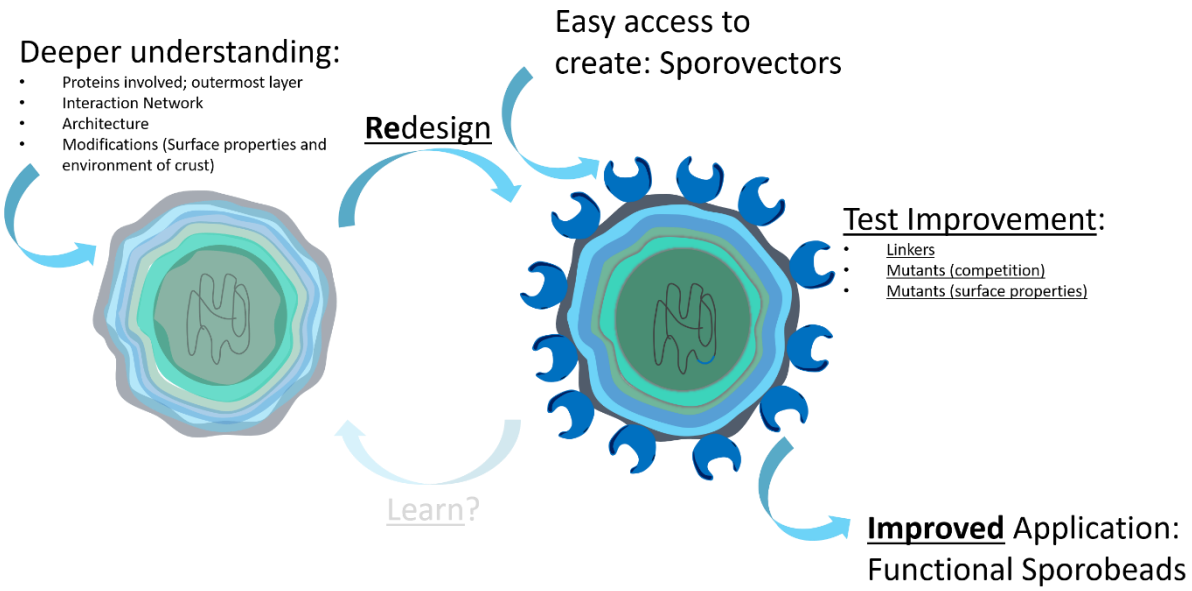
Additional supporting information may be found online in the Supporting Information section at the end of the article

Chapter 4: Strategies for the improvement of the Sporobead display system

This chapter presents the unpublished work (Manuscript I):

Strategies for the improvement of the Sporobead display system. **Bartels J**, Mascher T, unpublished

Background:



The newly gained, deeper understanding of the nature of the crust as well as the previously collected data on the applicability of the respective crust proteins as anchors for protein display create a new foundation for redesigning the application of the Sporobeads. This study aims to improve this system even further by utilizing different strategies including linkers or mutants to either remove native competition or to change the surface properties of the spore. In this process, the previously collected information is of great help in choosing the best candidates for these studies and explaining the outcome. This could then give the basis for the next create-test-learn cycle.

Strategies for the improvement of the Sporobead display system

Bartels J, *Mascher T*

Abstract

Sporobeads are functionalized endospores of *Bacillus subtilis* displaying a protein of choice on the crust utilizing a convenient vector collection (Sporovectors). Even though the applicability of this system was already evinced with GFP and two different laccases, there still is room for improvement. This study makes use of the arising opportunity to test some strategies to potentially improve these points. The resulting data shows that linkers can slightly improve the performance of the already well-performing variants and significantly improve some of the poorly performing variants (like the C-terminal variants). As maturation could play a role in stability and performance, the stability of the Sporobeads in the *cgeA* mutant background (perturbed crust polysaccharides) were tested. There was no difference in stability, maybe due to sugars still being present in the crust, but the change in surface properties led to an elevated activity. Therefore, more research in this regard could be productive for enzymes requiring a more hydrophobic environment. To eliminate potential competition in the structure, *cotZ* mutants were tested, and some showed slight improvements in activity, though the variation was quite high, and reusability suffered to some degree as the crust detaches from the spores. Despite this strategy leading to such poor improvements, it still provides the basis for utilizing the detached crust fragments as a potential and novel application for this system: spore-derived self-assembled non-GMO including particles (SporoSNIPs).

Introduction

The soil bacterium *Bacillus subtilis* has been of interest for synthetic biology and biotechnological applications, as under starvation it can form very long-lasting and resistant biological particles: endospores. Previous studies have already shown the ability to utilize the protective proteinaceous coat of the spore as a platform for the self-immobilization of proteins of interest onto the surface of these particles[44, 47]. The immobilization of proteins has various advantages regarding the stability of the enzyme during storage or reaction, the separation of the end product from the enzyme, the possibility of applications such as a fixed bed or cascade reactions, to name just a few[43]. Self-immobilization has the additional

advantage of being less costly in terms of both time and money. The already published Sporobead system[48] showed that it is also feasible to utilize the outermost layer, the crust[15, 16], as a platform for protein display. Though the system worked well with two laccases and GFP, there were some pitfalls: The C-terminal versions were mostly poor in their performance, the stability of BpuL (*Bacillus pumilus* laccase) dropped significantly after one day, and the total capacity reached by GFP was not matched for the enzymatic activity. Additionally, some future applications such as more bulky or demanding enzymes might fail in this system. Therefore, in order to make this system feasible for real-world applications and possibly fine-tune it in future, the goal of this study is to test different strategies for improvement. Conveniently, the methodology of the Sporovectors facilitates the testing of various possible improvement strategies, showing the advantages of this vector collection.

Methods

Bacterial growth conditions

B. subtilis were routinely grown in lysogeny broth (LB medium) (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) at 37 °C with agitation (220 rpm). All strains used in this study are listed in Supplemental Table S2. Selective media for *B. subtilis* contained chloramphenicol (5 µg ml^{-1}). Solid media additionally contained 1.5% (w/v) agar.

Cloning Procedures

General cloning procedures, such as endonuclease restriction digestion, ligation and PCR, were performed with enzymes and buffers from New England Biolabs (NEB; Ipswich, MA, USA) according to the respective protocols. Plasmid preparation was performed with alkaline lysis plasmid preparation. In order to produce the Sporovectors containing the linkers to N- or C-terminally clone a gene of interest with a linker in between, these linkers were cloned into the Sporovectors adhering to the RFC25, then the RFP cassette was cloned into place to enable red-white screening. For N-terminal variants, the linker was cloned by digesting with XbaI and AgeI and cloned into the respective Sporovector cut with XbaI and NgoMIV. The RFP cassette was cloned into the resulting linker-Sporovector with the restriction sites XbaI and NgoMIV.

For the C-terminal variants the linker was cloned by digesting with NgoMIV and SpeI and cloned into the respective Sporovector cut with AgeI and SpeI. The RFP cassette was cloned into the resulting linker-Sporovector with the restriction sites AgeI and SpeI. The respective gene of interest was cloned according to the description for the Sporovectors[48]. Allelic replacement mutagenesis of the *cotA* gene using long flanking homology (LFH-)PCR was performed as described previously[49]. All plasmids generated during this study are listed in Supplemental Table S1, all primer sequences are given in Supplemental Table S3.

Transformation

Transformations of *B. subtilis* were carried out as described previously[50]. The integration of plasmids into the *B. subtilis* genome was verified on starch plates (*amyE*).

Spore preparation

The *B. subtilis* strains were inoculated in 50 mL Difco Sporulation Medium (DSM, 0.8% w/v Tryptone, 0.1% w/v KCl, 1 mM $MgSO_4$, 10 µM $MnCl_2$, 1 µM $FeSO_4$, 0.5 mM $CaCl_2$) and grown at 37 °C with agitation (220 rpm) for 48 hours to ensure sporulation. These spore cultures were harvested by centrifugation at 10,000 g for 8 minutes, resuspended in 10 mL dH_2O and treated for 1 hour at 37 °C with 75 µg/ml lysozyme to lyse remaining cells. Afterwards, the prepared spores were washed with 10 mL dH_2O, 10 mL 0.05% SDS and then three times with 10 mL dH_2O. The spores were then resuspended in either 2 mL dH_2O or the appropriate buffer (adapted from[50]).

Fluorescence microscopy

The GFP-displaying spores were viewed on an agarose pad (1% agarose in H_2O, cast in Press-to-seal™ silicone isolators with 20 mm diameter and 1 mm depth by life technologies) under the Olympus AX70 microscope utilizing the 100x objective (UPlanFl 100x/1,30 Oil). The spores were documented with phase contrast and with the GFP fluorescence channel utilizing the filter cube U-MNIB (IF blue long pass, Ex. 470-490 nm, Em. 515-∞). The pictures were taken with a XC10 color camera by Olympus with 1000 ms exposure time and saved as tagged image files (tif).

Laccase activity assay with ABTS

The assay measures the activity of laccases by their ability to oxidize the chromogenic substrate ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) (green) to $ABTS^+$ (blue), which leads to a shift of peak absorbance of light from the wavelength of 405 to 420 nm. The reaction is set up in the appropriate buffer (100 mM sodium acetate buffer pH=5.0, 0.4 mM $CuCl_2$, the latter ensuring the integrity of the copper-reactive center), with the final concentration of 5 mM of ABTS (ABTS® BioChemica, Applichem).

For the spores displaying the laccases, a host strain lacking the laccase CotA (TMB2131) was utilized to avoid background activity. This strain was also used as a reference for the laccase background activity. For the reaction, the optical density of the spore suspensions is adjusted to an OD_{600} of approximately 0.5 to ensure similar amounts of spores. The reaction with BpuL was incubated at room temperature, the reaction with EcoL at 50 °C. For determining the enzymatic activities on the spores, the OD_{420} was measured after 20 minutes and one hour discontinuously by centrifugation of the reaction, to measure the supernatant. The OD_{420} of the reference strain was subtracted. For determining the stability of the enzymes, reactions were set up with fresh, one-day old and 4-day old spores performing the same measurement as above.

To measure the reusability of the spores displaying the laccase, the reaction was set up with one-day old spores (to ensure no significant loss of activity) and measured after one hour five subsequent times with the same spores, with washing in between the reactions.

Results

Linkers improve performance, especially of previously poorly performing Sporobead variants

The proteins in the crust are probably quite densely packed. This is at least hinted by the self-assembling structures produced during overexpression in *Escherichia coli* from CotY[10]. Therefore, it might be productive to give the fusion protein integrated into the crust more "wiggle-room." The fusion partner might hinder either the folding, assembly or integration into the crust. This could lead to an ineffective display of the fusion-protein. To this end, the Sporovectors[48] containing CotZ were adapted to contain a collection of linkers which, together

with the cloning scar, result in unstructured linkers of 6, 10, 14, 17 and 19 amino acids, two different 38-amino acid linkers (all from the parts registry[51]) and a long 58-amino acid linker[52] as well as a structured alpha-helix[46]. For CgeA as the poorest performer, only the 58-amino acids linker as well as the structured alpha-helix were implemented.

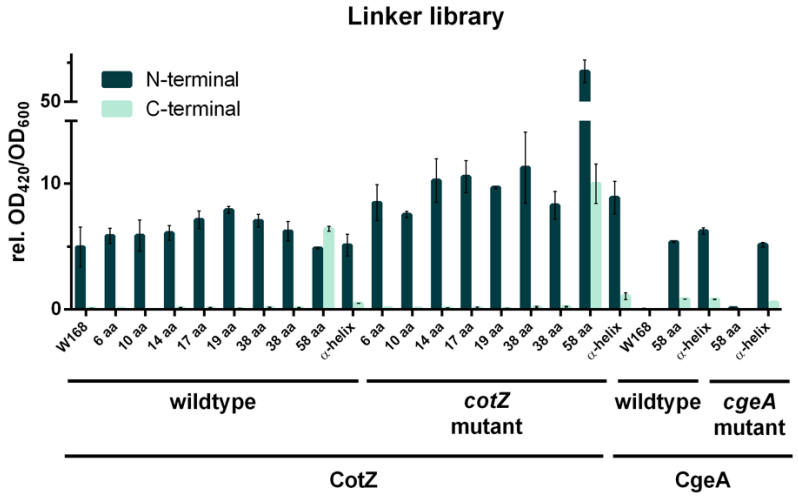


Figure 4: Performance of the Sporobeads with linkers
The activity of the laccase from B. pumilus *(BpuL) fused to the crust anchors CotZ and CgeA over different protein linkers N- or C-terminally (and thereby displayed on the spore surface) was determined by ABTS activity assays. Laccase activity from the reaction measured after 20 minutes is expressed in unit OD_{420}/OD_{600} as an indirect measure for the activity per spore. W68 refers to the version with no linker. No bar indicates no or very little activity (C-terminal and CgeA wildtype). For the linker amino-acid sequence, please refer to Supplemental Table S4.*

When these Sporobeads were tested (see Figure 4), the variants already performing reasonably before (N-terminal fusion to CotZ) only slightly improved due to some of the linkers. An exception to this is the 58-amino acid linker with CotZ as an anchor in the *cotZ* mutant, with an over 10-fold increase in activity. Maybe this long flexible linker gives CotZ the freedom it needs to completely replace the missing native CotZ proteins in the structure, which would be the desirable explanation. Another explanation could be that the linker is long enough for the BpuL laccase, which is closely related to CotA (a laccase found in the coat of the *Bacillus subtilis* spores and missing in all the strains), to integrate into the coat and replace the CotA missing there, whilst the CotZ remains in the crust. The performance in the natural environment of the coat might be higher than in the unnatural context of the crust, potentially explaining this improved activity.

Some variants that performed poorly before, exhibiting virtually no or only very little activity, like some of the C-terminal versions or the ones utilizing CgeA as an anchor, were improved

quite significantly to the point that they matched the best versions (N-terminal to CotZ): The 58-amino acid linker significantly improved the C-terminal fusion to CotZ, and both the 58-amino acid variant as well as the alpha-helical linker improved the variants with CgeA as an anchor. Interestingly, the 58-amino acid variant did not work as well in a c*geA* mutant though. This suggests that this very long linker might destabilize the integration of the fusion protein to CgeA when the native CgeA is missing.

Even though it is not possible to create a rule of thumb for which protein of interest to use with which anchor and in which mutant background, it is possible to improve poorly performing variants with this strategy. Due to the possibility of easily creating and testing a vast number of these Sporobeads through the Sporovectors, this is a feasible way to improve functional systems that might be performing subpar.

Stability does not improve if the crust polysaccharide is perturbed, but activity may

One of the two previously tested laccases (BpuL) lost approximately 80 percent of its original activity in one day[48]. This could potentially be due to the fact that the spore is still in the process of maturation and that the crust in its mature form presents a less optimal environment for this laccase normally found in the coat of *Bacillus pumilus*[53], which is more hydrophobic than the crust. The only maturation step influencing this property is the presumed glycosylation of the crust[17, 36-39]. One of the mutants with a perturbed crust polysaccharide layer is the *cgeA* mutant[54, 55]. Therefore, the performance and stability of the respective Sporobeads were tested in this mutant background (see Figure 5). Indeed, the performance was enhanced, which might be due to the more hydrophobic nature of the crust in this mutant. But the stability was not improved. Due to the high redundancies of the crust glycosylation[55], the crust still contains sugars, even though the polysaccharide structure is impaired. This partial maturation of the crust with sugars might still lead to a hydrophilic micro-environment. Potentially enough to perturb the activity of the laccase, even though the spores are still more hydrophobic overall.

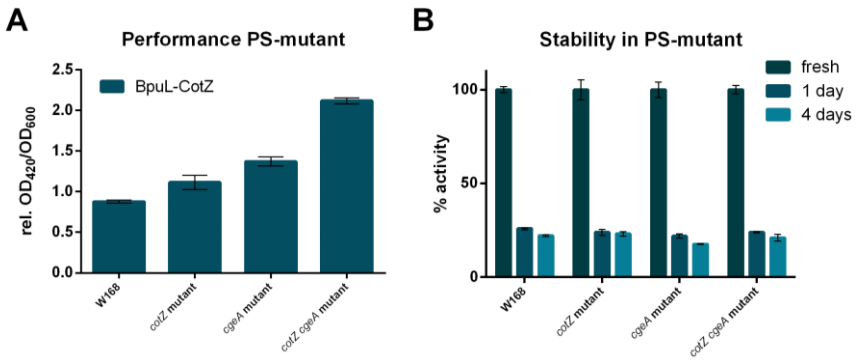


Figure 5: Performance and stability of the crust polysaccharide mutant CgeA
The activity of the laccase from B. pumilus *(BpuL) fused to the crust anchor CotZ (and thereby displayed on the spore surface) was determined by ABTS activity assays.* ***A****: Laccase activity from the reaction measured after 20 minutes is expressed in unit* OD_{420}/OD_{600} *as an indirect measure for the activity per spore.* ***B****: Stability of the laccase: the activity (assayed after 1 hour of reaction time) of the spore-displayed enzyme (relative activity in %) from freshly prepared spores and after one day and four days of storage at room temperature in the appropriate buffer.*

Eliminating native competition does not necessarily increase the performance (much)

In order to increase the capacity of the spore for the integration of the ectopic fusion protein, it might be promising to eliminate the native competition. To evaluate this strategy, *cotZ* mutant spores were tested for their performance, stability (not shown) and their reusability (Figure 6). Some of the variants are slightly increased in their activity (BpuL-CgeA/CotV/CotW/CotX/CotZ) but have a higher variance, except the variant which directly competes with the native CotZ (BpuL-CotZ). Some are similar in their performance or even slightly decreased (EcoL-CotX, EcoL-CotY). The negative effect of the mutants might be due to the change in the surface properties of the spore to a more hydrophobic state, or due to negative dependencies of the proteins in the crust. Nevertheless, the reusability is slightly less efficient. This is probably due to the fact that the crust detaches[15, 16, 55] in this mutant, and even though most fragments are still attached to the poles of the spore[55], some are detached completely (see Figure 7) and therefore successively lost during the recycling steps. The only exception: CotW, which remains on the spore's surface, explaining the smaller variance. Nevertheless, the clumping of the spores, and therefore the differential sedimentation and pellet integrity, seems to influence the first step of reusability. Surprisingly, the fact that the crust (except for CotW) is detached in the *cotZ* mutant does not diminish the stability of the enzyme during storage though (data not shown).

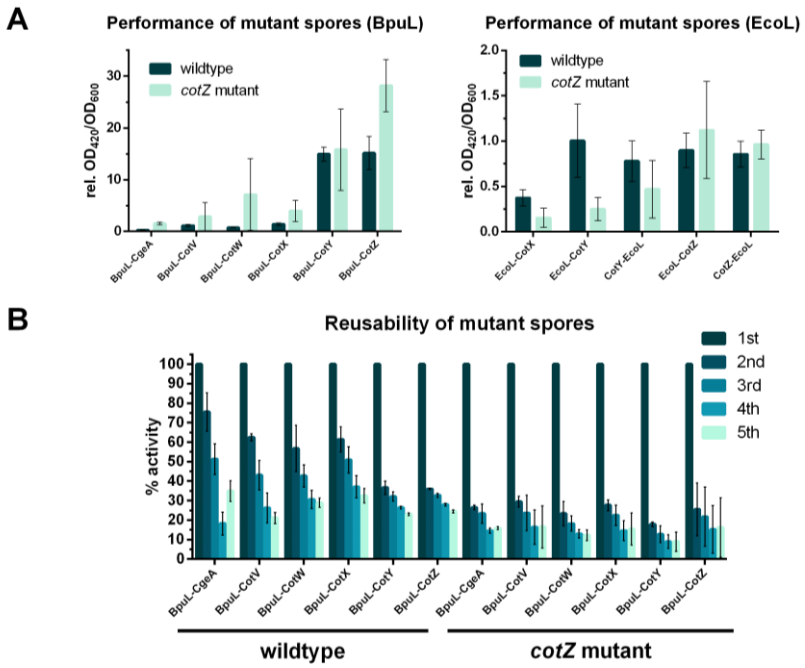


Figure 6: Performance of the cotZ *mutant spores and the reusability*

The activity of the two different laccases from E. coli *(EcoL) and* B. pumilus *(BpuL) fused to the crust anchor CotZ was determined by ABTS activity assays.* ***A****: Laccase activity from the reaction measured after 1 hour is expressed in unit* OD_{420}/OD_{600} *as an indirect measure for the activity per spore.* ***B****: Remaining activity after recycling and washing the spores after each reaction. The relative activity (in %) after one hour is depicted after each successive reaction over four recycling steps.*

The crust fragments could potentially be utilized as a novel application: SporoSNIPs

The fact that the crust fragments seem to be lost during the recycling process (see Figure 6B), leads to the preposition that some fragments are completely detached from the spore. These could potentially be separated and concentrated to give rise to functional spore-derived self-assembled non-GMO including particles (SporoSNIPs). To find out if this might be possible, the Sporobead probe of the *cotZ* mutant displaying GFP-CotY was centrifuged and subsequently visualized under the microscope to see if there are detached fragments and if these could potentially be enriched (Figure 7). This process would need to be optimized and there would have to be an established quality control system in place to ascertain that these concentrated fragments are actually GMO-free (as some spores were still visible after centrifugation, data not shown) prior to possible medical application or release into the environment. However, this could be a promising strategy to create cheap, convenient yet GMO-free, enzymatically functionalized biological particles (SporeSNIPs) without the laborious and expensive protein purification and immobilization procedures[43]. As the mutant spores showed no diminished stability in storage (data not shown), these fragments most likely still stabilize the respective integrated enzyme.

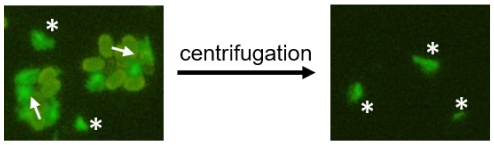


Figure 7: Separation of the detached crust fragments visualized with GFP-CotY
The detachment of the spore crust fragments is shown with a GFP-CotY translational fusion. Many crust fragments are still attached to the poles of the spore (white arrows), but some fragments seem to have detached completely from the spore (white asterisks) and can be enriched by a simple centrifugation step, shown on the right.

Discussion

This study shows that there still is room for improvement in the Sporobead display system[48]. The linkers were very promising at vastly improving poorly performing variants like the C-terminal variants or CgeA as the poorest performer. This strategy was already shown to be productive with CgeA and the alpha-helical linker[46]. Two mutants were also evaluated with different ends in mind: The *cotZ* mutant should eliminate potential native competition in the crust while the *cgeA* mutant should perturb the maturation step of glycosylation. This maturation step could be the potential reason for the considerable loss of activity after one day of storage encountered for the BpuL laccase[48]. The glycosylation mutant does not improve storage stability, but this might be due to the fact that sugars are still added to the spore surface during maturation, even though the polysaccharide structure itself is perturbed in such a way that the spores themselves are more hydrophobic and tend to clump together[54, 55]. The added sugars might still influence the micro-environment in such a way that the performance of the laccase (which is normally active in the more hydrophobic context of the spore coat[53]) drops during maturation. Nonetheless, this mutant has a slightly higher level of activity, indicating that the change in the surface properties might benefit the performance of some enzymes, including BpuL. The elimination of native competition for the *cotZ* mutant did not produce such promising results: Even though some variants were slightly improved, the variance was mostly considerably higher and the reusability was not as efficient. The stability, was not impaired, however, even though the crust detaches in this mutant[15, 16, 55]. The fact that the crust detaches might give rise to a new possibility for this application: It was shown that these fragments could be separated and enriched to acquire cheap, functional biological particles (SporoSNIPs). This system does not require expensive and laborious protein purification and successive immobilization steps[43]. Additionally, the crust fragments still seem

to stabilize the integrated enzyme during storage. If these fragments could be effectively separated from the spores, this system could potentially be GMO-free.

This study not only provides a novel possible application of the Sporobead system (SporoSNIPs), but also takes the first steps towards further optimizing the Sporobead system using various strategies, such as linkers and mutants. In future, further mutants and linkers or a combination of the two should be tested and evaluated for the rest of the crust anchors in order to get a complete picture of the true optimization potential.

Chapter 5: Discussion

The crust of *Bacillus subtilis* was the main focus of this thesis: On the one hand, the aim was to fundamentally understand the nature of this glycoprotein layer, and on the other, it was utilized as a platform for protein display. These two parts of the thesis benefit from each other: The fundamental study was facilitated a great deal by the possibility to easily manipulate the spore crust by ectopically introducing fusion proteins with the Sporovectors from the application of the Sporobeads. In return, the findings regarding the nature of the crust help explain shortcomings and give insights into potentially improving the display of the proteins.

The clear intersection of these two projects is how the knowledge of the nature of the crust helps explain the performance of the different crust proteins as an anchor. Accordingly, it also lays the groundwork for designing and explaining different improvement strategies, which in part were already tested in Chapter 4 (Manuscript I). The overarching discussion will therefore take the interaction network as well as the architecture and the roles of the crust proteins as the basis to explain the performance of the anchors and the preliminary improvement strategies. It will also use this knowledge to predict the performance of future improvement strategies as well as shed light on special cases in which the inferior anchors might still be of interest.

The respective improvement strategies, of which some have already been tested in Chapter 4 (Manuscript I), are shown in Figure 8. These strategies are aimed at improving either

(I) the **integration** into the crust structure with **linkers**, as the fusion protein might hinder the integration of the crust protein if it is too stringently linked (especially on the C-terminus, as this seems to be an important attachment/interaction point for most of the crust proteins),

(II) the **activity** of the enzyme integrated by adjusting the **surface properties** of the spore, therefore potentially improving or enabling the activity of enzymes requiring a more hydrophobic environment,

(III) or the **capacity** of the fusion proteins on the spore by making more room with crust mutants and therefore eliminating the **native competition**.

The discussion will first explain the performance of the six anchors on the basis of the knowledge about the crust and then illustrate these three improvement strategies in detail.

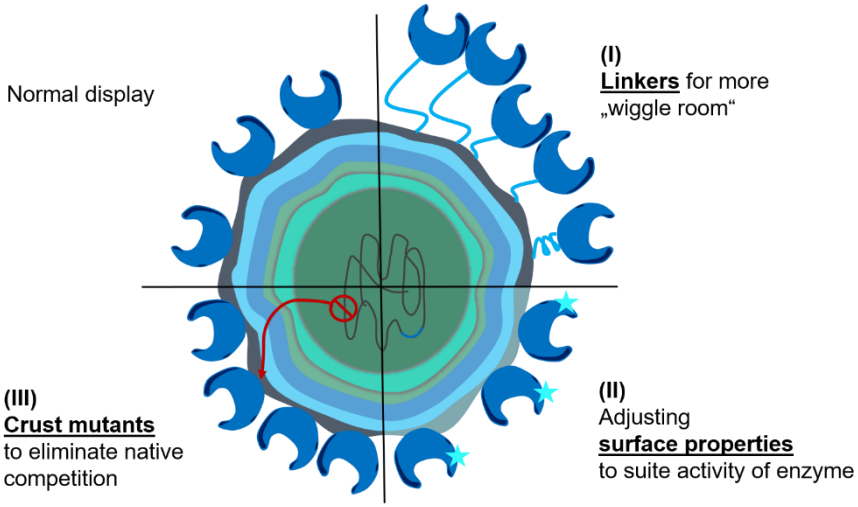


Figure 8: Possible strategies for the improvement of the Sporobead system
The possible strategies for improving the spore display involve (I) linkers, which might give the protein fusion more "wiggle room" and therefore provide benefits during the folding of the crust protein or the integration of the fusion protein into the crust; (II) adjusting surface properties to be more hydrophobic and therefore improving or enabling potential enzymatic reactions; (III) eliminating native competition by utilizing crust mutants as background strains. The rationale: The less native protein is incorporated into the crust, the more of the ectopic fusion protein might be integrated into the crust structure.

5.1 The abundance and potential role of the crust proteins infer how promising these are as anchors

Publication II (Chapter 3) showed[55] that the crust proteins can be divided into different classes: structural proteins (CotY and CotZ), supportive structural proteins (CotX and CotV), supportive proteins (CotW) and a probable glycosylation hub (CgeA). These roles. as well as the respective abundance and location of the proteins, already creates a good foundation for explaining their performance as an anchor for the Sporobeads: CotY and CotZ being the most promising, followed by CotX, then CotV and CotW and the least promising, CgeA[48]. This section will take a detailed look at this interrelation.

CotY and CotZ are the main structural components of the crust[55]. As such, they are the most abundant proteins. They are also homologues to some extent, in sequence and in function, as CotY can seemingly replace CotZ in the structure. The high abundance as well as the partial redundancy make them very interesting targets for the Sporobead application, as the performance will be high and any hindrance in the structure or function might be tolerated due to the homologous counterpart. These two proteins are not

identical, as CotZ is vital in anchoring the crust structure to the middle part of the spore, a function which cannot be assumed by CotY[55]. If the anchoring function is impaired by the fusion partner, some problems in the integration of the fusion protein or the structural integrity of the crust might arise when utilizing CotZ as an anchor. Therefore, CotY seems to be the best anchor in terms of abundance and function.

The supporting structural proteins CotX and CotV seem to be less abundant than CotY and CotZ. In addition, they are not distributed evenly over the spore, but rather preferentially found at the poles of the spore. This fact makes them inferior to CotY and CotZ. As they are still quite abundant, however, they should not be completely omitted from future Sporobead applications, as some fusion partners might be more suitable for these anchors. Nevertheless, some points have to be kept in mind when utilizing these two proteins as anchors. Firstly, as these two proteins contain conserved glycosylation motifs at a conserved position in the CotX superfamily domain, they might be targets for glycosylation[55]. If the fusion partner hinders or is intolerant to this modification, the performance might suffer. Secondly, these same CotX superfamily domains contain quite hydrophobic stretches, which might also represent a problematic micro-environment for some fusion proteins for folding or activity. But as the glycosylation at the proposed glycosylation motifs might counteract this hydrophobic property, these pitfalls might have less impact on the fusion partner than stated. These points could still be disadvantageous for some applications, but on the other hand, might also be advantageous for some fringe cases. The stated special role of these two proteins should also be kept in mind for cases in which the surface property of the spore is changed deliberately (by creating glycosylation mutants): These protein anchors might act completely differently as anchors without the proposed modification. Even though these proteins seem the most likely candidates for glycosylation, especially CotX[55], it is still unclear if any of the other crust proteins might not also be targets of glycosylation: Most of them contain more or less stringent glycosylation motifs or the necessary amino acid (N) for glycosylation[55]. Shuster et al. 2019[56] accordingly proposed CgeA as a target for glycosylation. Therefore, these considerations might also apply to the other anchors.

CotW is not very abundant, seems to fold as an alpha-helix, and supports the CotV and CotX structure or integration into the crust[55]. in addition to its low abundance, its supportive

function could make its utilization as an anchor problematic. The fusion partner might impair its supportive role. But as the low abundance already makes this anchor a less favorable candidate, this consideration might not be worth noting. The only specialty of this protein making it worthwhile to carry out further investigation is that it is uninfluenced in a *cotZ* mutant (and generally in all other crust (detachment) mutants), remaining on the spore[55]. Some applications might benefit from this characteristic. These applications could include those requiring the clumping phenotype of the *cotZ* mutant, yet still requiring the activity on the spore itself. This could include pickering solutions[57], where the clumping phenotype facilitates the interphase accumulation and stabilization. In such a case, the crust fragments might not be at the interphase, were the activity is desired. As such, CotW might be the anchor of choice. Additionally, as CotW remains on the spore in the *cotZ* mutant whereas the entire rest of the crust structure detaches, testing the performance in this mutant might be promising: The fusion protein might have more space to assemble onto the spore or be more accessible than when the spore crust is intact. In Chapter 4 (Manuscript I), this is the case, even though there is a very high variance. This might indicate that the incorporation of CotW in the *cotZ* mutant is random or affected by untested parameters (such as impurities, growth conditions, shaking rates). Nevertheless, as the performance seems to be elevated in the *cotZ* mutant, this might also be true for all other crust mutants where the crust structure is perturbed. That makes this anchor the most promising for the strategy (III), as will be discussed in that respective section in more detail.

The least promising crust protein (CgeA) is probably a coordinator of glycosylation. This is most likely the reason for its very low abundance and why it also seems to be lost over time (or otherwise modified)[55]. Like CotV and CotX, it is also preferentially located at the poles of the spore, which might support the theory that CotX and CotV are the targets of glycosylation. The fact that CgeA is not highly abundant, might be lost/modified over time and might play a (vital) role in spore maturation makes it a very interesting protein for further research, but unappealing as an anchor in the Sporobead application. However, as it does not seem to play a structural role, some applications might still be suited to this anchor, which might otherwise incur too much structural strain on the other anchors.

Uniquely, the C-terminal fusion is accumulated in the crust fragments in all mutants lacking CotZ ([15] and data not shown). The N-terminal version, on the other hand, seems to remain on the spore[55]. This might be explained by the fact that CgeA interacts with almost all crust proteins[19]: This interaction might be driven by the free N-terminus, whereas the (blocked) C-terminus is required for recruitment to the spore surface. Accordingly, if the C-terminus is blocked, CgeA is targeted to the crust fragments instead of remaining on the spore. The quantity seen for the accumulated CgeA in the crust fragments (data not shown) exceeds the quite low abundance of CgeA on the mature spore. Therefore, the motif responsible for this accumulation could be quite potent for recruiting fusion proteins to the spore fragments (such as in the application as SporoSNIPs, see Chapter 4: Manuscript I).

Taken together, the abundance, the function or structural role, the position of the crust protein on the spore, the possible modifications and the interdependencies of the different crust proteins already provide some rules of thumb as to which anchors are the most promising candidates in the Sporobead application. Additionally, it might give a rationale in which cases some of the less promising anchors might still be relevant:

CotY is the most promising.

CotZ is almost as promising as CotY. Some cases might lead to subpar performance: if the fusion partner impairs the crust anchoring function of CotZ, and the detachment of the crust is of hindrance to the application, i.e. fixed bed applications (where the crust fragments might be lost over time to the continuous flow).

CotV and CotX are less abundant and not evenly distributed over the spore, already making them inferior as anchors. As these proteins might be targets of glycosylation, there still might be some fringe cases in which these anchors perform better: Some applications might benefit from the micro-environment created by the glycosylation modification. For cases in which the spore surface properties are changed on purpose (with glycosylation mutants), these anchors might react differentially than the other anchors (which might or might not be affected by the glycosylation). In these mutants, they could still be superior to the other anchors.

CotW is less abundant than all of the above-named anchors combined. It is therefore a less favorable option. But it is the only protein that is independent from all other crust proteins. In

conclusion, testing this anchor against a crust mutant background might yield Sporobeads that are superior to the other anchors.

CgeA is a very interesting crust protein, as it might coordinate the glycosylation of the crust[55], yet as an anchor, it is least favorable due to the small amount found on the spore, as well as due to the fact that it seems to be lost/modified over time. In fringe cases where the anchor's structural fold or integration into the crust structure cannot be acquired due to the bulky or otherwise disturbing characteristic of the fusion partner, this anchor might still be of interest, as it does not seem to play any structural role. Moreover, the N-terminus of CgeA might contain a potent motif for the recruitment of fusion proteins to the crust fragments. This unknown motif could be of further interest in the SporoSNIPs application (see Chapter 4: Manuscript I).

5.2 (I) Linkers improve some variants, as they might eliminate hindrance in protein interaction and structure

Many studies have shown, that linkers can improve the expression, folding or function of fusion proteins (reviewed in [58]). In the case of spore display, there is even a direct example of an alpha-helical linker enabling the display of a fusion protein to CgeA[46]. On this basis, one of the best performing anchors (CotZ) as well as the worst anchor (CgeA) were tested with added linkers, including the above-mentioned alpha-helical linker, and is discussed in Chapter 4: Manuscript I.

The data (Chapter 4: Manuscript I) shows that in subpar performing cases, linkers are indeed promising. For all variants which already performed effectively (N-terminally to CotZ), there is only a mild improvement with a peak at around 19 amino acids. This peak might change with the nature of the linkers, as most of the linkers tested were unstructured GS linkers. The effect of the linkers is remarkable for variants not performing adequately (like the CgeA anchor or the C-terminal variants): CgeA performs subpar, as it is not very abundant on the spore and might be lost/modified over time, while the C-terminal variants perform subpar because the C-terminus proved to be vital in protein interaction and function for most of the crust proteins (except CotY and CotZ, where the C-terminus was important, but not vital)[55]. Seeing as

inadequate performance is triggered by different factors, the reason the linkers improve the effectiveness might be explained by varying mechanisms as well.

For CgeA, the linkers might lead to a more effective incorporation of the fusion protein, which might exploit the already quite low capacity to the fullest. It might also stabilize the protein or hinder the mechanism in which CgeA is lost or modified over time, so that the fusion protein accumulates on the spore over time instead of being lost/modified and therefore inactivated.

For the C-terminal variants, the linkers might give the C-terminus enough freedom to still fulfill its vital/important role in the crust structure or protein interaction network. This seems to be in line with the fact that only the very long linker (58 amino acids) and the structured alpha-linker work for these variants. The very long linker could give the C-terminus enough freedom to fold correctly, or it might enable the interaction partner to access the otherwise blocked C-terminus of the crust protein. The alpha-linker might facilitate the folding of the C-terminus in the fusion protein, as it might not be as destabilizing as an unstructured linker or the fusion partner. Additionally, it might also improve accessibility: A rigid linker, if folded in the correct orientation, might clear the way to the interaction point. The unstructured longer linker or the fusion partner might, on the other hand, wiggle around and block the access point in most of the orientations.

Future improvement of the system with linkers should be possible for the other anchors. Due to the fact that the worst anchor, CgeA, was improved by both tested linkers to the point that its performance was comparable to the best anchors CotZ and CotY, this should be all the more true for the other anchors, which have a higher abundance than CgeA. This alone shows how promising linkers are, especially for those anchors not performing adequately.

Taken together, linkers seem to be more of a strategy for rescuing low performers rather than vastly improving anchors that already show high performance. If the anchor of choice is already performing well, utilizing linkers might be less promising than one of the other improvement strategies. But for cases in which an inadequately working anchor or fusion site is necessary for the application, due to reasons discussed above, this strategy might make the utilization of this particular anchor or fusion site feasible.

5.3 (II) Adjusting surface properties might be vital for some applications, but the vast number of genes involved and the redundancies might pose a challenge

Some real-world applications, such as lipases[59], might require anchoring special enzymes to the hydrophilic surface of the spore, and these enzymes can be more active or accustomed to a hydrophobic environment. They might function in a subpar manner or not at all due to the hydrophilic micro-environment, even though the appropriate hydrophobic buffer is utilized for the reaction. This might change if the surface properties of the spore are adjusted to better accommodate the needs of these enzymes.

Some of the crust proteins are, without further modifications, quite hydrophobic, like CotX and CotV, which have elaborate hydrophobic stretches in the CotX superfamily domain[20, 55]. The conserved N-glycosylation motifs are also found in these hydrophobic stretches, indicating that these might be glycosylated. This glycosylation could therefore counteract the hydrophobic property of these stretches and lead to the overall hydrophilic nature of the crust. If the crust itself is disturbed, the spores already act more hydrophobic, clumping extensively[15, 16, 55]. When any of the known glycosylation genes are affected, this phenotype is even more pronounced. Not only do the spores clump together, but they also adhere to some plastic surfaces[54, 55]. When comparing these two phenotypes, it is unclear if crust mutants already give rise to a micro-environment that would suit these enzymes. This phenotype is most likely due to the partial or complete exposure of the more hydrophobic coat. On the other hand, the micro-environment of the crust parts or fragments on or attached to the spore (where the enzyme of choice is located) are presumably as hydrophilic as before, still probably glycosylated. This is shown by the data: Even though these mutants act more hydrophobic (clump together), they still produce a mucus-like shine in sporulated colonies, which is lost in those mutants involving glycosylation[55]. Consequently, this strategy requires an understanding of the players involved in glycosylation as well as the respective redundancies in order to produce the adequate mutants for a glycosylation-devoid micro-environment. First insights on the mode of glycosylation and the players involved is elucidated in Chapter 3: Publication II[55]. This study proposes two polysaccharide variants that are cross-linked. These two variants contain at least six different sugar species, of which some could even be rare or unique to *B. subtilis*. Even though the attachment sites or pathways might be

distinct, the involved players are highly redundant in producing the same sugar species. Due to this high level of redundancy and the many players, it might be more challenging to achieve a glycosylation-devoid strain than to simply create a single mutant.

Nonetheless, first insights into the effects accomplished by changing the surface property were shown in Chapter 4 (Manuscript I). For this purpose, the *cgeA* mutant was utilized. The respective mutant spores presumably produce a highly disturbed polysaccharide structure, as CgeA is the proposed glycosylation hub[55]. The activity of the laccase from *B. pumilus* (BpuL) was improved in this mutant background. BpuL is not per se an enzyme, which strictly requires a very hydrophobic environment. Nevertheless, it normally acts in the more hydrophobic context of the coat and already showed improved activity in this presumed glycosylation mutant *cgeA*. Even though the improvement is not as marked (probably due to the fact that a hydrophobic micro-environment is not vital to this enzyme), it does show that this strategy might prove promising for enzymes that would otherwise be intolerant to the micro-environment of glycosylation. Moreover, this strategy might also provide spores that are applicable for some special processes, such as interphase reactions between hydrophilic and hydrophobic solvents or even pickering emulsions (droplets of hydrophobic/hydrophilic solvents in hydrophilic/hydrophobic solvents, respectively). In this application, the stabilizing particles need to be more hydrophobic than particles that readily disperse in water, such as the wildtype spore. This already proved feasible with the more hydrophobic crust mutant *cotZ*[57], but might be even more promising with a complete glycosylation mutant.

This knowledge might also be of value for applications related to spore display and Sporobeads: spore absorption[37, 60]. This is the phenomenon where proteins expressed in the mother cell during sporulation are absorbed into the spore envelope without a specific anchor, merely due to protein interactions. That these interactions are mostly hydrophobic is elucidated by to the fact that this strategy is vastly improved in mutants, both of the crust[60] and the presumed glycosylation[37]. Accordingly, this information might even help improve this alternative application to some extent.

For this purpose, it could be interesting to produce mutants that are defective in the polysaccharide structure to desired points of hydrophobicity or even completely missing the glycosylation. This spectrum might give each specific application the desired surface property.

Regardless, as the glycosylation of the crust involves many players, high levels of redundancy and probably a high level of structural complexity[55], it might prove challenging to produce a mutant completely lacking this modification, or better yet, even fine-tuning the surface properties through this strategy. Most of the mutants involving glycosylation still have sugars added to the crust, but the structure of the polysaccharide is impaired[55]. Some glycosylation mutants even produce contrary phenotypes of an extended polysaccharide layer and an impaired sedimentation[55, 56]. This only shows how complex the mechanism of glycosylation is. Accordingly, in order to achieve a glycosylation-devoid mutant or fine-tune the surface properties, the nature and mechanism of this modification has to be understood completely. At the same time, already possessing knowledge of the proteins involved as well as proposed redundancies and modes of action of the players involved might provide enough information to attempt improving the micro-environment for some applications. This might prove challenging and require a great amount of trial and error due to the vast amount of proteins involved, but could be promising and, thanks to the easy and convenient Sporovector collection, a feasible alternative. As always in synthetic biology, these attempts could then provide more information on the nature of this modification, which in turn might help improve this strategy even further.

5.4 (III) The protein interaction network is so strongly interleaved that mutants seem less promising unless detached crust fragments are produced (SporoSNIPs)

The data on the protein interaction network[55] illustrates that the proteins in the crust have strong interdependencies between each other: CotY and CotZ represent one pillar of the structure and CotV and CotX another, supported by CotW. These two pillars are, to some extent, co-dependent and presumably linked over CotX. CotY and CotZ provide the main structure, whereas CotX and CotV probably play supportive roles in the propagation from the poles. CotZ is not only a highly abundant structural protein, but is also responsible for linking the crust tightly to the middle part of the spore. In the respective mutant, the spore crust is therefore detached from the majority of the body but remains loosely attached at the poles of the spore. Surprisingly, despite the high abundance of CotZ in the structure of the crust, at least major parts can be constructed without it being in place. This is probably due to the structural replacement in parts by the homologous protein CotY.

Due to the fact that the crust protein interdependencies and redundancies are so high[55], most mutants will have negative effects on the respective anchors or be compensated by the homologous protein rather than give space for the fusion protein. Nevertheless, there are still possibilities to improve the display. These possibilities will be discussed in the following and include mutants where the anchor is not dependent on the missing protein(s). It will also include mutants where the anchor is dependent on the missing protein(s), but the respective anchor self-assembles to crust fragments in the mother cell (such as SporoSNIPs introduced in Chapter 4 (Manuscript I) for the *cotZ* mutant). Aside from these mutants, all other possible mutants can already be omitted from further research.

Indicated by the preliminary data in Chapter 4 (Manuscript I), this strategy might not be as promising as hoped, leading only in some cases to marginal improvements (1.5-fold at best). Additionally, it might come with some pitfalls, such as poorer recycling capabilities of the spores. This is the case for the mutant where the direct native competitor is eliminated (EcoL/BpuL-CotZ in a *cotZ* mutant). This variant should theoretically lead to spores, where the ectopic fusion protein occupies its complete native capacity. But even in this theoretically optimal case, the performance was only marginally increased (for BpuL-CotZ) or not affected at all (for EcoL-CotZ). This can be explained by the aforementioned redundancy of the crust proteins. The native space won by eliminating CotZ was presumably replaced with the native, structurally redundant CotY instead of the bulkier fusion protein. As these two homologous proteins constitute the most important structural pillar of the crust, yet seem to be able to replace each other, any other variant might fall victim to the same effect. This would include CotY in a *cotY* mutant or those variants where the redundant competition is eliminated (CotY in a *cotZ* mutant or CotZ in a *cotY* mutant).

When regarding the second structural pillar of the crust, it must be noted that these two proteins, CotX and CotV, also share some homology (homologous CotX superfamily domain)[55]. Therefore, this partial redundancy might also lead to these two proteins replacing each other rather than giving more space for the fusion protein. But on top of this consideration and unlike CotZ and CotY, these two proteins are far more reliant on each other: If CotV is missing, CotX is perturbed, and if CotX is missing, CotV seems to self-assemble as fragments, but not on the spore. On top of that, these self-assembled fragments also emerge for the fusion

protein with CotX in a *cotX* mutant, indicating that this fusion protein might not be able to entirely compensate its own function. This might make mutants (direct native competition or redundancy competition) in this structural pillar even less promising. As already discussed above, the variant which might be of further interest is *cotX* and related mutants, because for these mutants the fusion protein self-assembles into crust fragments detached from the spore (similar to SporoSNIPs, discussed in Chapter 4 (Manuscript I)). These could theoretically include more protein than when assembled onto the spore, as they form in the mother cell. The mother cell gives these emerging fragments more space to assemble than the two-dimensional spore surface. Therefore, the size of the mother cell and the expression rate of the fusion protein are the limiting factors for the potentially elevated performance in these mutants. The expression is already higher for the fusion protein than for native crust protein, as the strongest crust gene promoter P_{cotYZ} was utilized for the Sporobead application[48]. The expression could additionally be further optimized, as was shown in the literature with the help of inducible promoters[61]. For this to be a feasible approach, it must be possible to co-purify these fragments efficiently together with the spores, which was not always the case (data not shown). The best-case scenario might even constitute a method to separate these fragments from the spores altogether; but only if the storage stability does not suffer. Alternatively, the separation could be directly prior to the possible application.

Similarly, as already discussed in Chapter 4 (Manuscript I), SporoSNIPs are produced in the *cotZ* mutant for all anchors (except CotW): The complete crust is displaced from the spore but remains mostly attached to the poles[55]. As these fragments seem to be bigger in size and are still loosely attached to the spore, it is far more feasible to reliably co-purify them with the spores (see Chapter 4, Manuscript I). The disadvantage of these SporoSNIPs over the fragments found in *cotX*-related mutants with CotV and CotX (discussed in the prior paragraph) is the more challenging separation of these fragments from the spores, as most of the fragments are still attached to the spore pole[55] (see Chapter 4, Manuscript I). But this might be a question of methodology, as it is possible to enrich some of the completely detached fragments (shown in Chapter 4, Manuscript I). In addition to the desired enrichment method, another method for deliberately detaching these fragments at a chosen point in time (after storage, for example) would make the utilization of these crust fragments an even more promising approach. To accomplish this, more research is required regarding the nature of the

attachment of the crust to the poles: Is this mere protein interaction, or are the crust fragments attached over protein cross-linking or another covalent modification, for example over a polysaccharide? The involvement of glycosylation could be a possibility, as some glycosyltransferase mutants show an outer coat defect. This indicates that glycosylation might even play a role in the outer coat or the interface between outer coat and crust[55]. If the nature of this attachment is known, then it must be feasible to detach these fragments completely. This would make it possible to completely separate these crust fragments from the spores, which would in turn lead to the production of adequate quantities for a real-world application.

It might even be possible to expand this strategy from the mere native or homologous competition within the same structural pillar to the broader competition of all crust proteins. These two structural pillars might compete for the same assembly space or attachment points on the spore surface. For this approach, the interdependencies of these two structural pillars must also be accounted for: CotY is slightly negatively influenced when CotV is missing, and more severe when CotX is missing (though still present on the spore, to some extent). CotV and CotX, on the other hand, are highly perturbed (again self-assembling as fragments detached from the spore) when CotY and CotZ are missing, making CotX the probable link between these two pillars. As the two structural pillars are interdependent, the only mutants worth being investigated in further research are those in which detached crust fragments are produced. Those would be, as already mentioned, CotV and CotX in *cotYZ*-related mutants.

CotW as an anchor plays an exemptive role in this strategy, as natively it does not partake in the crust structure itself but rather plays a supportive role for the structural pillar of CotV and CotX at the intersection of outer coat and crust[55]. That suggests that this protein might be located underneath the crust and not in the crust itself, even though it is required for the crust structure to properly emerge. As CotW is proposed to be at the interface of outer coat and crust, the most promising mutants are those where the crust is disrupted or removed entirely. This could make CotW more accessible in its proposed location or lead to more time and space for CotW to assemble onto the spore surface, as the crust structure or parts of the crust structure are missing and therefore unable to compete. To this end, the most promising mutants to test with CotW should be any lacking CotZ/CotY as the major structural proteins.

Additionally, but probably to a lesser extent, mutants missing the second supportive structural pillar (CotV/CotX) might also lead to improvements.

It is tempting to think of the spore surface as a platform for self-assembly, where every other protein missing gives rise to more space for the other proteins to assemble. But the crust structure is highly interleaved, which in turn leads to mutants in which the other proteins rather inadequately assemble onto the spore, as the supportive partner is missing, or not assemble at all, as the vital partner is missing. Therefore, many mutants can already be omitted, as they should not increase the abundance of the other proteins. Due to the redundancy of CotY to CotZ and CotV to CotX, even the elimination of the native or homologous competition might be less promising than hoped (as shown in Chapter 4 (Manuscript I) with BpuL/EcoL-CotZ in the *cotZ* mutant). As discussed above in detail, the only variants which might prove promising are mutants where the respective anchor assembles into crust fragments produced in the mother cell rather than the spore surface itself (i.e. SporoSNIPs). An exception as discussed in the prior paragraph is CotW as an anchor, as this anchor is not dependent on other crust proteins and might benefit from the (partially) missing crust structure. For all other anchors, crust fragments arise in *cotZ*-related mutants, and for CotV and CotX in *cotX*-related mutants. These crust fragments might include a lot more of the fusion protein, as these crust fragments provide a lot more space in which to self-assemble than the two-dimensional spore surface ever could provide. Moreover, the resulting biological particles (SporoSNIPs) are free of living GMO material. SporoSNIPs could therefore be applied in a wider range of possible applications, where GMOs are less desirable. This includes medical or environmental applications. Even though these particles (SporoSNIPs) are not spore-displayed anymore, they still seem to keep a few of the advantages of the spore itself, such as the stabilization of the enzyme during storage (see Chapter 4, Manuscript I). Whether or not this also holds true when these particles are stored completely on their own remains a question to be addressed once the separation has been methodologically established. But even if this is not the case, the SporoSNIPs could be stored together with the spores and separation could occur shortly before utilization. The only negative point worth noting is that these fragments are not as easily separated from the reaction supernatant as spores would be. This not only complicates the separation of the end product, but also makes them less applicable for fixed-bed reactions, successive reactions with the same particles or different

enzymes as a cascade reaction, to name just a few examples. Therefore, even if this strategy proves highly superior to the traditional Sporobead approach, Sporobeads still have a raison d´être.

The initial strategy of utilizing mutants to eliminate competition might therefore prove to be a failure and a success at the same time: a failure, because the display may not be improved drastically with this strategy, as the co-dependencies and redundancies play a bigger role than the competition for space; yet also a success, because the alternative strategy of utilizing non-GMO crust particles (SporoSNIPs) emerged from this rationale. This could prove to be a method superior to spore display, at least in some applications.

5.5 Conclusion

The crust is a quite complex glycoprotein layer consisting of six proteins. These proteins can be divided into four classes. First, the main structural proteins CotY and CotZ, of which CotZ, as the morphogenetic protein of the crust, anchors the structure at the middle part of the spore. Then there are the supporting structural proteins (CotV and CotX), which guide structure initiation and/or propagation from the poles of the spore. CotW is a supporting protein that stabilizes or helps the assembly of the CotV/CotX structural pillar. Last but not least, CgeA presumably acts as a glycosylation hub to coordinate the polysaccharide addition to the crust layer. This polysaccharide layer is composed of two variants (rhamnose-related and galactose-related), which are cross-linked, presumably by a polyglycerol linker. The galactose-related variant might be very similar to teichoic acid, whereas the rhamnose-related variant could contain the rare sugar viosamine, or VioNAc, and might be capped by a unique sugar with a lysine-derived side chain. In spite of crust's complexity, it was still feasible to utilize the crust as a platform for protein display. The performance varied for the specific anchors, with CotY and CotZ performing best, followed by CotX, then CotV and CotW, and the least favorable anchor being CgeA.

As discussed in this section, knowledge of the nature of the crust as well as the preliminary data on the improvement strategies of the spore display clearly lead to the following recommendations: If the best anchors CotY and CotZ perform well, and there is no reason to believe that the enzyme might require a different (more hydrophobic) micro-environment, then it is best to further utilize this constellation and try optimizing reaction conditions, spore

purification or other possible improvement strategies on the side of the enzyme. The elimination of the native competition only slightly improved the performance by a factor of two at most (see Chapter 4 (Manuscript I), the value approximately reached for BpuL-CotZ in *cotZ*). Linkers worked even less effectively for those variants already performing well, improving by a factor of approximately 1.5 (see Chapter 4 (Manuscript I), BpuL-CotZ with an unstructured 19-amino acid linker).

Changing the surface properties to increase hydrophobia might also help for enzymes and proteins more accustomed to these conditions, but the performance during recycling steps does suffer to some extent. As this approach is more challenging than the others due to gaps in our knowledge of the nature of the crust polysaccharide, this strategy should only be worthwhile if there is reason to believe that the protein of interest is intolerant to the hydrophilic micro-environment. But if any improvement of performance is welcomed, single or combined strategies might slightly – but presumably not vastly – improve performance.

Conversely, if the anchor works inadequately in a specific case, or if for reasons discussed in this section a less promising anchor or fusion site (C-terminal) is required, these strategies might yield beneficial outcomes.

One of these strategies has nevertheless given rise to a novel application: spore-derived self-assembled non-GMO including particles (SporoSNIPs). This application could potentially be superior to spore display. This is a perfect example of cross-talk between basic research and application. Without the desire to understand the protein interaction network and thereby visualize the crust proteins with GFP in the crust gene mutants, this would have never come to light. Performance with the application-relevant enzyme would never have hinted at this peculiarity, because it provides no clues as to the location of the enzyme itself. Without this knowledge, the SporoSNIPs strategy would not have evolved from the Sporobead system.

References

1. Alexander, M. (1964) Biochemical ecology of soil microorganisms. *Annu Rev Microbiol. 18*, 217-252.

2. López, D., and Kolter, R. (2010) Extracellular signals that define distinct and coexisting cell fates in *Bacillus subtilis*. *FEMS Microbiol Rev. 34(2)*, 134-149.

3. Höfler, C., Heckmann, J., Fritsch, A., Popp, P., Gebhard, S., Fritz, G., and Mascher, T. (2016) Cannibalism stress response in *Bacillus subtilis*. *Microbiology 162(1)*, 164-176.

4. Higgins, D., and Dworkin, J. (2012) Recent progress in *Bacillus subtilis* sporulation. *FEMS Microbiol Rev. 36(1)*, 131-148.

5. Barák, I., and Muchová, K. (2018) The positioning of the asymmetric septum during sporulation in *Bacillus subtilis*. *PLoS One 13(8)*, e0201979.

6. Ojkic, N., López-Garrido, J., Pogliano, K., and Endres, R. G. (2016) Cell-wall remodeling drives engulfment during *Bacillus subtilis* sporulation. *Elife 5*, e18657.

7. Ramamurthi, K. S., and Losick, R. (2008) ATP-driven self-assembly of a morphogenetic protein in *Bacillus subtilis*. *Mol Cell 31(3)*, 406-414.

8. Dworkin, J. (2014) Protein targeting during *Bacillus subtilis* sporulation. *Microbiol Spectr. 2(1)*.

9. Popham, D. L. (2002) Specialized peptidoglycan of the bacterial endospore: the inner wall of the lockbox. *Cell Mol Life Sci. 59(3)*, 426-433.

10. Jiang, S., Wan, Q., Krajcikova, D., Tang, J., Tzokov, S. B., Barak, I., and Bullough, P. A. (2015) Diverse supramolecular structures formed by self-assembling proteins of the *Bacillus subtilis* spore coat. *Mol Microbiol. 97(2)*, 347-359.

11. McKenney, P. T., Driks, A., and Eichenberger, P. (2013) The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nat Rev Microbiol. 11(1)*, 33-44.

12. Driks, A., and Eichenberger, P. (2016) The spore coat. *Microbiol Spectr. 4(2)*.

13. Plomp, M., Monroe Carroll, A., Setlow, P., and J Malkin, A. (2014) Architecture and assembly of the *Bacillus subtilis* spore coat. *PLoS One. 9(9)*.

14. McKenney, P. T., and Eichenberger, P. (2012) Dynamics of spore coat morphogenesis in *Bacillus subtilis*. *Mol Microbiol. 83(2)*, 245-260.

15. Imamura, D., Kuwana, R., Takamatsu, H., and Watabe, K. (2011) Proteins involved in formation of the outermost layer of *Bacillus subtilis* spores. *J Bacteriol. 193(16)*, 4075-4080.

16. McKenney, P. T., Driks, A., Eskandarian, H. A., Grabowski, P., Guberman, J., Wang, K. H., Gitai, Z., and Eichenberger, P. (2010) A distance-weighted interaction map reveals a previously uncharacterized layer of the *Bacillus subtilis* spore coat. *Curr Biol. 20(10)*, 934-938.

17. Waller, L. N., Fox, N., Fox, K. F., Fox, A., and Price, R. L. (2004) Ruthenium red staining for ultrastructural visualization of a glycoprotein layer surrounding the spore of *Bacillus anthracis* and *Bacillus subtilis*. *J Microbiol Methods. 58(1)*, 23-30.

18. Terry, C., Jiang, S., Radford, D. S., Wan, Q., Tzokov, S., Moir, A., and Bullough, P. A. (2017) Molecular tiling on the surface of a bacterial spore - the exosporium of the *Bacillus anthracis/cereus/thuringiensis* group. *Mol Microbiol. 104(4)*, 539-552.

19. Krajčíková, D., Forgáč, V., Szabo, A., and Barák, I. (2017) Exploring the interaction network of the *Bacillus subtilis* outer coat and crust proteins. *Microbiol Res. 204*, 72-80.

20. Krajčíková, D., Lukáčová, M., Müllerová, D., Cutting, S. M., and Barák, I. (2009) Searching for protein-protein interactions within the *Bacillus subtili*s spore coat. *J Bacteriol. 191(10)*, 3212-3219.

21. Liu, H., Krajcikova, D., Zhang, Z., Wang, H., Barak, I., and Tang, J. (2015) Investigating interactions of the *Bacillus subtilis* spore coat proteins CotY and CotZ using single molecule force spectroscopy. *J Struct Biol. 192(1)*, 14-20.

22. Liu, H., Qiao, H., Krajcikova, D., Zhang, Z., Wang, H., Barak, I., and Tang, J. (2016) Physical interaction and assembly of *Bacillus subtilis* spore coat proteins CotE and CotZ studied by atomic force microscopy. *J Struct Biol. 195(2)*, 245-251.

23. Liu, H., Krajcikova, D., Wang, N., Zhang, Z., Wang, H., Barak, I., and Tang, J. (2016) Forces and kinetics of the *Bacillus subtilis* spore coat proteins CotY and CotX binding to CotE inspected by single molecule force spectroscopy. *J Phys Chem B. 120(6)*, 1041-1047.

24. Zhang, J., Fitz-James, P. C., and Aronson, A. I. (1993) Cloning and characterization of a cluster of genes encoding polypeptides present in the insoluble fraction of the spore coat of *Bacillus subtilis*. *J Bacteriol. 175(12)*, 3757-3766.

25. Shuster, B., Khemmani, M., Abe, K., Huang, X., Nakaya, Y., Maryn, N., Buttar, S., Gonzalez, A. N., Driks, A., Sato, T., and Eichenberger, P. (2019) Contributions of crust proteins to spore surface properties in *Bacillus subtilis*. *Mol Microbiol. 111(3)*, 825-843.

26. Paidhungat, M., Setlow, B., Driks, A., and Setlow, P. (2000) Characterization of spores of *Bacillus subtilis* which lack dipicolinic acid. *J Bacteriol. 182(19)*, 5505-5512.

27. Setlow, P. (1988) Small, acid-soluble spore proteins of *Bacillus* species: structure, synthesis, genetics, function, and degradation. *Annu Rev Microbiol. 42*, 319-338.

28. Abhyankar, W., Pandey, R., Ter Beek, A., Brul, S., de Koning, L. J., and de Koster, C. G. (2015) Reinforcement of *Bacillus subtilis* spores by cross-linking of outer coat proteins during maturation. *Food Microbiol. 45(Pt A)*, 54-62.

29. Pandey, N. K., and Aronson, A. I. (1979) Properties of the *Bacillus subtilis* spore coat. *J Bacteriol. 137(3)*, 1208-1218.

30. Hosoya, S., Lu, Z., Ozaki, Y., Takeuchi, M., and Sato, T. (2007) Cytological analysis of the mother cell death process during sporulation in *Bacillus subtilis*. *J Bacteriol. 189(6)*, 2561-2565.

31. Setlow, P. (2006) Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J Appl Microbiol. 101(3)*, 514-525.

32. Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J., and Setlow, P. (2000) Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol Mol Biol Rev. 64(3)*, 548-572.

33. Riesenman, P. J., and Nicholson, W. L. (2000) Role of the spore coat layers in *Bacillus subtilis* spore resistance to hydrogen peroxide, artificial UV-C, UV-B, and solar UV radiation. *Appl Environ Microbiol. 66(2)*, 620-626.

34. Klobutcher, L. A., Ragkousi, K., and Setlow, P. (2006) The *Bacillus subtilis* spore coat provides “eat resistance” during phagocytic predation by the protozoan *Tetrahymena thermophila*. *Proc Natl Acad Sci U S A. 103(1)*, 165-170.

35. Laaberki, M.-H., and Dworkin, J. (2008) Role of spore coat proteins in the resistance of *Bacillus subtilis* spores to *Caenorhabditis elegans* predation. *J Bacteriol. 190(18)*, 6197-6203.

36. Abe, K., Kawano, Y., Iwamoto, K., Arai, K., Maruyama, Y., Eichenberger, P., and Sato, T. (2014) Developmentally-regulated excision of the SPβ prophage reconstitutes a gene required for spore envelope maturation in *Bacillus subtilis*. *PLoS Genet. 10(10)*, e1004636.

37. Cangiano, G., Sirec, T., Panarella, C., Isticato, R., Baccigalupi, L., De Felice, M., and Ricca, E. (2014) The *sps* gene products affect the germination, hydrophobicity, and protein adsorption of *Bacillus subtilis* spores. *Appl Environ Microbiol. 80(23)*, 7293-7302.

38. Wunschel, D., F. Fox, K., E. Black, G., and Fox, A. (1995) Discrimination among the *B. cereus* group, in comparison to *B. subtilis*, by structural carbohydrate profiles and ribosomal RNA spacer region PCR. *Syst Appl Microbiol.* 17(4), 625-635.

39. Arrieta-Ortiz, M. L., Hafemeister, C., Bate, A. R., Chu, T., Greenfield, A., Shuster, B., Barry, S. N., Gallitto, M., Liu, B., Kacmarczyk, T., Santoriello, F., Chen, J., Rodrigues, C. D., Sato, T., Rudner, D. Z., Driks, A., Bonneau, R., and Eichenberger, P. (2015) An experimentally supported model of the *Bacillus subtilis* global transcriptional regulatory network. *Mol Syst Biol. 11(11), 839*.

40. Luft, J. H. (1971) Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. *Anat Rec. 171(3)*, 347-368.

41. Plata, G., Fuhrer, T., Hsiao, T. L., Sauer, U., and Vitkup, D. (2012) Global probabilistic annotation of metabolic networks enables enzyme discovery. *Nat Chem Biol. 8(10)*, 848-854.

42. Oliva, C. R., Swiecki, M. K., Griguer, C. E., Lisanby, M. W., Bullard, D. C., Turnbough, C. L., Jr., and Kearney, J. F. (2008) The integrin Mac-1 (CR3) mediates internalization and directs *Bacillus anthracis* spores into professional phagocytes. *Proc Natl Acad Sci U S A. 105(4)*, 1261-1266.

43. Sheldon, R. A. (2007) Enzyme immobilization: the quest for optimum performance. *Adv Synth Catal. 349(8-9)*, 1289-1307.

44. Isticato, R., and Ricca, E. (2014) Spore surface display. *Microbiol Spectr. 2(5)*.

45. Setlow, P. (2003) Spore germination. *Curr Opin Microbiol. 6(6)*, 550-556.

46. Iwanicki, A., Piątek, I., Stasiłojć, M., Grela, A., Łęga, T., Obuchowski, M., and Hinc, K. (2014) A system of vectors for *Bacillus subtilis* spore surface display. *Microb Cell Fact. 13(1)*, 30.

47. Wang, H., Wang, Y., and Yang, R. (2017) Recent progress in *Bacillus subtilis* spore-surface display: concept, progress, and future. *Appl Microbiol Biotechnol. 101(3)*, 933-949.

48. Bartels, J., López Castellanos, S., Radeck, J., and Mascher, T. (2018) Sporobeads: the utilization of the *Bacillus subtilis* endospore crust as a protein display platform, *ACS Synth Biol. 7(2)*, 452-461.

49. Mascher, T., Margulis, N. G., Wang, T., Ye, R. W., and Helmann, J. D. (2003) Cell wall stress responses in *Bacillus subtilis*: the regulatory network of the bacitracin stimulon, *Mol Microbiol. 50(5)*, 1591-1604.

50. Harwood C. R., and Cutting S. (1990) Molecular biological methods for *Bacillus*. *John Wiley & Sons Ltd. Chichester, England*.

51. Registry of Standard Biological Parts, http://parts.igem.org/Main_Page.

52. Liew, P. X., Wang, C. L. C., and Wong, S.-L. (2012) Functional characterization and localization of a B*acillus subtilis* sortase and its substrate and use of this sortase system to covalently anchor a heterologous protein to the *B. subtilis* cell wall for surface display. *J Bacteriol. 194(1)*, 161-175.

53. Reiss, R., Ihssen, J., and Thöny-Meyer, L. (2011) *Bacillus pumilus* laccase: a heat stable enzyme with a wide substrate spectrum. *BMC Biotechnol. 11*, 9.

54. Roels, S., and Losick, R. (1995) Adjacent and divergently oriented operons under the control of the sporulation regulatory protein GerE in *Bacillus subtilis*. *J Bacteriol. 177(21)*, 6263-6275.

55. Bartels, J., Blüher, A., López Castellanos, S., Richter, M., Günther, M., and Mascher, T. (2019) The *Bacillus subtilis* endospore crust: protein interaction network, architecture and glycosylation state of a potential glycoprotein layer. *Mol Microbiol.* 112(5), 1576-1592.

56. Shuster, B., Khemmani, M., Nakaya, Y., Holland, G., Iwamoto, K., Abe, K., Imamura, D., Maryn, N., Driks, A., Sato, T., and Eichenberger, P. (2019) Expansion of the spore surface polysaccharide layer in *Bacillus subtilis* by deletion of genes encoding glycosyltransferases and glucose modification enzymes. *J Bacteriol.* 201(19), e00321-19

57. Plikat, C. (2019) Optimierte w/o Pickering Emulsionen für Mehrphasen-Biokatalyse. *TU Dresden, Professur für Molekulare Biotechnologie*.

58. Chen, X., Zaro, J. L., and Shen, W.-C. (2013) Fusion protein linkers: property, design and functionality. *Adv Drug Deliv Rev. 65(10)*, 1357-1369.

59. Manoel, E. A., dos Santos, J. C. S., Freire, D. M. G., Rueda, N., and Fernandez-Lafuente, R. (2015) Immobilization of lipases on hydrophobic supports involves the open form of the enzyme. *Enzyme Microb Technol. 71*, 53-57.

60. Sirec, T., Strazzulli, A., Isticato, R., De Felice, M., Moracci, M., and Ricca, E. (2012) Adsorption of β-galactosidase of *Alicyclobacillus acidocaldarius* on wild type and mutants spores of *Bacillus subtilis. Microb Cell Fact. 11*, 100.

61. Nguyen, Q. A., and Schumann, W. (2014) Use of IPTG-inducible promoters for anchoring recombinant proteins on the *Bacillus subtilis* spore surface. *Protein Expr Purif. 95*, 67-76.

Appendix

Supplemental Material for Publication I: Sporobeads: The Utilization of the *Bacillus subtilis* Endospore Crust as a Protein Display Platform

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Table S1: Plasmids created and used in this study

Plasmid[§]	Reference[%]	Backbone	Integration Site	Antibiotic resistance		Order[1]
				E. coli	*B. subtilis*	
pSB1C3-[RFP]	Parts registry	-	-	Cm	-	Parts registry
pSB1K3-[RFP]	Parts registry	-	-	Kan	-	Parts registry
pBS1C-[RFP]	Radeck et al. 2013	-	*amyE*	Amp	Cm	Parts registry
pSB1C3-B0014	Parts registry	pSB1C3	-	Cm	-	BBa_B0014 (Parts registry)
pSB1C3-GFP	This study	pSB1C3	-	Cm	-	BBa_K823039 (Parts registry)
pSB1C3-BpuL	This study	pSB1C3	-	Cm	-	BBa_K863001 (Parts registry)
pSB1C3-T7-BpuL-6xhis	Parts registry	pSB1C3	-	Cm	-	BBa_K863000 (Parts registry)
pSB1C3-EcoL	This study	pSB1C3	-	Cm	-	BBa_K863006 (Parts registry)
pSB1C3-T7-EcoL-6xhis	Parts registry	pSB1C3	-	Cm	-	BBa_K863005 (Parts registry)
pBS3C*lux*-P_{cotVWX}	This study	pBS3C*lux*	*sacA*	Amp	Cm	-
pBS3C*lux*-P_{cotX}	This study	pBS3C*lux*	*sacA*	Amp	Cm	-
pBS3C*lux*-P_{cotYZ}	This study	pBS3C*lux*	*sacA*	Amp	Cm	-
pBS3C*lux*-P_{cgeA}	This study	pBS3C*lux*	*sacA*	Amp	Cm	-
p1CSV-CgeA-N	This study JPUB_009501	pBS1C	*amyE*	Amp	Cm	**ECE363**
p1CSV-CgeA-C	This study JPUB_009502	pBS1C	*amyE*	Amp	Cm	**ECE364**
p1CSV-CotV-N	This study JPUB_009503	pBS1C	*amyE*	Amp	Cm	**ECE365**
p1CSV-CotV-C	This study JPUB_009504	pBS1C	*amyE*	Amp	Cm	**ECE366**
p1CSV-CotW-N	This study JPUB_009505	pBS1C	*amyE*	Amp	Cm	**ECE367**
p1CSV-CotW-C	This stud JPUB_009506	pBS1C	*amyE*	Amp	Cm	**ECE368**
p1CSV-CotX-N	This study JPUB_009507	pBS1C	*amyE*	Amp	Cm	**ECE369**
p1CSV-CotX-C	This study JPUB_009508	pBS1C	*amyE*	Amp	Cm	**ECE370**
p1CSV-CotY-N	This study JPUB_009509	pBS1C	*amyE*	Amp	Cm	**ECE371**
p1CSV-CotY-C	This study JPUB_009510	pBS1C	*amyE*	Amp	Cm	**ECE372**
p1CSV-CotZ-N	This study JPUB_009511	pBS1C	*amyE*	Amp	Cm	**ECE373**
p1CSV-CotZ-C	This study JPUB_009512	pBS1C	*amyE*	Amp	Cm	**ECE374**
p1CSV-CgeA-N-GFP	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CgeA-C-GFP	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotV-N-GFP	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotV-C-GFP	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotW-N-GFP	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotW-C-GFP	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotX-N-GFP	This study	pBS1C	*amyE*	Amp	Cm	-

p1CSV-CotX-C-GFP	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotY-N-GFP	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotY-C-GFP	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotZ-N-GFP	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotZ-C-GFP	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CgeA-N-BpuL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CgeA-C-BpuL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotV-N-BpuL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotV-C-BpuL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotW-N-BpuL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotW-C-BpuL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotX-N-BpuL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotX-C-BpuL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotY-N-BpuL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotY-C-BpuL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotZ-N-BpuL	This study	*pBS1C*	*amyE*	Amp	Cm	-
p1CSV-CotZ-C-BpuL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CgeA-N-EcoL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CgeA-C-EcoL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotV-N-EcoL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotV-C-EcoL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotW-N-EcoL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotW-C-EcoL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotX-N-EcoL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotX-C-EcoL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotY-N-EcoL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotY-C-EcoL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotZ-N-EcoL	This study	pBS1C	*amyE*	Amp	Cm	-
p1CSV-CotZ-C-EcoL	This study	pBS1C	*amyE*	Amp	Cm	-

§ The Plasmids are annotated according to the backbone and the insert connected with a hyphen, which is also annotated in the according column. The Sporovectors (p1CSV-*crust gene*-N/C) are readily available from the BGSC. The denomination derives from a shortened code for the pBS1C (p1C) as the backbone, SV for Sporovector, followed by the crust protein coded in the Sporovector and the translational fusion site (N=N-terminal, C=C-terminal). They all confer ampicillin resistance in *E. coli* and chloramphenicol resistance in *B. subtilis*. In *B. subtilis* they integrate into the genome at the *amyE*-locus, which can be verified by the inability to degrade starch on starch minimal plates.

% The JPUB-Numbers refer to the accession number at the JBEI ICE Registry[1]

[1] BBa-number refers to the parts registry[2], whereas the ECE number is derived from the *Bacillus* genetic stock center (BGSC)[3]

Table S2: Strains created and used in this study

Strain	Genotyp[§]	Antibiotic resistance (*B. subtilis*)
TMB1854	W168, *sacA::*P_{cotVWX}*-lux*	Cm
TMB4581	W168, *sacA::*P_{cotX}*-lux*	Cm
TMB1853	W168, *sacA::*P_{cotYZ}*-lux*	Cm
TMB1899	W168, *sacA::*P_{cgeA}*-lux*	Cm
TMB4519	W168, *amyE::*P_{cotYZ}*-gfp-cgeA*	Cm
TMB4521	W168, *amyE::*P_{cotYZ}*-cgeA-gfp*	Cm
TMB4858	W168, *amyE::*P_{cotYZ}*-gfp-cotV*	Cm
TMB4859	W168, *amyE::*P_{cotYZ}*-cotV-gfp*	Cm
TMB4511	W168, *amyE::*P_{cotYZ}*-gfp-cotW*	Cm
TMB4513	W168, *amyE::*P_{cotYZ}*-cotW-gfp*	Cm
TMB4854	W168, *amyE::*P_{cotYZ}*-gfp-cotX*	Cm
TMB4855	W168, *amyE::*P_{cotYZ}*-cotX-gfp*	Cm
TMB4523	W168, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm
TMB4525	W168, *amyE::*P_{cotYZ}*-cotY-gfp*	Cm
TMB4515	W168, *amyE::*P_{cotYZ}*-gfp-cotZ*	Cm
TMB4517	W168, *amyE::*P_{cotYZ}*-cotZ-gfp*	Cm
TMB2131	W168, *cotA::mls*^{r}	MLS
TMB5066	W168, *amyE::*P_{cotYZ}*-cotA-cgeA; cotA::mls*^{r}	Cm, MLS
TMB4306	W168, *amyE::*P_{cotYZ}*-cgeA-cotA; cotA::mls*^{r}	Cm, MLS
TMB4982	W168, *amyE::*P_{cotYZ}*-cotA-cotV; cotA::mls*^{r}	Cm, MLS
TMB4984	W168, *amyE::*P_{cotYZ}*-cotV-cotA; cotA::mls*^{r}	Cm, MLS
TMB4543	W168, *amyE::*P_{cotYZ}*-cotA-cotW; cotA::mls*^{r}	Cm, MLS
TMB4545	W168, *amyE::*P_{cotYZ}*-cotW-cotA; cotA::mls*^{r}	Cm, MLS
TMB4974	W168, *amyE::*P_{cotYZ}*-cotA-cotX; cotA::mls*^{r}	Cm, MLS
TMB4976	W168, *amyE::*P_{cotYZ}*-cotX-cotA; cotA::mls*^{r}	Cm, MLS
TMB4547	W168, *amyE::*P_{cotYZ}*-cotA-cotY; cotA::mls*^{r}	Cm, MLS
TMB4549	W168, *amyE::*P_{cotYZ}*-cotY-cotA; cotA::mls*^{r}	Cm, MLS
TMB4338	W168, *amyE::*P_{cotYZ}*-cotA-cotZ; cotA::mls*^{r}	Cm, MLS
TMB4314	W168, *amyE::*P_{cotYZ}*-cotZ-cotA; cotA::mls*^{r}	Cm, MLS
TMB5022	W168, *amyE::*P_{cotYZ}*-cueO-cgeA; cotA::mls*^{r}	Cm, MLS
TMB5024	W168, *amyE::*P_{cotYZ}*-cgeA-cueO; cotA::mls*^{r}	Cm, MLS
TMB5006	W168, *amyE::*P_{cotYZ}*-cueO-cotV; cotA::mls*^{r}	Cm, MLS
TMB5008	W168, *amyE::*P_{cotYZ}*-cotV-cueO; cotA::mls*^{r}	Cm, MLS
TMB5032	W168, *amyE::*P_{cotYZ}*-cueO-cotW; cotA::mls*^{r}	Cm, MLS
TMB5034	W168, *amyE::*P_{cotYZ}*-cotW-cueO; cotA::mls*^{r}	Cm, MLS
TMB4998	W168, *amyE::*P_{cotYZ}*-cueO-cotX; cotA::mls*^{r}	Cm, MLS
TMB5000	W168, *amyE::*P_{cotYZ}*-cotX-cueO; cotA::mls*^{r}	Cm, MLS
TMB5028	W168, *amyE::*P_{cotYZ}*-cueO-cotY; cotA::mls*^{r}	Cm, MLS
TMB5030	W168, *amyE::*P_{cotYZ}*-cotY-cueO; cotA::mls*^{r}	Cm, MLS
TMB4322	W168, *amyE::*P_{cotYZ}*-cueO-cotZ; cotA::mls*^{r}	Cm, MLS
TMB5026	W168, *amyE::*P_{cotYZ}*-cotZ-cueO; cotA::mls*^{r}	Cm, MLS

§ The strains all derive from the *B. subtilis* wildtype W168 or the strain TMB2131 in case of the laccases. The strain TMB2131 derives from a genomic replacement of the laccase gene *cotA* with the *mls*^{r}-cassette in the wildtype W168. The first four strains (TMB1854, TMB4581, TMB1853, TMB1899) derive from the transformation of *B. subtilis* wildtype W168 with the pBS3C*lux* variants with the different crust gene promoters, that integrated into the *sacA*-locus. All of the other strains derived from the transformation of the respective Sporovectors containing GFP (*gfp*), the laccase from *Bacillus pumilus* BpuL (*cotA*) or the laccase from *Escherichia coli* EcoL (*cueO*), integrated into the amyE-locus.

Table S3: Primers used in this study

Primer number	Primer name	Sequence (5‘ to 3‘)[§]
TM2889	[RFP]-fwd	TGCCACCTGACGTCTAAG
TM3000	NgoMIV-[RFP]-rev	GATCGCCGGCTATAAACGCAGAAAGGCCC
TM2995	AgeI-[RFP]-fwd	GATCACCGGTGCAATACGCAAACCGCCTC
TM2890	[RFP]-rev	ATTACCGCCTTTGAGTGA
TM3001	[RFP]-AgeI1-mut-fwd	CCTACATGGCTAAAAAACCTGTTCAGCTGCCGGGTGC
TM3002	[RFP]-AgeI1-mut-rev	GCACCCGGCAGCTGAACAGGTTTTTTAGCCATGTAGG
TM3003	[RFP]-AgeI2-mut-fwd	GGTCGTCACTCCACCGGAGCTTAATAACGCTGATAGTGC
TM3004	[RFP]-AgeI2-mut-rev	GCACTATCAGCGTTATTAAGCTCCGGTGGAGTGACGACC
TM2994	EcoRI-P_{cotYZ}-fwd	GATCGAATTCGACAGCAACAAATACACTCG
TM2999	XbaI-P_{cotYZ}-rev	GATCTCTAGAATTATAGGGTATTTGACTTTAGTC
TM2916	P_{cotYZ} -EcoRI-NotI-XbaI (ENX) -fwd	GATCGAATTCGCGGCCGCTTCTAGAGACAGCAACAAATACACTCGTAG
TM2917	P_{cotYZ}-SpeI-rev	GATCACTAGTATTATAGGGTATTTGACTTTAGTCC
TM2918	P_{cotV}-ENX-fwd	GATCGAATTCGCGGCCGCTTCTAGAGTGCCAATCATGATCCCTTCAC
TM2919	P_{cotV}-SpeI-rev	GATCACTAGTATCTTATCCATACTCTATATAATGC
TM5020	P_{cotX}-ENX-fwd	TGCAGAATTCGCGGCCGCTTCTAGAGCCAATCATAAAAAATAGGGTTCTTCATCAG
TM5021	P_{cotX}-SpeI-rev	GATCACTAGTATTTCTTTTACTGTTATTAAATGAGCGAGCC
TM2914	P_{cgeA}-ENX-fwd	GATCGAATTCGCGGCCGCTTCTAGAGATATGTGCGACTTCGATTTATGG
TM2915	P_{cgeA}-SpeI-rev	GATCACTAGTAATTCGATAGTGAACTCACGTTATTC
TM2996	B0014-SpeI-fwd	GATCACTAGTTCACACTGGCTCACCTTC
TM2997	B0014-PstI-rev	GATCCTGCAGAAATAATAAAAAAGCCGGATTA
TM2932	*cgeA*-replaced- ENX-SD-NgoMIV-fwd	GATCGAATTCGCGGCCGCTTCTAGAGAAT***AGGAGG***TGTGTGTAATGGCCGGCGAAAATGCACAGTTAAAAAAGG
TM2966	*cgeA*-AgeI-mut-fwd	GGTGACATGATCGGACCTGTTGTTTTCGTTGCTTTC
TM2967	*cgeA*-AgeI-mut-rev	GAAAGCAACGAAAACAACAGGTCCGATCATGTCACC
TM2933	*cgeA*-AgeI-SpeI-rev	GATCACTAGTATTAACCGGTTGAAAAGAACGTAACGCTTTCTAC
TM4902	*cotV*-ENX-SD-NgoMIV-fwd	GATCGAATTCGCGGCCGCTTCTAGAA***AGGAGG***TCGCCGGCATGTCATTTGAAGAAAAAGTCGAATC
TM4903	*cotV*-EcoRI-mut-fwd	GAGCAATTATCAAGCGAGTTCGAACAGCAGATCGAAG
TM4904	*cotV*-EcoRI-mut-rev	CTTCGATCTGCTGTTCGAACTCGCTTGATAATTGCTC
TM4905	*cotV*-SpeI-NotI-PstI (SNP)-AgeI-rev	AGCTCTGCAGCGGCCGCTACTAGTATTAACCGGTAAGGACGTCAAGTTCACTAAGAATG
TM3062	*cotW*-ENX-fwd	GATCGAATTCGCGGCCGCTTCTAGAA***AGGAGG***TGGCCGGCATGTCAGATAACGATAAATTCAAAG
TM3063	*cotW*-AgeI-SpeI-rev	AATTACTAGTATTAACCGGTATTGTTATCGTTTTTCCGTCTTTG
TM3505	*cotX*-ENX-SD-NgoMIV-fwd	GATCGAATTCGCGGCCGCTTCTAGAA***AGGAGG***TCGCCGGCATGGAAAGCAGACCATATTCTTG
TM3506	*cotX*-SNP-AgeI-rev	AGCTCTGCAGCGGCCGCTACTAGTATTAACCGGTGAGGACAAGAGTGATAACTAGGATG
TM3503	*cotY*-ENX-SD-NgoMIV-NgoMIVmut-fwd	GATCGAATTCGCGGCCGCTTCTAGAA***AGGAGG***TCGCCGGCATGAGCTGCGGAAAAACCCATGGCCGACATGAGAACTGTGTATGCG

TM3504	*cotY*-SNP-AgeI-rev	AGCTCTGCAGCGGCCGCTACTAGTATTAACCGGTT CCATTGTGATGATGCTTTTTATC
TM2929	*cotZ*-replaced-ENX-SD-NgoMIV-fwd	GATCGAATTCGCGGCCGCTTCTAGAGGC***AGGAGG*** GATAATATGGCCGGCAAAACATCAAGCTG
TM2930	*cotZ*-AgeI-SpeI-rev	GATCACTAGTATTAACCGGTATGATGATGTGTACGA TTGATTAATCG
TM3087	BpuL-X-SD-AvaI-fwd	CGCTTCTAGATA***AGGAGG***AA**CCCGGG**ATGAACCTA GAAAAATTTGTTGACG
TM3088	BpuL-SNP-BspEI-rev	GATCCTGCAGCGGCCGCTACTAGTATTA**TCCGGA**C TGGATGATATCCATCG
TM3020	EcoL-ENX-SD-NgoMIV-fwd	GATCGAATTCGCGGCCGCTTCTAGATA***AGGAGG***AA CTACTATGGCCGGCCAACGTCGTGATTTCTTAAAAT ATTCCG
TM3021	EcoL-SNP-AgeI-rev	GATCCTGCAGCGGCCGCTACTAGTATTAACCGGTT ACCGTAAACCCTAACATCATCC
TM3089	*cotA*-del-up-BglII	GATC**AGATCT**GTACAAGCCATTGTGGC
TM3165	*cotA*-up-rev-mls	CCTATCACCTCAAATGGTTCGCTGTTTCATCTGTCCT TATCTAAATTTTC
TM3166	*cotA*-do-fwd-mls	CGAGCGCCTACGAGGAATTTGTATCGCCCGACAAA CTTGCCTCTAG
TM3090	*cotA*-del-do-SalI-rev	GATC**GTCGAC**CCTTCTGTAGCGCCTGC
TM0139	*mls*-fwd	CAGCGAACCATTTGAGGTGATAGGGATCCTTTAACT CTGGCAACCCTC
TM0140	*mls*-rev	CGATACAAATTCCTCGTAGGCGCTCGGGCCGACTG CGCAAAAGACATAATCG

§ The restriction sites in the sequence were marked as follows: AgeI (red), NgoMIV (light blue), EcoRI (dark green), NotI (grey and underlined), XbaI (orange), SpeI (purple), PstI (light green), RBS (dark blue and italic). All other sites for restriction enzymes were marked bold und underlined.

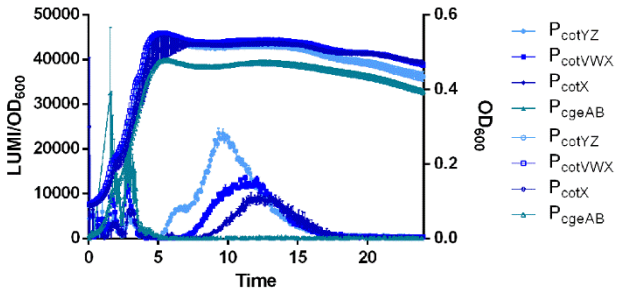


Figure S1: Promoter strength measurement
Crust gene promoters (P_{cotYZ}, P_{cotVWX}, P_{cotX} and P_{cgeAB}) were transcriptionally fused to the *lux* operon and luminescence was measured in a microtiter plate reader to determine luciferase activity. Cell growth was monitored by optical density at a wavelength of 600 nm (OD600). Raw luminescence output (relative luminescence units, RLU) was first corrected by the RLU of a strain harboring an empty pBS3C-*lux* and then normalized to cell density by dividing each data point by its corresponding corrected OD600 value. The maximum of the resulting graph, ignoring the initial fluctuation due to the low OD600, was utilized as a measure of promoter strength.

References

1. JBEI ICE Registry, *https://public-registry.jbei.org/folders/330*.

2. Knight, T. (2007) Draft Standard for Biobrick Biological Parts, http://hdl.handle.net/1721.1/45138.

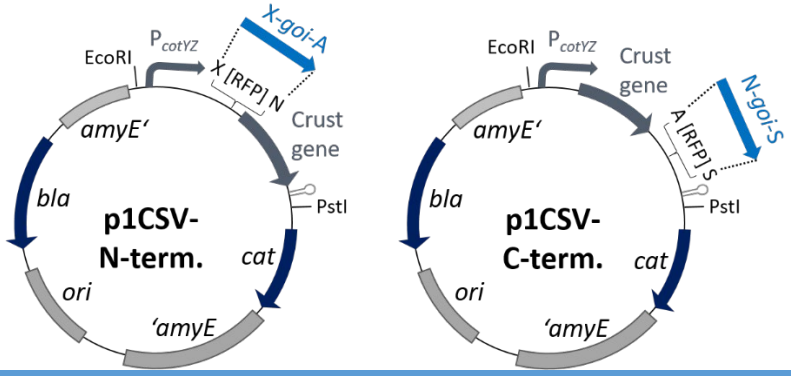
3. Columbus, O. *Bacillus* Genetic Stock Center, http://www.bgsc.org/

How-to-use guide: Sporovectors

A convenient system to produce spores displaying a protein of interest on their surface

The genetic components of the Sporovectors

The figure depicts the vector maps of the two archetypes of Sporovectors: On the left for N-terminal fusions and on the right for C-terminal fusions to the respective crust gene. The part between the EcoRI and the PstI restriction sites is the Sporovector specific part, located on the backbone pBS1C from the *Bacillus* BioBrick Box. Underneath is a tabular explanation of the genetic components found on the Sporovectors.



Genetic component	description	function
pBS1C		
ori	Origin of replication (*E. coli*)	Amplification of vector in *E. coli* (For **cloning in *E. coli***)
bla	Codes for ampicillin resistance (*E. coli*)	Selection marker for *E. coli* (For **cloning in *E. coli***)
amyE';'amyE	Homologues regions to the *amyE* gene and adjacent regions (*B. subtilis*)	Homologous **integration** of the inlying part into the *amyE* gene in *B. subtilis* (For stable transformation in *B. subtilis*)
cat	Codes for chloramphenicol resistance (*B. subtilis*)	Selection marker for *B. subtilis* (For stable **transformation in *B. subtilis***)
Sporovector specific part		
EcoRI, PstI	Restrictions sites from the BioBrick RFC10 standard	Enables the **subcloning** of the Sporovector specific part into a different backbone
P_{cotYZ}	The strongest crust gene promoter	Drives the **expression** of the fusion-crust-protein at the time point of sporulation
Crust gene	The corresponding proteins build up the outermost proteinaceous layer of the spore: the crust. (CgeA, CotV, CotW, CotX, CotY and CotZ)	**Anchoring** the fusion partner to the crust
X-[RFP]N/A-[RFP]-S	An RFP cassette flanked by the appropriate restriction sites from the RFC25 (XbaI=X and NgoMIV=N for N-terminal fusion/ AgeI=A and SpeI=S for C-terminal fusion)	**Multiple Cloning site** for a translational fusion of a gene of interest flanked by the appropriate restriction sites from the RFC25 (XbaI=X and AgeI=A for N-terminal fusion/ NgoMIV=N and SpeI=S for C-terminal fusion) **Enables Red-White-screening**
B0014 (shown as hairpin)	Double terminator	**Transcriptional Insulation**

Required steps to utilize the Sporovectors for a selected gene of interest

1. Make sure the gene of interest is appropriate for the system
2. Design primers for the gene of interest to fit the cloning standard
3. Clone the gene of interest into the Sporovector (a subcloning step is recommended for the cloning into multiple versions of the Sporovectors)
4. Transformation of the construct into *B. subtilis*
5. Produce and purify spores to test application

These steps will be described in detail in the following section. The goal is to provide a guide to be used in the laboratory, to aid in the practical utilization of this system.

1. Make sure the gene of interest is appropriate for the system

The gene of interest should be appropriate for the expression in *B. subtilis*. A codon optimisation is not obligatory, but it should be kept in mind, that if the codon usage is non-optimal, the system might not work without.
Additionally, if the function of the protein of interest is linked with a multimerization, requires a chaperone for folding or is a membrane protein, the function might be limited or even impaired.

2. Design primers for the gene of interest to fit the cloning standard

- The gene of interest must be in the RFC25 Freiburg cloning standard, including a RBS for *B. subtilis*. In this section the recommended sequence is provided (see Table below) to most conveniently design the primers.
- A more detailed explanation as well as options to shorten these sequences (in specific cases), are given afterwards and is not strictly required.
- The gene of interest can be any gene, that *B. subtilis* is able to express, but should not include the respective restriction sites XbaI and AgeI for N-terminal or NgoMIV and SpeI for C-terminal variants, optimally also not the additional restriction sites required for the RFC10 standard (EcoRI and PstI), to still enable subcloning into a BioBrick backbone.
- When cloning with the RFC25 standard, a scar from the AgeI/NgoMIV mixed site in between the translational fused genes remains (ACCGGC), leading to a two amino acid linker (Thr-Gly) between the protein of interest and the crust protein.
- When utilizing this standard, it should also be stated, that in the C-terminal fusion there is a two amino acid (Thr-Gly) addition to the protein due to the AgeI site at the C-terminus.

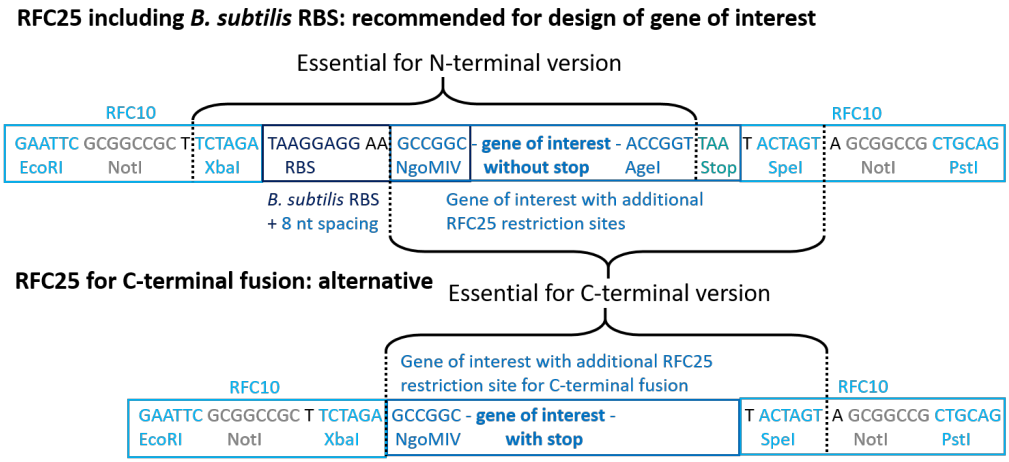
Primer overhang	Sequence (from 5' to 3')
Foward Primer	GATCGAATTCGCGGCCGCTTCTAGAA*AGGAGG*TGGCCGGC
Reverse Primer (attention! Exclude the stop codon!)	AGCTCTGCAGCGGCCGCTACTAGTATTAACCGGT

AgeI, NgoMIV, EcoRI, NotI, XbaI, SpeI, PstI, *RBS*

Detailed explanation

In addition to the recommended sequences, an explanation of the standard is given with the required restriction sites and their specific functions. In some cases, this this knowledge can be used to reduce the primer sequence length, in order to save money. If the recommended sequence is utilized, then the next section is not required.
The figure below depicts the proposed standard for the gene of interest (top sequence), which is based on the BioBrick RFC25 cloning standard, but includes a ribosome binding site (RBS)

for B. subtilis (already described in the *Bacillus* BioBrick Box). The light blue part shows the normal BioBrick RFC10 standard: EcoRI-NotI-XbaI for the prefix and SpeI-NotI-PstI for the suffix. These additions are convenient for subcloning of the gene of interest into a BioBrick backbone. The NgoMIV and the AgeI followed by the stop codon adheres to the BioBrick RFC25 cloning standard. In between the XbaI and the NgoMIV, the optimal RBS for B. subtilis has been included with the optimal 8 nucleotide spacing to the start codon of the gene of interest. These restriction sites should not be present in the gene of interest, but if these restriction sites cannot be removed, this standard can be altered utilizing restriction sites that yield compatible overhangs.



The part of this standard that is required for the N-terminal or C-terminal version is highlighted and can be shortened if only one variant is desired. For the C-terminal variant, the AgeI followed by the stop codon can also be replaced by the native stop codon, to prevent the C-terminal two amino acid (Thr-Gly) addition to the protein due to the AgeI site. This alternative is shown at the bottom of the figure. **But if both variants are desired, it is convenient to apply the complete standard.**

3. Clone the gene of interest into the Sporovector

First a step-by-step guide is given, followed by a more detailed explanation. If the step-by-step guide is followed, it might not be required to also read the detailed explanation.

1. Produce the PCR-product of the gene of interest with the appropriate restriction sites
2. Optional step: Subclone into a compatible BioBrick backbone (pSB1C3 is recommended), gel purification is recommended to reduce red colonies. Ligate, transform and plate on the appropriate selection plate (Chloramphenicol for pSB1C3, Kanamycin for pSB1K3). Screen the positive white colonies to check for the gene of interest by sequencing (see table below)

Primer	Sequence (from 5' to 3')
pSB1C/K3 Forward sequencing Primer	TGCCACCTGACGTCTAAG
pSB1C/K3 Reverse sequencing Primer	ATTACCGCCTTTGAGTGA

3. Digest the positive plasmid or the PCR product for N-terminal variants with XbaI and NgoMIV or for C-terminal variants with NgoMIV and SpeI and the respective Sporovector for N-terminal fusion with XbaI and AgeI or for C-terminal fusion with AgeI and SpeI (the RFP-cassette should drop out, which has a size of approximately 1,2 kb). Gel purification is recommended to reduce red colonies (or the integration of the BioBrick backbone pSB1C3).

Variant	Gene of interest	Sporovector
N-terminal	XbaI + NgoMIV	XbaI + AgeI
C-terminal	NgoMIV + SpeI	AgeI + SpeI

Ligate the Sporovector and the gene of interest (AgeI and NgoMIV produce compatible overhangs and therefore a mixed site, which links the two parts in-frame together with a Thr-Gly linker), transform and plate on Ampicillin-selection plates. Replica plates should be on Ampicillin, but additionally can also be done on the selection marker of the BioBrick backbone utilized for the subcloning (Chloramphenicol for pSB1C3), if applicable (positive colonies should not grow). Screen the positive white colonies to check for the gene of interest by sequencing (see table below).

Primer	Sequence (from 5' to 3')
pBS1C Forward sequencing Primer	AAAGGTCATTGTTGACGCGG
pBS1C Reverse sequencing Primer	GAGCGTAGCGAAAAATCC

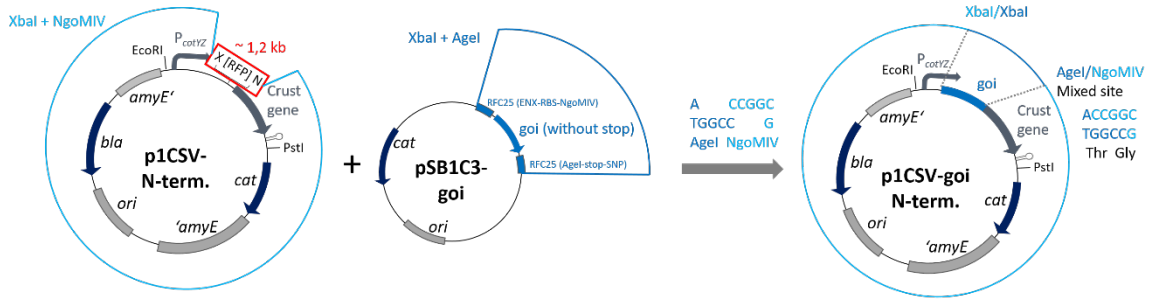
Detailed explanation

Subcloning: In order to utilize the gene of interest for more than one variant, it is recommended to subclone the PCR-product into a BioBrick backbone, like the pSB1C3 (parts registry, recommended) or the pSB1K3 (parts registry). It is essential to subclone into a backbone without an ampicillin selection marker, as else a selection for the Sporovector backbone is not possible. For subcloning, the RFC10 restriction sites EcoRI and PstI are included in the recommended Primer sequence. These are also present in every BioBrick backbone, and mostly flank a RFP-cassette for red-white screening. If the gene of interest is subcloned into the multiple cloning site, then the resulting colonies are white (dependent on the strain of *E. coli* used for the cloning, the colour can be more or less visible, sometimes only visible on replica plates or in the (pelleted) overnight cultures).

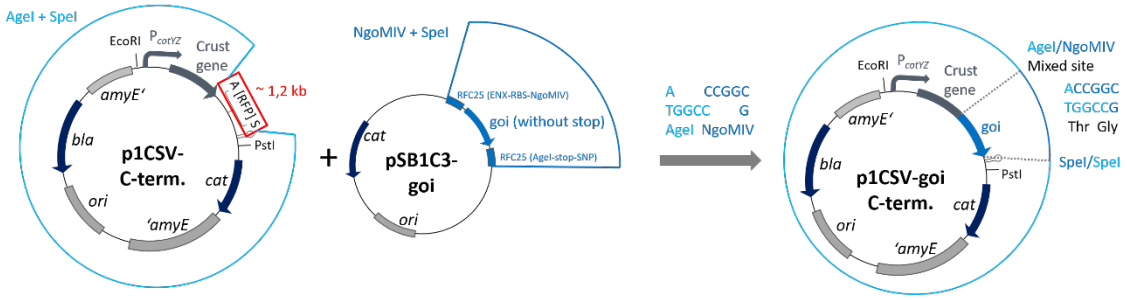
Cloning gene of interest into the Sporovectors:

See the figure below for a graphic illustration of how the cloning works.

N-terminal



C-terminal



- **N-terminally**: To clone the gene of interest N-terminally, utilize the appropriate Sporovector. The appropriate digest is named above and shown in the figure above. Through the digest of the gene of interest with XbaI and AgeI, the RBS of the gene of interest is still present and the stop codon for the gene of interest found after the AgeI site is lost, so that the translation can continue. The AgeI overhang from the gene of interest is compatible with the NgoMIV of the Sporovector. The mixed site (ACCGGC) leads to a translational fusion of the gene of interest with the crust gene with a two amino acid linker (Thr-Gly).
- **C-terminally**: To clone the gene of interest C-terminally, utilize the appropriate Sporovector. The appropriate digest is named above and shown in the figure above. Through the digest of the gene of interest with NgoMIV and SpeI, the RBS of the gene of interest is lost and the stop codon for the gene of interest found after the AgeI site is still present, so that the translational stops after the gene of interest. The NgoMIV overhang from the gene of interest is compatible with the AgeI of the Sporovector. The mixed site (ACCGGC) leads to a translational fusion of the gene of interest with the crust gene with a two amino acid linker (Thr-Gly).
- **Screening**: Utilize the negative marker RFP, which produces red colonies. Therefore, if the gene of interest is integrated into the multiple cloning site, the colony will appear white. If the backbone of the subcloning (if applicable) is instead integrated, the colony will still appear white, but will grow on Chloramphenicol (or the respective resistance marker). With these convenient screening methods, it is quite easy to find positive colonies.

4. Transformation of the construct into *B. subtilis*

As this system should be applicable by a wide audience and not only by laboratories competent in *B. subtilis* methodology, the *B. subtilis* specific methods required to utilize this system will be explained in the following sections.
For the transformation, the Sporovector must be linearized. For this **digest 1-2 µg of the Sporovector with ScaI**. ScaI cuts in the *bla*-gene and therefore linearizes the Plasmid without destroying the *B. subtilis* specific part. This is required, as the integration of the Plasmid follows a double cross-over event at the homologous parts of the *amyE*-gene, leading to an integration of the part inbetween. Without linearization, only a single cross-over event is possible, leading to an instable integration of the whole plasmid and the doubling of the homologous parts (one from the genome and one from the backbone). This opens up the possibility of the loss of the integrated plasmid via homologous recombination. In case the gene of interest contains a ScaI site, alternatively BsaI can be used for linearization.

***Bacillus subtilis* Transformation**

- inoculate 10 ml MNGE (freshly prepared) or simply 1/50 from overnight culture
- let grow to OD_{600} = 1.1-1.3 at 37 °C with agitation (at least 200 rpm!)
- use 400 l cells or trans or ation (in test-tube, not eppendorf!):
    - add DNA (ca. 1-2 µg linearized plasmid **digested with ScaI**)
    - let grow for 1 hour
    - add 100 µl Expression Mix
    - let grow for 1 hour
    - plate on selective media (Chloramphenicol 5 µg/ml)

1 Liter 10 X MN-Medium: (autoclaved)	136 g K_2HPO_4 (x 3 H_2O) 60 g KH_2PO_4 10 g Na-citrat (x 2 H_2O)

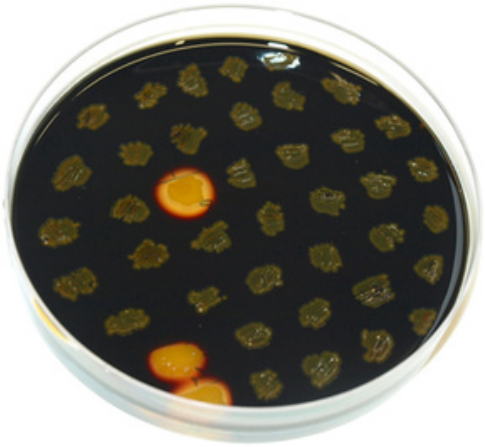
10 ml	**20 ml**	**MNGE-Medium:**
920 µl	1,84 ml	10xMN-Medium
8,28 ml	16,56 ml	Sterile water
1 ml	2 ml	Glucose (20 %)
50 µl	100 µl	K-Glutamat (40 %)
50 µl	100 µl	Fe[III]- ammonium-citrate (2,2 mg/ml)
100 µl	200 µl	Tryptophan (5 mg/ml)
30 µl	60 µl	$MgSO_4$ (1 M)
(100 µl	200 µl	threonine (5 mg/ml) for strains carrying an insertion in thrC)

Expression Mix:	500 µl yeast extract (5 %) 250 µl casamino-acids (CAA) (10 %) 250 µl H_2O (sterile) 50 µl Tryptophan (5 mg/ml)

Screening of the Transformants

The colonies growing on the selective media are mostly positive, though there still is the possibility, that they are either spontaneously resistant or that the *cat*-gene integrated spontaneously at some other locus in the genome. To establish whether the colonies on the plate are positive or not, a screening determining the integrity of the *amyE*-gene is performed, as this is the integration point of the Sporovectors. The gene *amyE* codes or an -Amylase which degrades starch. Disruption of amyE disables *B. subtilis* of degrading starch.

For the screening, produce Replica-plates on Cm5 and on starch plates. Usually 4-8 colonies are sufficient to identify two independent positive colonies. To help with the interpretation, it is advisable to also add the wildtype (W168) to the starch plate as a negative control. Incubate the plates over night at 37 °C. Starch is usually visualized by the starch-iodine reaction with Lugol's iodine that reveals a dark blue color. Pour Lugol's iodine on the plate so that it is covered with a thin film. Around colonies which can degrade starch (WT and wrong colonies), there should be a bright zone around the colony. Correct clones do not show this bright zone. The figure depicts such a starch test plate, which has three negative colonies, seen as bright orange (the color of the starch agar in this case).



Starch plates:		
	Nutrient Broth (Difco)	7,5 g
	Starch	5 g
	Agar	15 g
	H_2O (dest)	ad 1.000 ml

5. Produce and purify spores to test application

- Inoculate the respective *B. subtilis* strain in 50 mL Difco Sporulation Medium (DSM) and let the cultures grow at 37 °C with agitation (220 rpm) for 48 hours to ensure sporulation (the sporulation onsets at approximately 16 hours, but the highest spore titer is reached with 48 hours).
- Then centrifuge spore cultures at 10 000 g for 8 minutes in 50 mL Falcons.
- Resuspend in 10 mL dH_2O and treat for 1 hour at 37 °C with 75 µg/ml lysozyme to lyse remaining cells.
- Wash with 10 mL dH_2O, 10 mL 0.05 % SDS and then three times with 10 mL dH_2O (centrifugation at 10 000 g for 8 minutes). Be careful to decant the supernatant very quickly, almost immediately tilting on the head, to prevent high loss of spores from the pellet. The pellet is not very stable, so it is normal to lose some of it. But with this technique the best results were obtained.
- Resuspend in either 2 mL dH_2O or the appropriate buffer (required for your application).

Difco Sporulation Medium (DSM):

Nutrient Broth	8 g
KCl	1 g
$MgSO_4$ (1 M)	1 ml
$MnCl_2$ (10 mM)	1 ml
H_2O (bidest)	ad 1.000 ml
Add after autoclaving:	
CaCl2 (1 M)	0,5 ml
FeSO4 (1 mM)	1 ml

How to further handle the spores depends on the application at hand. But if colorimetric measurements are desired, measure only the supernatant after pelleting the spores, as they might interfere with the measurement. It is best to test this beforehand.

Supplemental Material for Publication II: "The Bacillus subtilis endospore crust: protein interaction network, architecture and glycosylation state of a potential glycoprotein layer"

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Table of Contents:

Table S1: Strains created and used in this study

Strain	Reference	Genotyp[§]	Antibiotic resistance (*B. subtilis*)
TMB4519	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-gfp-cgeA*	Cm
TMB4521	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cgeA-gfp*	Cm
TMB4858	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-gfp-cotV*	Cm
TMB4859	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotV-gfp*	Cm
TMB4511	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-gfp-cotW*	Cm
TMB4513	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotW-gfp*	Cm
TMB4854	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-gfp-cotX*	Cm
TMB4855	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotX-gfp*	Cm
TMB4523	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm
TMB4525	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotY-gfp*	Cm
TMB4515	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-gfp-cotZ*	Cm
TMB4517	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotZ-gfp*	Cm
TMB1886	iGEM LMU 2012[2]	*ΔcgeA*	none
TMB1891	iGEM LMU 2012[2]	*ΔcotZ*	none
TMB4632	This study	W168, *cotVW::kan*^{r}	Kan
TMB5224	This study	W168, *cotW::kan*^{r}	Kan
TMB4588	This study	W168, *cotVWX::kan*^{r}	Kan
TMB4633	This study	W168, *cotX::kan*^{r}	Kan
TMB4634	This study	W168, *cotXYZ::kan*^{r}	Kan
TMB4587	This study	W168, *cotYZ::kan*^{r}	Kan
TMB4635	This study	W168, *cotVWXYZ::kan*^{r}	Kan
TMB4641	This study	*ΔcgeA*, *cotVW::kan*^{r}	Kan
TMB5226	This study	*ΔcgeA*, *cotW::kan*^{r}	Kan
TMB4640	This study	*ΔcgeA*, *cotVWX::kan*^{r}	Kan
TMB4642	This study	*ΔcgeA*, *cotX::kan*^{r}	Kan
TMB4643	This study	*ΔcgeA*, *cotXYZ::kan*^{r}	Kan
TMB4645	This study	*ΔcgeA*, *cotYZ::kan*^{r}	Kan
TMB4644	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}	Kan
TMB4636	This study	*ΔcotZ*, *cotVW::kan*^{r}	Kan
TMB5225	This study	*ΔcotZ*, *cotW::kan*^{r}	Kan
TMB5237	This study	*ΔcotZ*, *cotVWX::kan*^{r}	Kan
TMB4637	This study	*ΔcotZ*, *cotX::kan*^{r}	Kan
TMB5227	This study	W168, *cotHBywrJ::kan*^{r}	Kan
TMB4520	This study	*ΔcgeA*, *amyE::*P_{cotYZ}*-gfp-cgeA*	Cm
TMB4522	This study	*ΔcgeA*, *amyE::*P_{cotYZ}*-cgeA-gfp*	Cm
TMB4960	This study	*ΔcgeA*, *amyE::*P_{cotYZ}*-gfp-cotV*	Cm
TMB4961	This study	*ΔcgeA*, *amyE::*P_{cotYZ}*-cotV-gfp*	Cm
TMB4830	This study	*ΔcgeA*, *amyE::*P_{cotYZ}*-gfp-cotW*	Cm
TMB4831	This study	*ΔcgeA*, *amyE::*P_{cotYZ}*-cotW-gfp*	Cm
TMB4956	This study	*ΔcgeA*, *amyE::*P_{cotYZ}*-gfp-cotX*	Cm
TMB4957	This study	*ΔcgeA*, *amyE::*P_{cotYZ}*-cotX-gfp*	Cm
TMB4834	This study	*ΔcgeA*, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm
TMB4835	This study	*ΔcgeA*, *amyE::*P_{cotYZ}*-cotY-gfp*	Cm
TMB4832	This study	*ΔcgeA*, *amyE::*P_{cotYZ}*-gfp-cotZ*	Cm
TMB4833	This study	*ΔcgeA*, *amyE::*P_{cotYZ}*-cotZ-gfp*	Cm
TMB4840	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-gfp-cgeA*	Cm
TMB4841	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cgeA-gfp*	Cm
TMB4966	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-gfp-cotV*	Cm
TMB4967	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotV-gfp*	Cm
TMB4512	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-gfp-cotW*	Cm
TMB4514	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotW-gfp*	Cm
TMB4962	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-gfp-cotX*	Cm
TMB4963	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotX-gfp*	Cm
TMB4524	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm

TMB4526	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotY-gfp*	Cm
TMB4516	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-gfp-cotZ*	Cm
TMB4518	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotZ-gfp*	Cm
TMB4666	This study	*cotVW::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cgeA*	Cm, Kan
TMB4667	This study	*cotVW::kan*^{r}, *amyE::*P_{cotYZ}*-cgeA-gfp*	Cm, Kan
TMB4876	This study	*cotVW::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotV*	Cm, Kan
TMB4877	This study	*cotVW::kan*^{r}, *amyE::*P_{cotYZ}*-cotV-gfp*	Cm, Kan
TMB4662	This study	*cotVW::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotW*	Cm, Kan
TMB4663	This study	*cotVW::kan*^{r}, *amyE::*P_{cotYZ}*-cotW-gfp*	Cm, Kan
TMB4872	This study	*cotVW::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotX*	Cm, Kan
TMB4873	This study	*cotVW::kan*^{r}, *amyE::*P_{cotYZ}*-cotX-gfp*	Cm, Kan
TMB4668	This study	*cotVW::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Kan
TMB4669	This study	*cotVW::kan*^{r}, *amyE::*P_{cotYZ}*-cotY-gfp*	Cm, Kan
TMB4664	This study	*cotVW::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotZ*	Cm, Kan
TMB4665	This study	*cotVW::kan*^{r}, *amyE::*P_{cotYZ}*-cotZ-gfp*	Cm, Kan
TMB5253	This study	*cotW::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cgeA*	Cm, Kan
TMB5254	This study	*cotW::kan*^{r}, *amyE::*P_{cotYZ}*-cgeA-gfp*	Cm, Kan
TMB5255	This study	*cotW::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotV*	Cm, Kan
TMB5256	This study	*cotW::kan*^{r}, *amyE::*P_{cotYZ}*-cotV-gfp*	Cm, Kan
TMB5257	This study	*cotW::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotW*	Cm, Kan
TMB5258	This study	*cotW::kan*^{r}, *amyE::*P_{cotYZ}*-cotW-gfp*	Cm, Kan
TMB5259	This study	*cotW::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotX*	Cm, Kan
TMB5260	This study	*cotW::kan*^{r}, *amyE::*P_{cotYZ}*-cotX-gfp*	Cm, Kan
TMB5261	This study	*cotW::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Kan
TMB5262	This study	*cotW::kan*^{r}, *amyE::*P_{cotYZ}*-cotY-gfp*	Cm, Kan
TMB5263	This study	*cotW::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotZ*	Cm, Kan
TMB5264	This study	*cotW::kan*^{r}, *amyE::*P_{cotYZ}*-cotZ-gfp*	Cm, Kan
TMB4605	This study	*cotVWX::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cgeA*	Cm, Kan
TMB4606	This study	*cotVWX::kan*^{r}, *amyE::*P_{cotYZ}*-cgeA-gfp*	Cm, Kan
TMB4870	This study	*cotVWX::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotV*	Cm, Kan
TMB4871	This study	*cotVWX::kan*^{r}, *amyE::*P_{cotYZ}*-cotV-gfp*	Cm, Kan
TMB4601	This study	*cotVWX::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotW*	Cm, Kan
TMB4602	This study	*cotVWX::kan*^{r}, *amyE::*P_{cotYZ}*-cotW-gfp*	Cm, Kan
TMB4866	This study	*cotVWX::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotX*	Cm, Kan
TMB4867	This study	*cotVWX::kan*^{r}, *amyE::*P_{cotYZ}*-cotX-gfp*	Cm, Kan
TMB4607	This study	*cotVWX::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Kan
TMB4608	This study	*cotVWX::kan*^{r}, *amyE::*P_{cotYZ}*-cotY-gfp*	Cm, Kan
TMB4603	This study	*cotVWX::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotZ*	Cm, Kan
TMB4604	This study	*cotVWX::kan*^{r}, *amyE::*P_{cotYZ}*-cotZ-gfp*	Cm, Kan
TMB4678	This study	*cotX::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cgeA*	Cm, Kan
TMB4679	This study	*cotX::kan*^{r}, *amyE::*P_{cotYZ}*-cgeA-gfp*	Cm, Kan
TMB4882	This study	*cotX::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotV*	Cm, Kan
TMB4883	This study	*cotX::kan*^{r}, *amyE::*P_{cotYZ}*-cotV-gfp*	Cm, Kan
TMB4674	This study	*cotX::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotW*	Cm, Kan
TMB4675	This study	*cotX::kan*^{r}, *amyE::*P_{cotYZ}*-cotW-gfp*	Cm, Kan
TMB4872	This study	*cotX::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotX*	Cm, Kan
TMB4873	This study	*cotX::kan*^{r}, *amyE::*P_{cotYZ}*-cotX-gfp*	Cm, Kan
TMB4680	This study	*cotX::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Kan
TMB4681	This study	*cotX::kan*^{r}, *amyE::*P_{cotYZ}*-cotY-gfp*	Cm, Kan
TMB4676	This study	*cotX::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotZ*	Cm, Kan
TMB4677	This study	*cotX::kan*^{r}, *amyE::*P_{cotYZ}*-cotZ-gfp*	Cm, Kan
TMB4690	This study	*cotXYZ::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cgeA*	Cm, Kan
TMB4691	This study	*cotXYZ::kan*^{r}, *amyE::*P_{cotYZ}*-cgeA-gfp*	Cm, Kan
TMB4888	This study	*cotXYZ::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotV*	Cm, Kan
TMB4889	This study	*cotXYZ::kan*^{r}, *amyE::*P_{cotYZ}*-cotV-gfp*	Cm, Kan
TMB4686	This study	*cotXYZ::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotW*	Cm, Kan
TMB4687	This study	*cotXYZ::kan*^{r}, *amyE::*P_{cotYZ}*-cotW-gfp*	Cm, Kan
TMB4884	This study	*cotXYZ::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotX*	Cm, Kan
TMB4885	This study	*cotXYZ::kan*^{r}, *amyE::*P_{cotYZ}*-cotX-gfp*	Cm, Kan
TMB4692	This study	*cotXYZ::kan*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Kan
TMB4693	This study	*cotXYZ::kan*^{r}, *amyE::*P_{cotYZ}*-cotY-gfp*	Cm, Kan

TMB4688	This study	*cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB4689	This study	*cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB4593	This study	*cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB4594	This study	*cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB4864	This study	*cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB4865	This study	*cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB4589	This study	*cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB4590	This study	*cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB4860	This study	*cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB4861	This study	*cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB4595	This study	*cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB4596	This study	*cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB4591	This study	*cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB4592	This study	*cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB4702	This study	*cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB4703	This study	*coVWtXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB4894	This study	*cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB4895	This study	*cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB4698	This study	*cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB4699	This study	*cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB4890	This study	*cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB4891	This study	*cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB4704	This study	*cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB4705	This study	*cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB4700	This study	*cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB4701	This study	*cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB4774	This study	*ΔcgeA*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB4775	This study	*ΔcgeA*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB4930	This study	*ΔcgeA*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB4931	This study	*ΔcgeA*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB4770	This study	*ΔcgeA*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB4771	This study	*ΔcgeA*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB4926	This study	*ΔcgeA*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB4927	This study	*ΔcgeA*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB4776	This study	*ΔcgeA*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB4777	This study	*ΔcgeA*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB4772	This study	*ΔcgeA*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB4773	This study	*ΔcgeA*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB5277	This study	*ΔcgeA*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB5278	This study	*ΔcgeA*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB5279	This study	*ΔcgeA*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB5280	This study	*ΔcgeA*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB5281	This study	*ΔcgeA*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB5282	This study	*ΔcgeA*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB5283	This study	*ΔcgeA*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB5284	This study	*ΔcgeA*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB5285	This study	*ΔcgeA*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB5286	This study	*ΔcgeA*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB5287	This study	*ΔcgeA*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB5288	This study	*ΔcgeA*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB4762	This study	*ΔcgeA*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB4763	This study	*ΔcgeA*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB4924	This study	*ΔcgeA*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB4925	This study	*ΔcgeA*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB4758	This study	*ΔcgeA*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB4759	This study	*ΔcgeA*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB4920	This study	*ΔcgeA*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB4921	This study	*ΔcgeA*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB4764	This study	*ΔcgeA*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB4765	This study	*ΔcgeA*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB4760	This study	*ΔcgeA*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan

TMB4761	This study	*ΔcgeA*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB4786	This study	*ΔcgeA*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB4787	This study	*ΔcgeA*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB4936	This study	*ΔcgeA*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB4937	This study	*ΔcgeA*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB4782	This study	*ΔcgeA*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB4783	This study	*ΔcgeA*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB4932	This study	*ΔcgeA*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB4933	This study	*ΔcgeA*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB4788	This study	*ΔcgeA*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB4789	This study	*ΔcgeA*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB4784	This study	*ΔcgeA*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB4785	This study	*ΔcgeA*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB4798	This study	*ΔcgeA*, *cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB4799	This study	*ΔcgeA*, *cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB4942	This study	*ΔcgeA*, *cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB4943	This study	*ΔcgeA*, *cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB4794	This study	*ΔcgeA*, *cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB4795	This study	*ΔcgeA*, *cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB4938	This study	*ΔcgeA*, *cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB4939	This study	*ΔcgeA*, *cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB4800	This study	*ΔcgeA*, *cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB4801	This study	*ΔcgeA*, *cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB4796	This study	*ΔcgeA*, *cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB4797	This study	*ΔcgeA*, *cotXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB4822	This study	*ΔcgeA*, *cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB4823	This study	*ΔcgeA*, *cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB4954	This study	*ΔcgeA*, *cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB4955	This study	*ΔcgeA*, *cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB4818	This study	*ΔcgeA*, *cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB4819	This study	*ΔcgeA*, *cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB4950	This study	*ΔcgeA*, *cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB4951	This study	*ΔcgeA*, *cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB4824	This study	*ΔcgeA*, *cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB4825	This study	*ΔcgeA*, *cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB4820	This study	*ΔcgeA*, *cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB4821	This study	*ΔcgeA*, *cotYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB4810	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB4811	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB4948	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB4949	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB4806	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB4807	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB4944	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB4945	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB4812	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB4813	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB4808	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB4809	This study	*ΔcgeA*, *cotVWXYZ::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB4714	This study	*ΔcotZ*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB4715	This study	*ΔcotZ*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB4900	This study	*ΔcotZ*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB4901	This study	*ΔcotZ*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB4710	This study	*ΔcotZ*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB4711	This study	*ΔcotZ*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB4896	This study	*ΔcotZ*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB4897	This study	*ΔcotZ*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB4716	This study	*ΔcotZ*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB4717	This study	*ΔcotZ*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB4712	This study	*ΔcotZ*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB4713	This study	*ΔcotZ*, *cotVW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan

TMB5265	This study	*ΔcotZ*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB5266	This study	*ΔcotZ*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB5267	This study	*ΔcotZ*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB5268	This study	*ΔcotZ*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB5269	This study	*ΔcotZ*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB5270	This study	*ΔcotZ*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB5271	This study	*ΔcotZ*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB5272	This study	*ΔcotZ*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB5273	This study	*ΔcotZ*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB5274	This study	*ΔcotZ*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB5275	This study	*ΔcotZ*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB5276	This study	*ΔcotZ*, *cotW::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB5241	This study	*ΔcotZ*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB5242	This study	*ΔcotZ*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB5243	This study	*ΔcotZ*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB5244	This study	*ΔcotZ*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB5245	This study	*ΔcotZ*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB5246	This study	*ΔcotZ*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB5247	This study	*ΔcotZ*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB5248	This study	*ΔcotZ*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB5249	This study	*ΔcotZ*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB5250	This study	*ΔcotZ*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB5251	This study	*ΔcotZ*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB5252	This study	*ΔcotZ*, *cotVWX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB4726	This study	*ΔcotZ*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cgeA*	Cm, Kan
TMB4727	This study	*ΔcotZ*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cgeA-gfp*	Cm, Kan
TMB4906	This study	*ΔcotZ*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Kan
TMB4907	This study	*ΔcotZ*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotV-gfp*	Cm, Kan
TMB4722	This study	*ΔcotZ*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB4723	This study	*ΔcotZ*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotW-gfp*	Cm, Kan
TMB4902	This study	*ΔcotZ*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Kan
TMB4903	This study	*ΔcotZ*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotX-gfp*	Cm, Kan
TMB4728	This study	*ΔcotZ*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Kan
TMB4729	This study	*ΔcotZ*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotY-gfp*	Cm, Kan
TMB4724	This study	*ΔcotZ*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-gfp-cotZ*	Cm, Kan
TMB4725	This study	*ΔcotZ*, *cotX::kan*^{r}, *amyE::*P$_{cotYZ}$*-cotZ-gfp*	Cm, Kan
TMB5329	This study	W168, *cotHBywrJ::kan*^{r}*, amyE::*P$_{cotYZ}$*-gfp-cotW*	Cm, Kan
TMB5153	This study	W168, *cgeAB::mls*^{r}	Ery, linco
TMB5154	This study	*ΔcotZ*, *cgeAB::mls*^{r}	Ery, linco
TMB5155	This study	W168, *cgeB::mls*^{r}	Ery, linco
TMB5156	This study	*ΔcotZ*, *cgeB::mls*^{r}	Ery, linco
TMB5157	This study	W168, *cgeCDE::mls*^{r}	Ery, linco
TMB5158	This study	*ΔcotZ*, *cgeCDE::mls*^{r}	Ery, linco
TMB5163	This study	W168, *spsM::mls*^{r}	Ery, linco
TMB5164	This study	*ΔcotZ*, *spsM::mls*^{r}	Ery, linco
TMB5161	This study	W168, *yodU::mls*^{r}	Ery, linco
TMB5162	This study	*ΔcotZ*, *yodU::mls*^{r}	Ery, linco
TMB5177	This study	W168, *spsA-L::mls*^{r}	Ery, linco
TMB5178	This study	*ΔcotZ*, *spsA-L::mls*^{r}	Ery, linco
TMB5183	This study	W168, *cotSAcotSytxO::mls*^{r}	Ery, linco
TMB5184	This study	*ΔcotZ*, *cotSAcotSytxO::mls*^{r}	Ery, linco
TMB5179	This study	W168, *ytcABC::mls*^{r}	Ery, linco
TMB5180	This study	*ΔcotZ*, *ytcABC::mls*^{r}	Ery, linco
TMB5181	This study	W168, *ytcC::mls*^{r}	Ery, linco
TMB5182	This study	*ΔcotZ*, *ytcC::mls*^{r}	Ery, linco
TMB5185	This study	W168, *glgBCDAP::mls*^{r}	Ery, linco
TMB5186	This study	*ΔcotZ*, *glgBCDAP::mls*^{r}	Ery, linco
TMB5189	This study	W168, *yfnHGFED::mls*^{r}	Ery, linco
TMB5190	This study	*ΔcotZ*, *yfnHGFED::mls*^{r}	Ery, linco
TMB5191	This study	W168, *ykvP::mls*^{r}	Ery, linco
TMB5192	This study	*ΔcotZ*, *ykvP::mls*^{r}	Ery, linco

TMB5239	This study	W168, *spsMcgeB::mls^r, spsA-L::spec^r*	Ery, linco, spec
TMB5712	This study	W168, *cgeB::kan^r, yfnHGFED::mls^r*	Ery, linco, Kan
TMB5713	This study	W168, *cgeCDE::kan^r, yfnHGFED::mls^r*	Ery, linco, Kan
TMB5714	This study	W168, *spsA-L::spec^r, yfnHGFED::mls^r*	Ery, linco, spec
TMB5361	This study	W168, *cgeAB::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5362	This study	*ΔcotZ*, *cgeAB::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5363	This study	W168, *cgeB::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5364	This study	*ΔcotZ*, *cgeB::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5365	This study	W168, *cgeCDE::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5366	This study	*ΔcotZ*, *cgeCDE::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5371	This study	W168, *spsM::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5372	This study	*ΔcotZ*, *spsM::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5369	This study	W168, *yodU::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5370	This study	*ΔcotZ*, *yodU::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5385	This study	W168, *spsA-L::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5386	This study	*ΔcotZ*, *spsA-L::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5391	This study	W168, *cotSAcotSytxO::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5392	This study	*ΔcotZ*, *cotSAcotSytxO::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5387	This study	W168, *ytcABC::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5388	This study	*ΔcotZ*, *ytcABC::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5389	This study	W168, *ytcC::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5390	This study	*ΔcotZ*, *ytcC::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5393	This study	W168, *glgBCDAP::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5394	This study	*ΔcotZ*, *glgBCDAP::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5397	This study	W168, *yfnHGFED::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5398	This study	*ΔcotZ*, *yfnHGFED::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5399	This study	W168, *ykvP::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5400	This study	*ΔcotZ*, *ykvP::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotV*	Cm, Ery, linco
TMB5401	This study	W168, *cgeAB::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5402	This study	*ΔcotZ*, *cgeAB::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5403	This study	W168, *cgeB::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5404	This study	*ΔcotZ*, *cgeB::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5405	This study	W168, *cgeCDE::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5406	This study	*ΔcotZ*, *cgeCDE::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5616	This study	W168, *spsM::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5617	This study	*ΔcotZ*, *spsM::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5614	This study	W168, *yodU::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5615	This study	*ΔcotZ*, *yodU::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5630	This study	W168, *spsA-L::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5631	This study	*ΔcotZ*, *spsA-L::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5636	This study	W168, *cotSAcotSytxO::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5637	This study	*ΔcotZ*, *cotSAcotSytxO::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5632	This study	W168, *ytcABC::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5633	This study	*ΔcotZ*, *ytcABC::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5634	This study	W168, *ytcC::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5635	This study	*ΔcotZ*, *ytcC::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5638	This study	W168, *glgBCDAP::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5639	This study	*ΔcotZ*, *glgBCDAP::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5642	This study	W168, *yfnHGFED::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5643	This study	*ΔcotZ*, *yfnHGFED::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5644	This study	W168, *ykvP::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5645	This study	*ΔcotZ*, *ykvP::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotX*	Cm, Ery, linco
TMB5646	This study	W168, *cgeAB::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Ery, linco
TMB5647	This study	*ΔcotZ*, *cgeAB::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Ery, linco
TMB5648	This study	W168, *cgeB::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Ery, linco
TMB5649	This study	*ΔcotZ*, *cgeB::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Ery, linco
TMB5650	This study	W168, *cgeCDE::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Ery, linco
TMB5651	This study	*ΔcotZ*, *cgeCDE::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Ery, linco
TMB5656	This study	W168, *spsM::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Ery, linco
TMB5657	This study	*ΔcotZ*, *spsM::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Ery, linco
TMB5654	This study	W168, *yodU::mls^r, amyE::*P$_{cotYZ}$*-gfp-cotY*	Cm, Ery, linco

TMB5655	This study	*ΔcotZ*, *yodU::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5670	This study	W168, *spsA-L::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5671	This study	*ΔcotZ*, *spsA-L::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5676	This study	W168, *cotSAcotSytxO::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5677	This study	*ΔcotZ*, *cotSAcotSytxO::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5672	This study	W168, *ytcABC::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5673	This study	*ΔcotZ*, *ytcABC::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5674	This study	W168, *ytcC::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5675	This study	*ΔcotZ*, *ytcC::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5678	This study	W168, *glgBCDAP::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5679	This study	*ΔcotZ*, *glgBCDAP::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5682	This study	W168, *yfnHGFED::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5683	This study	*ΔcotZ*, *yfnHGFED::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5684	This study	W168, *ykvP::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco
TMB5685	This study	*ΔcotZ*, *ykvP::mls*^{r}, *amyE::*P_{cotYZ}*-gfp-cotY*	Cm, Ery, linco

§ The strains all derive from the *B. subtilis* wildtype W168 or the mutant strains. The mutant strains derive from a genomic replacement of the respective genes with the *mls*^{r}, kan^r or spec^r-cassette in the wildtype W168. All of the other strains derived from the transformation of the respective Sporovectors containing GFP (*gfp*) integrated into the amyE-locus.

Table S2: Plasmids used in this study

Plasmid§	Reference	Backbone	Integration Site	Antibiotic resistance	
				E. coli	*B. subtilis*
p1CSV-CgeA-N-GFP	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CgeA-C-GFP	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotV-N-GFP	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotV-C-GFP	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotW-N-GFP	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotW-C-GFP	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotX-N-GFP	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotX-C-GFP	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotY-N-GFP	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotY-C-GFP	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotZ-N-GFP	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotZ-C-GFP	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm

§ The Plasmids are annotated according to the backbone and the insert connected with a hyphen, which is also annotated in the according column. The denomination derives from a shortened code for the pBS1C (p1C) as the backbone, SV for Sporovector, followed by the crust protein coded in the Sporovector and the translational fusion site (N=N-terminal, C=C-terminal). They all confer ampicillin resistance in *E. coli* and chloramphenicol resistance in *B. subtilis*. In *B. subtilis* they integrate into the genome at the *amyE*-locus, which can be verified by the inability to degrade starch on starch minimal plates.

Table S3: Primers used in this study

Primer number	Primername	Sequence (5‘ to 3‘)§
TM0137	Kan-fwd	cagcgaaccatttgaggtgatagg
TM0138	Kan-rev	cgatacaaattcctcgtaggcgctcgg
TM0139	Mls-fwd	CAGCGAACCATTTGAGGTGATAGGgatcctttaactctggcaaccctc
TM0140	Mls-rev	CGATACAAATTCCTCGTAGGCGCTCGGgccgactgcgcaaaagacataatcg
TM0141	Spec-fwd	CAGCGAACCATTTGAGGTGATAGGgactggctcgctaataacgtaacgtgactggcaagag
TM0142	Spec-rev	CGATACAAATTCCTCGTAGGCGCTCGGcgtagcgagggcaaggggtttattgttttctaaaatctg
TM4906	CotVWX_LFH_up_fwd	gtggttcatatcttccttctatcc
TM4907	CotVWX_LFH_up_rev	CCTATCACCTCAAATGGTTCGCTGaaccttcactcctttcttatcc
TM4908	CotVWX_LFH_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGgacctaaaagcagagctaaaaac
TM4909	CotVWX_LFH_do_rev	tctggctcatattatccctcc
TM4910	CotYZ_LFH_up_fwd	gaacagatcagacaaagacgg
TM4911	CotYZ_LFH_up_rev	CCTATCACCTCAAATGGTTCGCTGtgatttcagctccttctttatagg
TM4912	CotYZ_LFH_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGtcataagctggaaaagcttgca
TM4913	CotYZ_LFH_do_rev	gatagaatatgacacaatcggagg
TM5022	LFH VW do fwd	CGAGCGCCTACGAGGAATTTGTATCGccaatcataaaaaatagggttctc
TM5023	LFH VW do rev	ACTGCATCGCATACACAGTTC
TM5024	LFH X up fwd	TGCACCCTGCAATATTTGAGC
TM5025	LFH X up rev	CCTATCACCTCAAATGGTTCGCTGATCTATTCCTCCTTTTCTTTTACTG
TM5026	LFH X down fwd	CGAGCGCCTACGAGGAATTTGTATCGgacctaaaagcagagctaaaaacg
TM5027	LFH X down rev	TTTTCTACAGCTTCACGCACGC
TM5879	CotW_up_fwd	gcaccaatagcagctcagc
TM5880	CotW_up_rev	CCTATCACCTCAAATGGTTCGCTGttgttttcacctcgtcttttaagtttg
TM5881	CotH_up_fwd	gttatgtcatgacatatatctaatacagggc
TM5882	CotH_up_rev	CCTATCACCTCAAATGGTTCGCTGccataatcctccttacaaatttaggc
TM5883	YwrJ_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGtgtatacaaaactgccctcttg
TM5884	YwrJ_do_rev	gtcttcccaaaagcctgc
TM5891	CgeA_up_fwd	ctgttaattaatgtggcataacggg
TM5892	CgeA_up_rev	CCTATCACCTCAAATGGTTCGCTGtacacacacctcctattcgatagtg
TM5893	CgeB_up_fwd	gaagtgtatctccgttgatcaaattg
TM5894	CgeB_up_rev	CCTATCACCTCAAATGGTTCGCTGcggatgttatgaaaagaacgtaacg
TM5895	CgeB_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGaaaatcgttcattgctatgaacg
TM5896	CgeB_do_rev	cattacagatccgaaccatcc
TM5897	CgeC_up_fwd	ccagtcgtgaggaacgc
TM5898	CgeC_up_rev	CCTATCACCTCAAATGGTTCGCTGatcatccctctcccttatccttatc
TM5899	CgeE_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGaataggcgtttgcacaaacc
TM5900	CgeE_do_rev	ctgagatagctggataccgc
TM5903	SpsA_up_fwd	cttcatcaaatgtaatgtaatcgagg
TM5904	SpsA_up_rev	CCTATCACCTCAAATGGTTCGCTGatcctacacctccttcttgg
TM5905	SpsL_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGaaataggaacgtgatcaatcgaag

TM5906	SpsL_do_rev	ctcagtctctgactacaaatctatcg
TM5907	YodU_up_fwd	taggatggttcggatctgtaatgc
TM5908	YodU_up_rev	CCTATCACCTCAAATGGTTCGCTGggcttatcctccaactctcg
TM5909	YodU_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGtattaagatacttactacatatctaacgaaaaaaagaagc
TM5910	YodU_do_rev	ttgggagcaaacaatcaaggg
TM5911	YpqP_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGaaacaagcctgtctatccattagg
TM5912	YpqP_do_rev	gatgtattcccttatgaaaaactgc
TM5913	YtcA_up_fwd	gctatcagcttctaatgcctcc
TM5914	YtcA_up_rev	CCTATCACCTCAAATGGTTCGCTGcacctgagctcctttacaagattac
TM5915	YtcC_up_fwd	ggaaaagcgatttacgttcataaagg
TM5916	YtcC_up_rev	CCTATCACCTCAAATGGTTCGCTGttttattccccctgatacagc
TM5917	YtcC_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGaacctacagctcgtgttgc
TM5918	YtcC_do_rev	gtcaagagaccttcgtattctgc
TM5919	CotSA_up_fwd	cagcccgtcttcaataaagc
TM5920	CotSA_up_rev	CCTATCACCTCAAATGGTTCGCTGacatagcctccattcctgc
TM5923	YtxO_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGagtcacatttatgccttttttaagttctc
TM5924	YtxO_do_rev	gatttaaactcttatccgcccg
TM5925	YfnH_up_fwd	cgcttgagcaattcttcaagg
TM5926	YfnH_up_rev	CCTATCACCTCAAATGGTTCGCTGctctacccctcatctctatgaatattg
TM5927	YfnD_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGaaaaccccctgccgcc
TM5928	YfnD_do_rev	tcaggccaggcgatgg
TM5929	GlgB_up_fwd	gtcggcggttcgattcc
TM5930	GlgB_up_rev	CCTATCACCTCAAATGGTTCGCTGgtaatcatcctttctgacatcatag
TM5933	GlgP_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGaaaagccgctgtataagcg
TM5934	GlgP_do_rev	gatgacgaaacatttaatgtcatgc
TM6038	YkvP_up_fwd	gctctactgctggatcaattgg
TM6039	YkvP_up_rev	CCTATCACCTCAAATGGTTCGCTGacatttccatcctctctttcac
TM6040	YkvP_do_fwd	CGAGCGCCTACGAGGAATTTGTATCGaggagagtaatatgaatgaagggg
TM6041	YkvP_do_rev	gctaaatactcgtaatcatatgatccg

§ Underlined: The primers for the resistance cassettes (mls^r, spec^r) include overhangs that match the kan^r cassette, as this was the best for the fusion PCR and additionally allows the utilization of all cassettes without changing the primer sequence. Uppercase: The primers for the up (reverse primer) and do (forward primer) fragments to produce the mutants, which include overhangs reverse complementary to the kan^r cassette overhang, to fuse these fragments with the resistance cassette in a fusion PCR. Lowercase: The part complementary to the gene.

Table S4: Clusters and operons of the SigK and SigE regulon containing glycosyltransferases

	Genetic context	Function (according to Subtiwiki)	Gene	Gene Product (according to Subtiwiki)	Pfam domains	Swissprot	Comments (Location etc.)	Genomic coordinates	Paralogues Proteins	Regulon	Known Phenotypes	References
cgeAB and *cgeCDE*	Next to *spsM* (split gene derived from *yodU* and *ypqP* by excision of SP Prophage) Next to *yodTSRQPkamA yokU*, which could potentially synthesize a side chain based on lysine	maturation of the outermost layer of the spore	*cgeA*	spore crust protein	-	-	outermost layer of the spore coat (the spore crust), more abundant at the mother cell-proximal pole of the forespore	2,148,676 2,149,077	-	SigK	Tendency for spores to clump	[3]
			cgeB	Unknown	Glycosyl transferases group 1	msrB Met sulfoxide reductase family	-	2,149,084 2,150,037	-	SigK		
			cgeC	Unknown	-	-	-	2,148,166 2,148,471	-	SigK	-	-
			cgeD	Unknown	Glycosyl transferase family 2	-	-	2,146,821 2,148,101	-	SigK		
			cgeE	Unknown	Acetyltransferase (GNAT) family	antibiotic N-acetyltransferase family	-	2,146,013 2,146,792	-	SigK		
spsABCDEFGIJKL	Nothing noticable	spore coat polysaccharide synthesis	*spsA*	nucleotide-sugar-dependent glycosyltransferase	Glycosyl transferase family 2	-	-	3,892,351 3,893,121	-	SigK, SigE	small germination defect, more hydrophobic, highly adhesive, form extensive clumps (spsIJKL: predicted to produce Rhamnose)	[4-7]
			spsB	Unknown	CDP-Glycerol:Poly(glycerophosphate) glycerophosphotransferase	-	Outer spore coat (requires CotE)	3,890,922 3,892,346	-	SigK, SigE		
			spsC	Unknown	DegT/DnrJ/EryC1/StrS aminotransferase family	degT/dnrJ/eryC1 family	binds the outer surface of the forming spore	3,889,732 3,890,901	-	SigK, SigE		
			spsD	Unknown	Acetyltransferase (GNAT) family	-	-	3,888,862 3,889,731	-	SigK, SigE		
			spsE	Unknown	NeuB (prokaryotic N-acetylneuraminic acid (Neu5Ac) synthase) family SAF domain	-	-	3,887,741 3,888,862	-	SigK, SigE		
			spsF	Unknown	Cytidylyltransferase	CMP-NeuNAc synthetase family	-	3,887,026 3,887,748	-	SigK, SigE		
			spsG	Unknown	Glycosyltransferase family 28 C-terminal domain	-	-	3,886,004 3,887,023	-	SigK, SigE		
		rhamnose biosynthesis, spore coat	*spsI*	glucose-1-phosphate thymidylyltransferase	Nucleotidyl transferase	glucose-1-phosphate thymidylyltransferase family	outer spore coat	3,885,239 3,885,979	-	SigK, SigE		

Table S4: Clusters and operons of the SigK and SigE regulon containing glycosyltransferases

		polysaccharide synthesis	*spsJ*	dTDP -glucose-4,6-dehydratase	GDP-mannose 4,6 dehydratase	sugar epimerase family	Mother cell cytoplasm	3,884,292 3,885,239	YfnG	SigK, SigE		
			spsK	dTDP-4-dehydrorhamnose reductase	RmlD substrate binding domain	dTDP-4-dehydrorhamnose reductase family	Outer spore coat (transient)	3,883,427 3,884,278	-	SigK, SigE		
			spsL	dTDP -4-dehydrorhamnose-3,5-epimerase	dTDP-4-dehydrorhamnose 3,5-epimerase	-	-	3,882,979 3,883,434	-	SigK, SigE		
spsM Splitgene (split by SP Prophage)	Next to *cgeAB* and cgeCDE	spore envelope polysaccharide biosynthesis	*yodU*	Unknown	Polysacc_synt_2	-	Mother cell cytoplasm, colocalizes with YfnH	2,151,626 2,152,045	EpsC	SigK	Less water dispersal more adhesion, loss of polysaccharide layer (Galactose, Rhamnose)	[7, 8]
			SPβ Prophage	n.A.	n.A.	n.A.	Genetic excision upon Sporulation	2,152,086 2,286,408	n.A.	SigA		
			ypqP	Unknown	Polysacc_synt_2	-	Mother cell cytoplasm, colocalizes with YfnH	2,286,448 2,287,053	EpsC	SigK		
ytcABC	Directly next to *cotSAcotSytxO* and near to *glgBCDAP* Next to *ytdA* (a putative UTP-glucose-1-phosphate uridylyltransferase locating to the spore coat)	Unknown	*ytcA*	Unknown	UDP-glucose/GDP-mannose dehydrogenase family, NAD binding domain / central domain / UDP binding domain	-	-	3,155,725 3,157,011	TuaD, Ugd	SigK	-	[7]
			ytcB	putative UDP-glucose epimerase	NAD dependent epimerase/dehydratase family	sugar epimerase family	Mother cell cytoplasm	3,157,008 3,157,958	YfnG	SigK		
		lipopolysaccharide biosynthesis	*ytcC*	sporulation protein	Glycosyltransferase Family 4 / Glycosyl transferases group 1	-	-	3,157,961 3,159,184	CotSA	SigK		
cotSAcotSytxO	Directly next to *ytcABC* and near to *glgBCDAP*	resistance of the spore	*cotSA*	spore coat protein	Glycosyltransferase Family 4 / Glycosyl transferases group 1	glycosyltransferase 1 family	-	3,160,761 3,161,894	YtcC	SigK	-	-
			cotS	spore coat protein	-	cotS family	outer spore coat, localization depends on CotE	3,159,691 3,160,746	-	SigK		
		protection of the spore	*ytxO*	spore coat protein	-	-	outer spore coat, localization depends on CotE	3,159,258 3,159,689	-	SigK		
yfnH GFED	Nothing noticable	Unknown	*yfnH*	Unknown	Nucleotidyl transferase	-	similar to glucose-1-phosphate cytidylyltransferase	798,469 799,233	-	SigK	-	[7, 9]

Table S4: Clusters and operons of the SigK and SigE regulon containing glycosyltransferases

							Mother cell cytoplasm, colocalizes with SpsM					
			yfnG	Unknown	GDP-mannose 4,6 dehydratase	sugar epimerase family	similar to CDP-glucose 4,6-dehydratase	799,303 800,208	SpsJ, YtcB	SigK		
			yfnF	Unknown	Nucleotide-diphospho-sugar transferase	-	-	800,232 801,143	-	SigK		
			yfnE	Unknown	Glycosyl transferase family 2	-	-	801,172 802,350	-	SigK, SigE		
			yfnD	Unknown	Nucleotide-diphospho-sugar transferase	-	-	802,351 803,286	-	SigK, SigE		
glgBCDAP	Near to *cotSAcotSytxO* and *ytcABC*	Glycogen Biosynthesis	*glgB*	1,4-alpha-glucan branching enzyme	CBM_48 Alpha-amylase Alpha-amylase_C	glycosyl hydrolase 13 family	-	3,169,763 3,171,646	AmyX	SigE	-	*10*
			glgC	glucose-1-phosphate adenylyltransferase	NTP_transferase	bacterial/plant glucose-1-phosphate adenylyltransferase family	-	3,168,624 3,169,766	GlgD	SigE		
			glgD	glucose-1-phosphate adenylyltransferase	NTP_transferase	bacterial/plant glucose-1-phosphate adenylyltransferase family	-	3,167,569 3,168,600	GlgC	SigE		
			glgA	glycogen synthase (ADPGlc)	Glyco_transf_5 (Starch synthase catalytic domain) Glycos_transf_1	Bacterial/plant glycogen synthase subfamily	-	3,166,118 3,167,572	-	SigE		
			glgP	glycogen phosphorylase	Phosphorylase	glycogen phosphorylase family	phosphorylation on (Thr-291 OR Ser-294)	3,163,735 3,166,131	-	SigE		
ykvPykzQykvQykzR	Nothing noticable	Unknown	*ykvP*	Unknown,	Glyco_trans_1_2 LysM	(localization spore wall)	-	1,444,099 1,445,298	-	SigK	-	*11, 12*
			ykzQ	Unknown,	LysM	-	Localization: outer spore coat	1,445,314 1,445,541	-	SigK		
			ykvQ	Unknown	Glyco_hydro_18	glycosyl hydrolase 18 family	-	1,445,638 1,446,336	-	SigK		
			ykzR	Unknown	-	-	-	1,446,317 1,446,568	-	-		

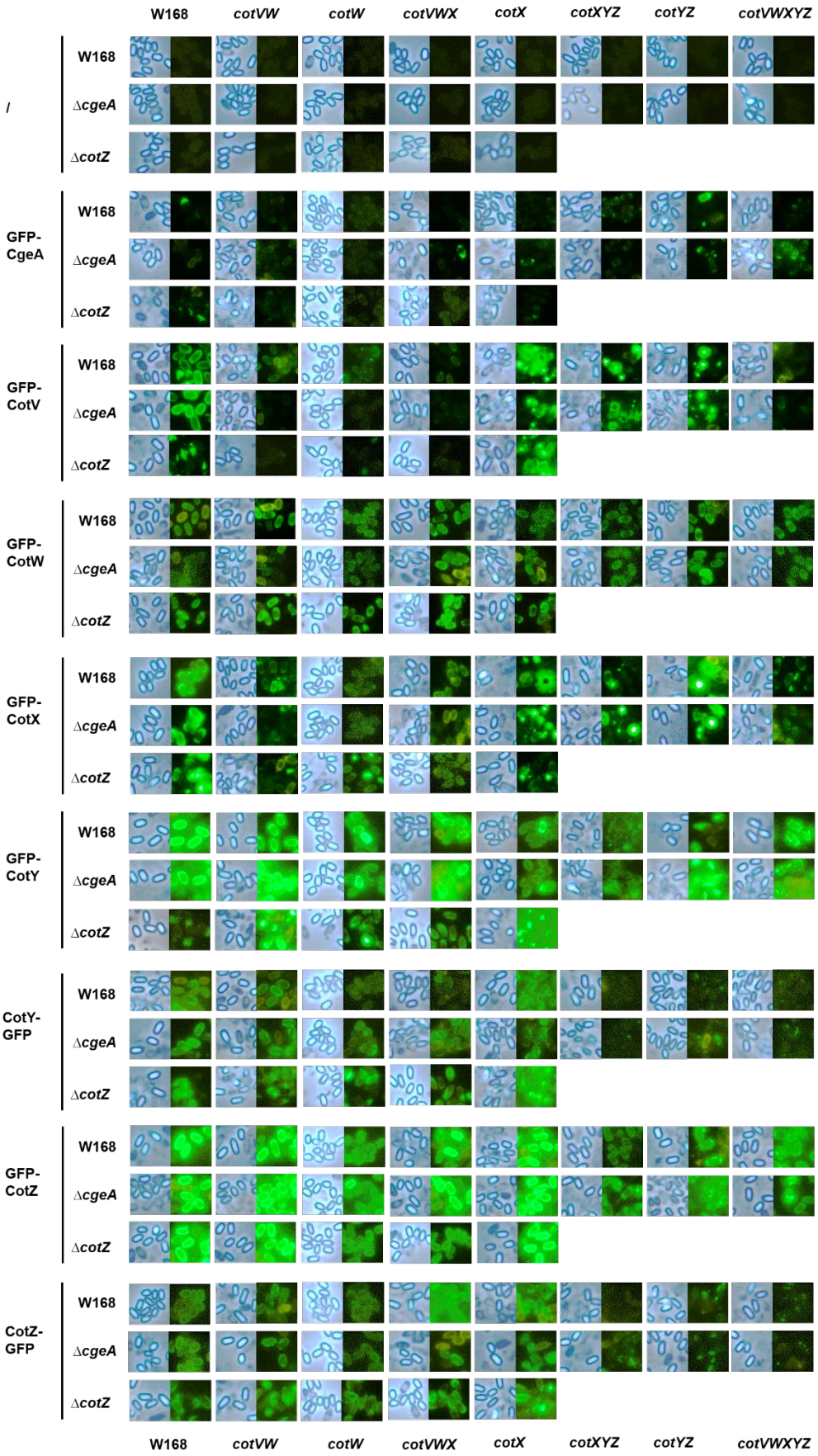

Figure S 1: GFP-Matrix of the genetic interaction between the crust genesThe figure shows the complete data set of the genetic interaction of the crust genes examined by expressing a GFP-fusion to a crust proteins in a collection of crust gene mutants. The settings of the microscope, as well as the editing oft he pictures were the same for all of the pictures.

Discussion on the limitation of ectopic GFP-fusions for the assessement of abundancy and distribution:

First, fluorescent microscopy is (as it was done here) only a semi-quantitative method and is not the best method of choice for the assessment of abundancies. Yet it was utilized here to give the reader a first impression of the following data collected with genetic interaction studies. The conclusions reached fit the model, as well as what is to be expected from the operon structure in combination with the promoter strength (published in Bartels et al. 20181). Any discrepancies to already published data[13-15] could come from differences in the fusion site, as this is the first study utilizing N-terminal GFP-fusions.

The fusion of GFP to a protein can always lead to it not behaving natively anymore, it might lose part of its affinity to parts of the spore, the stability or its functionality.

Affinity: This is the case for some of the C-terminal variants: The theory for at least some of the proteins, such as CotY and CotZ, but could also be the case for CotX and CotV, is that during the assembly of the structure there seems to be a necessity for at least some of the proteins to have a free C-terminus. This would fit the head-to-tail structure of the CotY when overexpressed: in one of the orientations the GFP can be incorporated, while in the other it has to be the native protein). Therefore, less of the GFP-fusion can be incorporated into the native structure, but if the native protein (and therefore the free C-terminus) is additionally missing, it cannot be incorporated into the structure at all. Therefore, the C-terminal CotY and CotZ fusions behave similarly in the genetic interaction than the N-terminal counterpart albeit in lower abundancies, except for the mutants lacking the native CotY/CotZ.

Stability: The stability seems not to be affected, as the GFP stays stable on the spores for at least one year (data not shown).

Functionality: The functionality (i.e. if it still behaves natively in the structure) is hard to assess due to a missing assay for this particular function, but the spores expressing the GFP-fusions behaved like the wildtype (appearance in microscopy, sedimentation during centrifugation, sporulated colony morphology, non-clumping or adhesion to diverse surfaces…). Nevertheless, this must be kept in mind, when interpreting the abundancies and the distribution of the GFP-fusions.

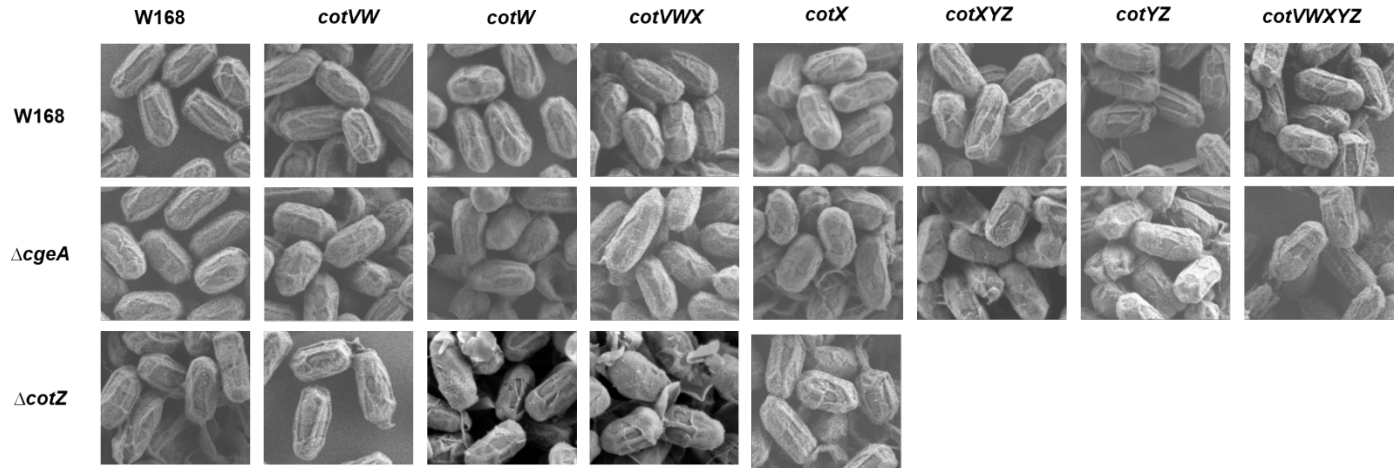

Figure S 2: Complete SEM dataset for crust gene mutants
This figure shows the complete dataset of the (native) SEM of all crust gene mutants. The differences in quality are due to different days of data collection.

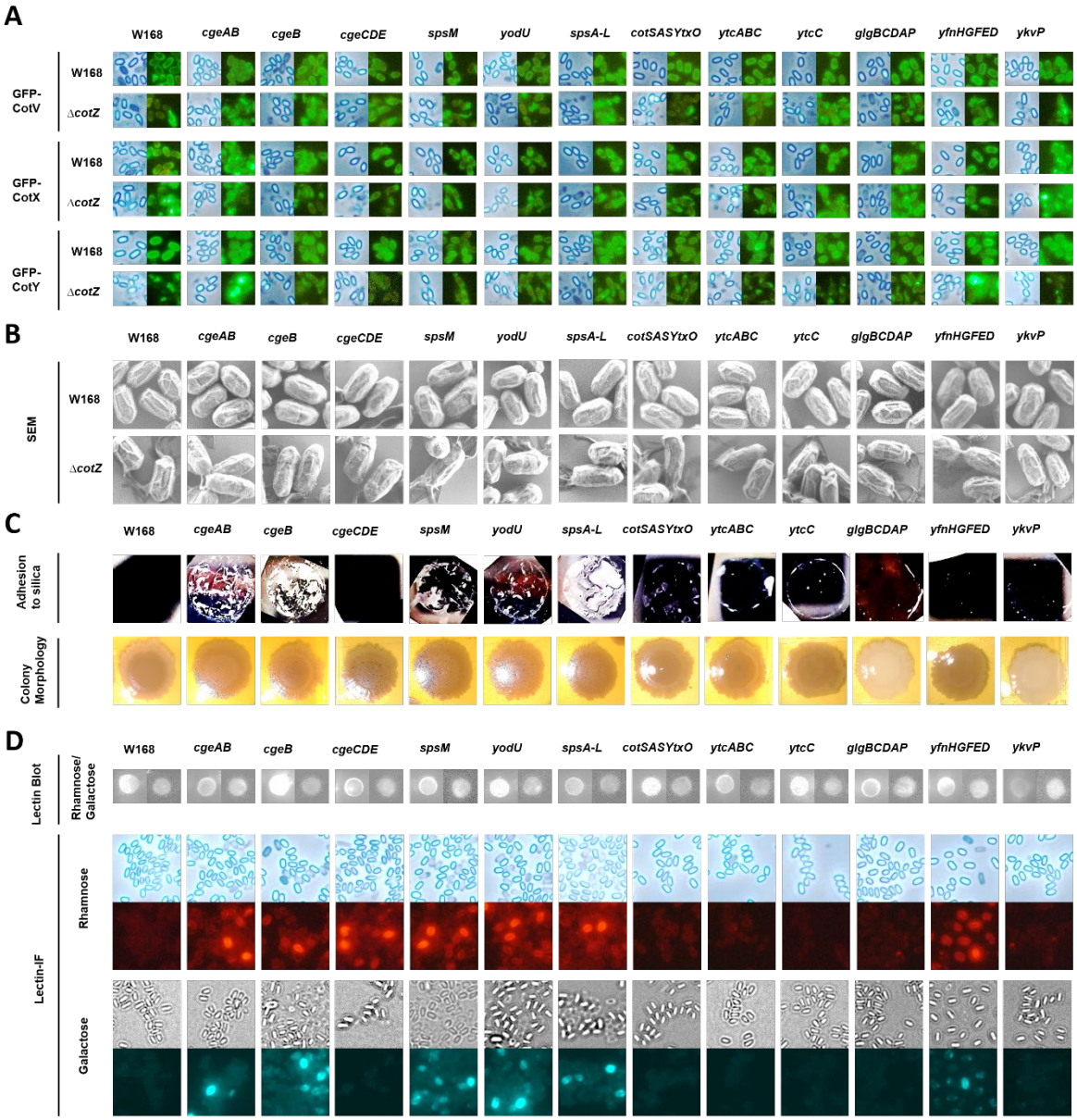

Figure S 3: Complete dataset for Glycosyltransferases

This figure shows the dataset for the complete collection of the glycosyltransferase mutants. This dataset shows the involvement of cgeB, cgeD, spsM, spsA-L and yfnHGFED in the glycosylation of the crust. ***A****: Glycosyltransferase mutants expressing GFP-fusions to the three major structural components of the crust. In the wildtype the data show that the crust is still present in these mutants. In the crust displacement mutant (cotZ) the data show, that the structure is still intact, as it displaces similiar to the control. Therefore the glycosylation of the crust is not essential fort he structural integrity oft he crust.* ***B****: Glycosyltransferase mutants in SEM showing, that the crust is still present and intact and the structure is not perturbed, as seen in the crust displacement mutant (cotZ). This supports the statement made from the GFP-data, that the glycosylation oft he crust is not essential fort he structural integrity of the crust.* ***C****: Phenotype of the glycosyltransferase mutants: on the top the adhesive properties to silica, which shows, that mutants involving cgeB, spsM and spsA-L are more hydrophobic, probably due to the loss of the polysaccharide layer. Mutants involving cgeD and yfnHGFED do not show the same effect. On the bottom: the colony morphology of the glycosyltransferase mutants. The wildtype shows a mucus like shine after sporulation. The mutants involving cgeB, cgeD, spsM and spsA-L have lost this, indicating a loss of the polysaccharide layer. This indicates, that the mutant missing cgeD has lost the polysaccaride layer, but not the determinant of hydrophilicity, as it does not adhere to silica.* ***D****: Data utilizing lectins against Galactose (AIA, lectin from Artocarpus integrifolia (Jacalin)) and Rhamnose (CSL, Oncorhynchus keta L-rhamnose binding lectin). On the top: lectin blot of spore surface extracts from the glycosyltransferase mutants showing that there seems to be no major changes in the quantity of the sugars on the spore. On the bottom: Lectin stain of the spores from the glycosyltransferase mutants. Contrary to the expectation the wildtype shows no significant signal for neither Galactose nor Rhamnose. But in the glysosyltransferase mutants involving cgeB, cgeD, spsM, spsA-L and yfnHGFED there is a signal for both (except for cgeD, which misses a signal for Galactose). When comparing this with the lectin stain of boiled probes (data shown in main paper Figure 3), which looks quite similiar, this could indicate, that the lectins bind monosaccharides in these mutants, but not the sugar moieties in the polysaccharide context (like structure or modifications). This indicates that in mutants missing cgeB, cgeD, spsM, spsA-L and yfnHGFED the polysaccharide structure is disturbed, but not the addition of these sugars. This could be the case due to the redundancy of these genes in the involvement of the addition of the sugars, but the joint action of these in the production of the polysaccharide layer. Additionally the mutant yfnHGFED still seems to produce the*

polysaccharide layer (see data from C), but has a strong signal for Rhamnose and a signal for Galactose. This indicates, that the polysaccharide structure or modification is somehow disturbed.

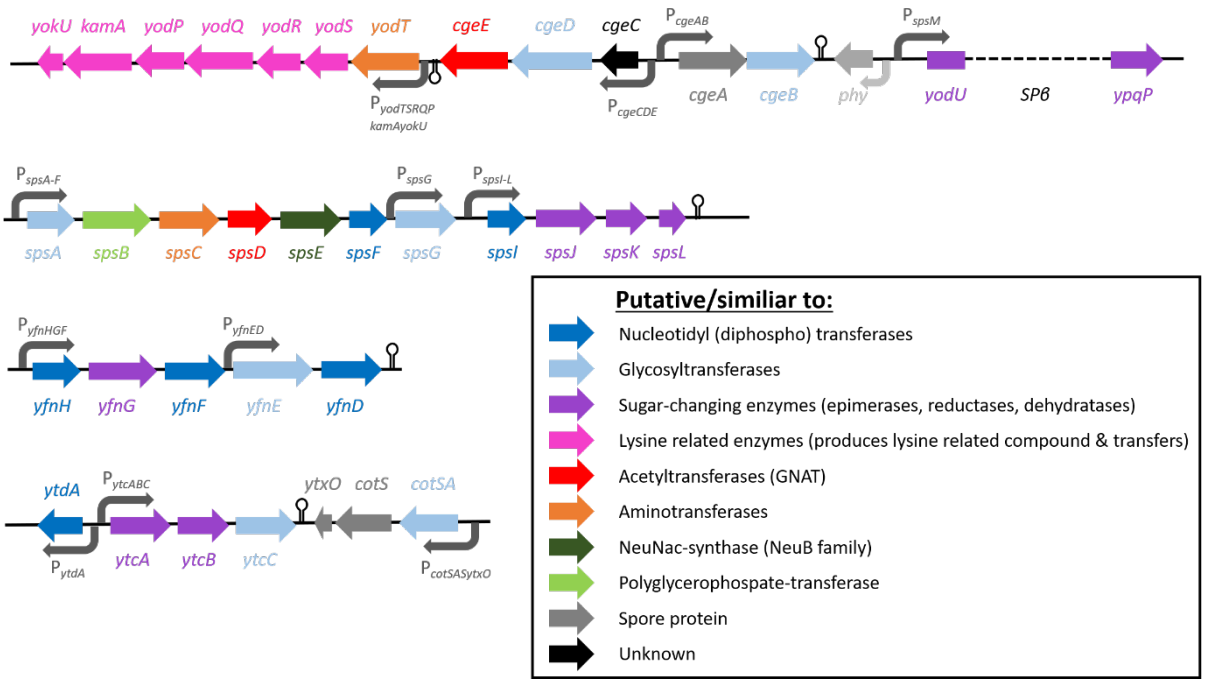

Figure S 4:Operon structure and predicted functions of the genes presumably involved with crust polysaccharides
Predicted functions of the gene products of all genes shown to be involved in the synthesis or the addition of the polysaccharides to the crust. The two operons ytcABC and cotSASytxO were only shown to produce a coat phenotype, but as these operons contain glycosyltransferases and sugar epimerases, as well as having a nucleotidyl transferase next to it (ytdA), which was shown to be phenotypically involved with the spore polysaccharides[7], it is tempting to think that there is an involvement of the crust polysaccharides at the intersection of outer coat and crust. Another operon yodTSRQPkamAyokU found next to cgeCDE is also shown, as it might also potentially be involved, as it also contains an aminotransferase, like spsC, which is required for explaining the high level of redundancy. It also contains putative enzymes which produce a lysine-related compound and potentially transfers it. This could play a role in an unusual capping sugar. The functions of the genes are color coded and this color code is utilized in all further figures.

Plausible sugar biosynthesis pathway of Rhamnose-related variant (including rare sugar Viosamine)

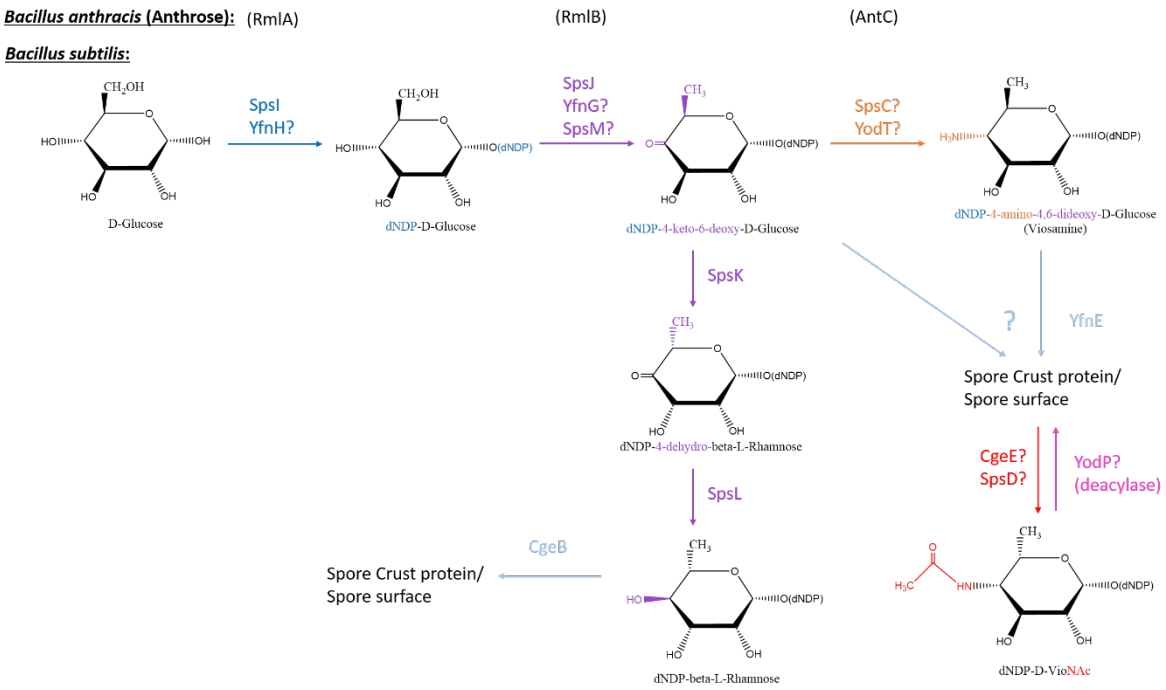

Figure S 5: Plausible sugar biosynthesis pathway of the rhamnose-related variant

Derivated from the data of this study there seems to be a joined biosynthesis pathway between spsA-L (where spsI-L were already predicted to produce rhamnose[5]) and the unidentified sugar transferred presumably by YfnE as well as the further involvement of cgeB and spsM (as this sugar also accumulates in these mutants). The pathway predicted for rhamnose is shown, which is over the sugar intermediate dNDP-4-keto-deoxy-D-glucose (for rhamnose it is predicted to be dTDP, but as it is not certain and also there might be redundancies involving other nucleotide carriers, this is held general). The first and second step of the pathway could also potentially be catalyzed by YfnH and YfnG/SpsM respectively, which explains the redundancy. The keto-group is a requirement for the aminotransferase activity predicted for SpsC and YodT, resulting in dNDP-4-amino-4,6-dideoxy-D-glucose, also known as the rare sugar viosamine[16]. This pathway is also predicted for the related species Bacillus anthracis (the respective enzymes are written on top over the corresponding pathway step) and also part of the rhamnose synthesis pathway[17, 18]. The aminogroup then again is a prerequisite for the predicted acetyltransferase activity of CgeE and SpsD, leading to the rare sugar N-acetyl-viosamine (VioNac), which is a plausible reaction also found in the mimivirus[19]. If this reaction occurs in the mother cell cytoplasm or already on the spore surface and at what point the sugar is transferred by (presumably) YfnE is not sure, but as SpsC was shown to bind the spore surface[6] it could be, that the sugar intermediate 4-keto-6-deoxy-D-Glucose is already transferred to the spore crust and then further modified. This would explain the accumulation of the unidentified sugar, which could either be viosamine or VioNac: If the 4-keto-6-deoxy-D-glucose is directly handed over by YfnG, but not by SpsM and SpsJ, these will compete directly over the dNDP-D-Glucose pool. If one of these other two are missing, the flux pulls the reaction towards the viosamine/VioNac. The HPLC data of this study shows, that CgeB transfers rhamnose or a precursor onto the spore surface, as rhamnose is missing in the yfnHGFEDcgeB mutant. If the transferase activity is missing, the flux of the prior rhamnose producing reactions will slow down also leading to a pull towards viosamine/VioNac and therefore an accumulation, which also happens if the enzymes producing rhamnose SpsK and SpsL are missing.

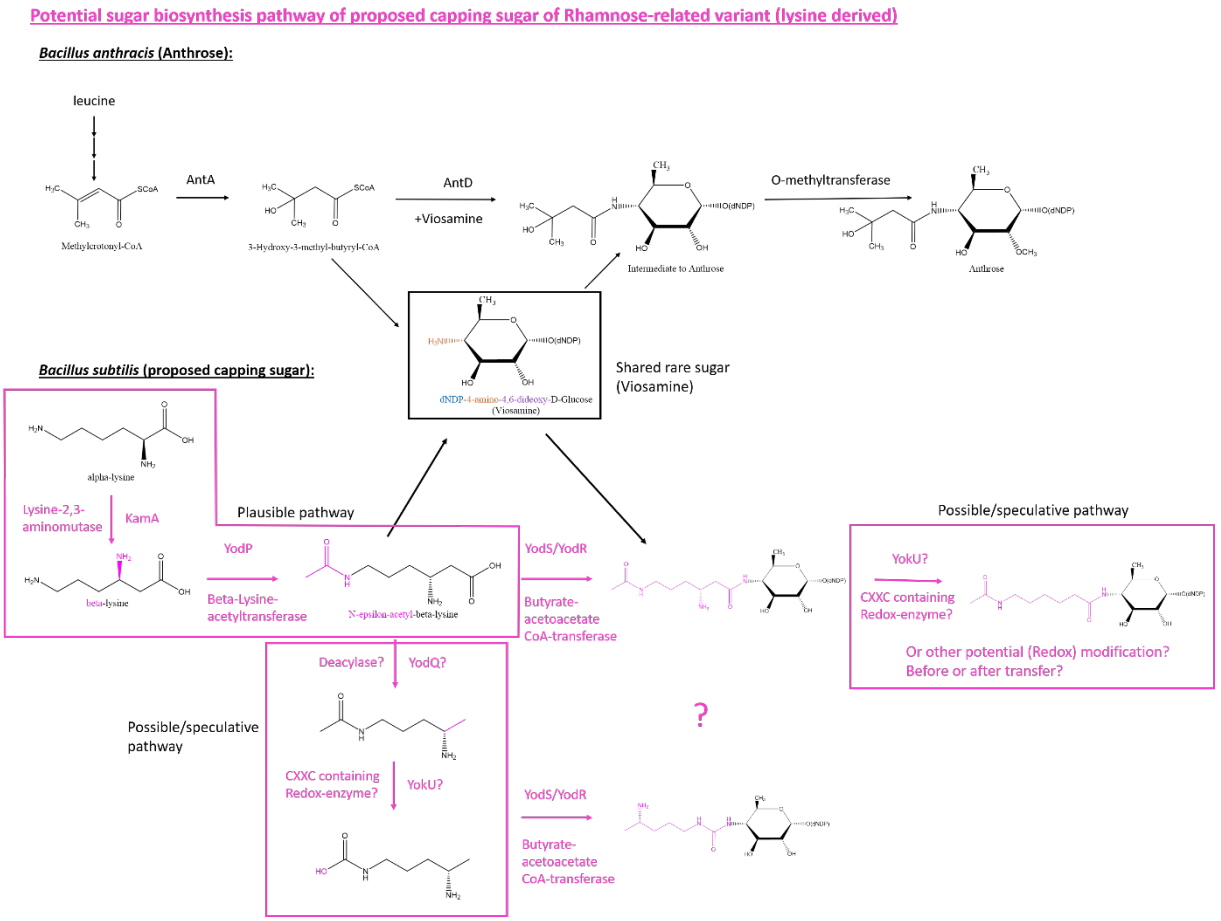

Figure S 6: Potential biosynthesis pathway of the proposed capping sugar

In the search for the redundancy for the aminotransferase activity of SpsC (as the sugar profile in the HPLC does not change in the spsA-L mutant) the operon yodTSRQPkamAyokU with the aminotransferase YodT was found directly next to cgeCDE. The other genes found in this operon have putative activities in lysine modification and transfer. As the relative Bacillus anthracis produces the unique sugar anthrose[17, 18] based on a modified leucine as an additional side group it is tempting to suggest something similar in B. subtilis. The biosynthetic pathway of B. anthracis as well as the transfer onto the rare sugar viosamine is shown on the top. As Bacillus subtilis also has all the prerequisites for the production of viosamine (see -figure S5) as well as a cluster putatively able to modify and transfer lysine as an additional side group, something quite similar could occur in B. subtilis and is shown on the bottom. The plausible first modification steps of lysine are shown (pink box, termed plausible pathway), which are derived from the predicted enzyme activity and known reactions catalyzed by these[20]. YodS and YodR as putative butyrate-acetoacetate-CoA transferases could be involved in transferring this side group, before or after final modification steps. YodQ is a putative deacylase and YokU is a CXXC containing enzyme, which can catalyze redox-reactions, which could be a wide range of reactions like for example a deamination[21] or the conversion of an acyl-group to a carboxylic acid group. If this putative enzyme is involved in the modification of the side chain cannot be predicted though. YodQ could be involved in the deacetylation of VioNac to free the necessary aminogroup for the transfer, or it could further modify the side group. Further shown (other pink boxes, termed possible/speculative pathway) are possible, yet highly speculative further modifications. The purpose of showing these highly speculative reactions is to illustrate that this side chain could differ highly from what is plausible due to the activity of further modifying enzymes, but the result is always a side group which (stereochemically) highly varies from the lysine from which it is derived. This suggested pathway could additionally explain the extended polysaccharide layer by the accumulation of rhamnose in the yfnHGFED mutant: YfnE is presumably the transferase for the unidentified sugar or its precursor, which could be viosamine or VioNac (see main Paper Figure 4) whereas CgeB transfers rhamnose or a precursor, probably on the same growing chain, whereas rhamnose is less abundant. If the unidentified sugar is missing, rhamnose is added uncontrollably leading to an extended polysaccharide layer. This unique sugar could act as an additional capping moiety, which prevents the uncontrollable addition of rhamnose. This proposed capping sugar could therefore not only constrict the uncontrolled polymerization of the polysaccharide chain, but also lead to additional protection against biological degradation of the spore envelope, as the base sugar is not only rare, but also contains a side chain not similar to anything generally found in nature and therefore not a target of general degradation enzymes.

Potential Galactose-related variant (teichoic acid derived?):

Polymer of GlcNac (or just precursor)/GalNAc (AIA lectin binds to this sugar)/ ManNAc and NeuNAc (SpsE proposed enzymatic function)?

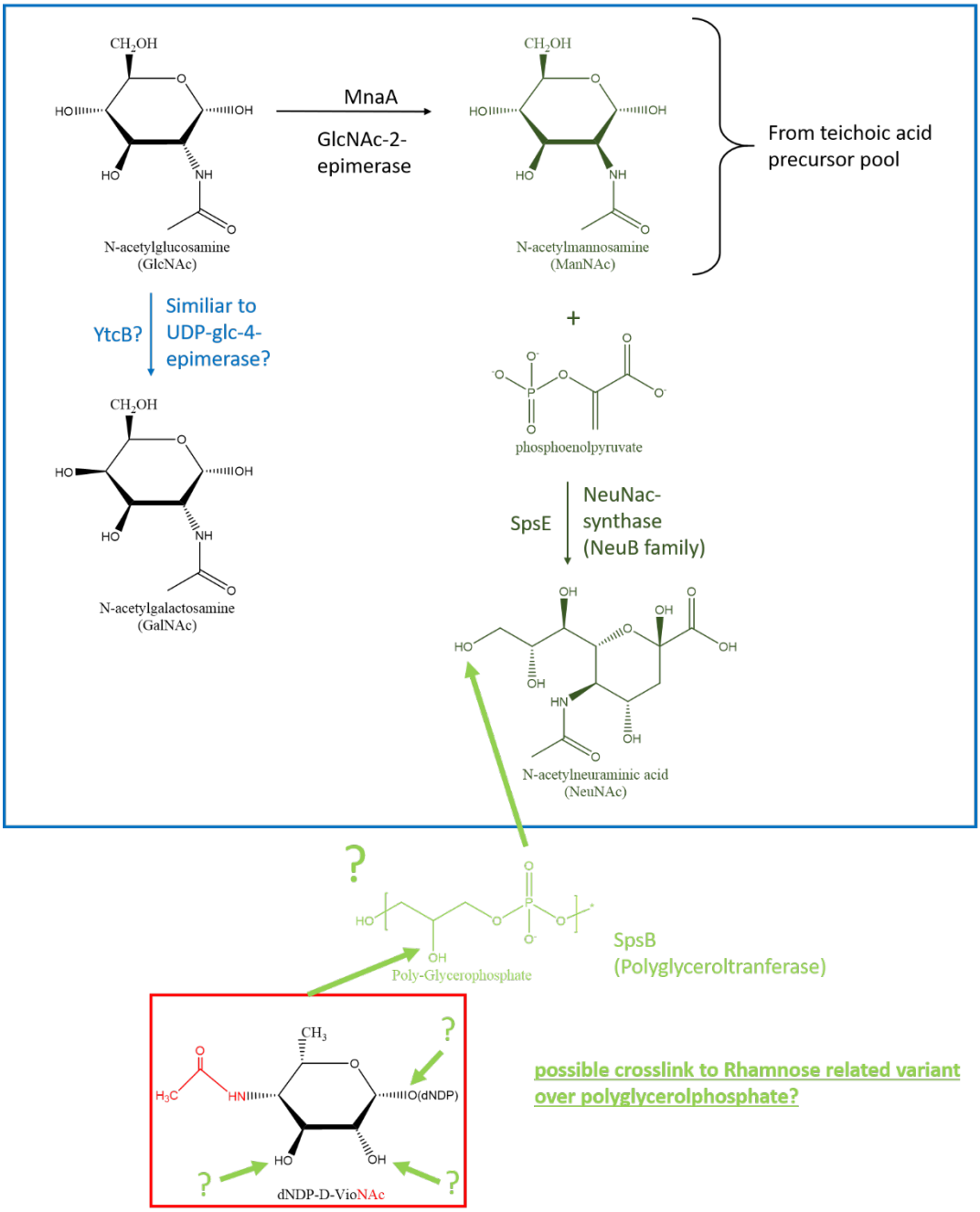

Figure S 7: Potential participants in the galactose-related variant and potential crosslinking

Shown are potential participants in the galactose-related variant. Little is known of this variant, as none of the studied enzymes are in any way predicted to produce galactose or a similar sugar. From the data it is known, that the variant is unique from the rhamnose-related variant, as it can be phenotypically separated (see main Paper Figure 3, cgeCDE mutant), even though often affected in conjunction (maybe therefore crosslinked). What is also known, is that the lectin AIA from Artocarpus integrifolia can bind to a part of this variant. This lectin binds to galactose or GalNac[22]. As galactose was not identified (at least in high amounts) in the HPLC study as being a part of the spore polysaccharide, it might well be GalNAc. GalNAc could be synthesized from GlcNAc by an enzyme similar to a Glucose-4-epimerase, of which the only predicted one in all studied genes is the ytcB (the other epimerases all being predicted 4,6-epimerases). But as these predictions might as well not be as reliable it could also be produced by another gene product. But this could also be an indication that there might even be a direct link between the crust glycosylation and the potential outer coat glycosylation inferred by the outer coat defect of the ytcABC and cotSASytxO mutants (see main Paper

Figure 3). Then there is another putative enzyme which could also be involved in this variant, as the sugars affected are also related teichoic acid like GalNAc: SpsE. SpsE is a putative NeuNac-synthase, which condensates Phosphoenolpyruvate and ManNAc to NeuNac. GlcNAc and ManNAc required for both these activities could be derived from the teichoic acid pool[23]. As both variants are phenotypically mostly affected in conjunction, it is tempting to think that these variants are somehow crosslinked. A possible crosslink could be over polyglycerophosphate, as SpsB is a putative polyglyceroltransferase. This could crosslink the NeuNAc, which has many possible hydroxy-groups as well as a somewhat similar structured sidechain, to VioNAc, which also has a few potential hydroxy-groups. The proposed linking point is thereby VioNAc and not viosamine or a potential other sugar of the rhamnose variant, as the only mutant with a "separated" phenotype is cgeCDE. CgeE is a putative acetyltransferase, which could produce VioNAc from viosamine, redundantly with SpsD, which could still act at a different position of the structure. In teichoic acid the added sugars often contain an acetylgroup, making this a possible necessity[24]. If the acetylgroup is required for the enzymatic function, then the crosslink will not be created in the cgeCDE mutant, but the polyglycerophosphate might still be transferred by SpsB to NeuNAc. This also could explain why spsA-L does not produce the same phenotype albeit also containing the putative acetyltransferase SpsD, as the polyglyceroltransferase is additionally missing. This could also explain the unique phenotype of the cgeCDE mutant: The polysaccharide structure is defect, leading to matt colonies, but polyglycerophosphate might be linked to the spore surface without creating the normal crosslink, which could explain the poor sedimentation properties of the spores. Therefore, the galactose-related variant might be a polysaccharide containing GlcNAc (which could also just act as a precursor for GalNAc and ManNAc), GalNAc, ManNAc (which again could just act as a precursor for NeuNAc) and NeuNAc, which is then linked to the rhamnose-related variant over polyglycerophosphate, similar maybe to teichoic acid.

Detailed discussion of the proposed pathways:

The data of this study showed that the proteins SpsM, SpsA-L, CgeAB and CgeCDE clearly act in conjunction (as they produce the same phenotype), but also execute a high level of redundancy: the spores of the respective mutants, though showing a phenotype related with the loss of polysaccharide (matt colonies, high amount of clumping and adhesion) still showed the same sugar profile in HPLC experiments. This discrepancy is explained by a defect in the structure and not a change in the composition of the polysaccharides in these mutants. This can clearly be demonstrated with the lectins: they bind the sugar moieties of the disturbed polysaccharides in the mutants more effectively than the native polysaccharide structure in the wildtype. The reported redundancies could still be only partial, as the same sugar moieties could be missing at one position, but still present at another position in the crust polysaccharide. One mutant demonstrated that there seems to be two independent polysaccharides: in the *cgeCDE* mutant. The signal for the galactose binding lectin was like the wildtype even though the signal for the rhamnose binding lectin was elevated (see main Paper Figure 3). This indicates that there seem to be two polysaccharides that can be phenotypically separated. The fact that they are mostly affected in conjunction indicates that these two variants might be crosslinked or otherwise functionally linked. This indicates that the spore polysaccharide layer is composed of (at least) two variants of polysaccharides, which will further be termed rhamnose-related variant and the galactose-related variant (due to the fact, that the respective lectins bind to a sugar component in this variant, even though no or only very little of these sugars seem to actually be part of these variants[25]). The *yfnHGFED* mutant had an opposing phenotype linked to an extended polysaccharide layer, but the polysaccharide structure also seemed to be additionally impaired, as the mutant showed elevated lectin binding signals (see main Paper Figure 3). This mutant additionally was missing the unidentified sugar (13,52 minutes) and accumulated rhamnose (13,98 minutes) instead (see main Paper Figure 4).

Taking the information won from this study, the predicted functions of the involved genes (see Figure S4) and known pathways involving similar enzymes or in related species the plausible or speculative pathways can be deduced. For the rhamnose-related variant the proposed pathway for rhamnose by *spsI-L*[5] was taken as the basis (see Figure S5). As the data suggest a joined pathway between SpsA-L and the unidentified sugar presumably transferred by YfnE as well as the further involvement of CgeB and SpsM, this pathway was extended to fit all the putative enzymatic activities to explain the data. The rhamnose pathway synthesizes rhamnose over the sugar intermediate 4-keto-6-deoxy-glucose. The keto-group of this sugar could be a prerequisite of the aminotransferase activity predicted for SpsC. As the sugar profiles in the mutants do not change in the HPLC data, there has to be redundancies for each of the steps. To explain the redundancies: the first step of the rhamnose-pathway could potentially also be catalyzed by YfnH and the second step by YfnG and SpsM. For the aminotransferase activity of SpsC, YodT, an aminotransferase located in an operon directly next to *cgeCDE*, was identified as a good candidate to explain the redundancy of this step. The resulting sugar 4-amino-4,6-dideoxy-D-glucose, also known as the rare sugar viosamine[16], could be the prerequisite for the putative acetyltransferase activities, which requires an aminogroup, of CgeE and SpsD, leading to N-acetyl-viosamine (VioNAc). The pathway from glucose to viosamine is also predicted to be performed by the close relative *Bacillus anthracis* during the production of the unique sugar anthrose[17, 18], making this proposed pathway more likely to exist

also in *B. subtilis*. The acetylation of this sugar is also something found in nature, though quite rarely[19]. This taken together illustrates the plausibility of this proposed pathway. As the unidentified sugar is missing in the *yfnHGFED* mutant, this sugar or its precursor is presumably transferred by YfnE. As it accumulates in the *spsA-L*, *spsM* and *cgeB* mutant, the unidentified sugar could be viosamine or VioNAc, due to the involvement of these (putative) enzymes in the respective pathway. As already discussed in the main Paper, CgeB seems to transfer rhamnose or its precursor and SpsK and SpsL produces rhamnose from 4-keto-6-deoxy-glucose, which would explain the accumulation of this unidentified sugar in these mutants: the flux towards rhamnose is slowed down pulling the pathway towards viosamine and VioNAc (which is the presumed unidentified sugar). For SpsM (as well as for SpsJ, even though the accumulation is already explained by SpsK and SpsL missing) the accumulation could be explained, if YfnE already transfers 4-keto-6-deoxy-glucose and not viosamine: YfnG could directly hand over this compound to YfnE, which could lead to a direct competition over the dNDP-glucose pool with SpsM and SpsJ. If one of these two enzymes are missing the flux is therefore pulled towards the direct transfer by YfnE and the sugar resulting in this transfer accumulates. That 4-keto-6-deoxy-glucose could be the target of transfer is underpinned by the fact, that the aminotransferase SpsC, which might act on the keto-group of this sugar, is located on the spore surface[6]. This pathway infers that the rhamnose-related variant,), could mostly be built of viosamine or VioNAc, though containing small amounts of rhamnose[8, 25]. The presence of small amounts of rhamnose seems necessary for the intact structure of the polysaccharide, as the polysaccharide structure is impaired in the *cgeB* mutant, which presumably transfers rhamnose or its precursor. As rhamnose accumulates in the *yfnHGFED* mutant leading to an extended polysaccharide layer (see main Paper Figure 3 and Figure 4), this rhamnose-related variant might as well be capped to protect against this uncontrollable polymerization of rhamnose.

The proposed capping sugar is shown in Figure S6 and is based on the rare sugar viosamine and the operon found next to *cgeCDE: yodTSRQPkamAyokU*, of which YodT was already discussed to be the redundant enzyme for the aminotransferase activity of SpsC. This operon additionally contains enzymes which could potentially modify and transfer lysine as a possible side chain onto the amino-group of the viosamine. The first two steps of the lysine modification can be inferred by the putative enzymatic activities of KamA and YodP and the known reactions catalyzed by these kind of enzymes[20]. YodS and YodR could transfer the outcome or any intermediate of the reaction to the aminogroup of viosamine. The exact outcome cannot be predicted, as YodQ as a deacylase and YokU as a CXXC-containing enzyme with a possible redox-activity could be catalyzing a greater number of reactions, of which some speculative possibilities are shown in Figure S6. YodQ could also not be involved in the modification of lysine but rather act on VioNAc to free the aminogroup of the acetylgroup (also shown in Figure S5) and therefore activate it for the transfer of the lysine derived side chain. The possibilities are not shown as an attempt to have the complete picture, but to illustrate possible outcomes for this capping sugar, of which all are (stereochemically) quite different to the lysine which it derived from. Something quite similar was already experimentally verified in the close relative *Bacillus anthracis*, which produces the unique sugar anthrose from the intermediate 4-amino-4,6-dideoxy-glucose (viosamine). This sugar is produced by adding a side chain to viosamine, which derives from but is highly variant from leucine. This is also shown as a comparison in Figure S6. The presence of a similar pathway in B. anthracis as well as the required putative enzymes in B. subtilis, underpins these two proposed pathways (see Figure S5 and S6).

In contrast very little is known of the galactose-related, as there is no putative enzymatic function predicted to produce galactose or a similar sugar of the gene products producing a phenotype. The only enzymatic function which could produce galactose/GalNAc from glucose/GlcNAc is YtcB which is similar to UDP-glucose-4-epimerase (see Table S4). The other epimerases are all putative 4,6-epimerases, which cannot produce galactose or a similar variant, as the glucose backbone will lose the hydroxy-group at the position six. As the lectin AIA from Artocarpus integrifolia can bind either galactose or GalNAc[22] and galactose was not identified (at least not in high amounts) it is quite plausible that GalNAc is part of the galactose-related variant. The *spsA-L* operon also contains a putative NeuNAc-synthase (SpsE), which condensates ManNAc and Phosphoenolpyruvate (PEP) to NeuNAc. GlcNAc as well as ManNAc (produced from GlcNAc by MnaA) could derive from the teichoic acid pool of the mother cell. The possible participants of the galactose-related variant are shown in Figure S7. The *spsA-L* operon also contains a polyglycerophosphate transferase, which could explain the proposed crosslink of the galactose-related variant to the rhamnose-related variant: like in

teichoic acid, this side chain could be transferred to sugars of the polysaccharide chain and link two sugars in that way. Possible links are between NeuNAc (which already contains a structurally related side chain with many possible hydroxy-groups) and VioNAc (which also contains many possible hydroxy-groups). The reason for VioNAc being the proposed linkage partner is due to the unique phenotype of the *cgeCDE* mutant, which contains the proposed acetyltransferase to produce the VioNAc. In teichoic acid the linked sugar is often acetylated, therefore this might be a plausible prerequisite[24]. If VioNAc is the linkage partner, the mutant *cgeCDE* would be missing this linkage point, but unlike the *spsA-L* mutant (which would also be missing this linkage point, maybe though at a different position due to the redundancy) still has the polyglycerophosphate transferase activity in place by the SpsE. This could lead to the shown "separation" phenotype (see main Paper Figure 3): a more accessible rhamnose-related variant, due to the missing crosslink to the VioNAc at this position, but an intact galactose-related variant (with the polyglycerophosphate linker attached). The disturbed rhamnose-related variant could lead to the matt colonies (see main Paper, Figure 3) of this mutant whereas the unlinked yet transferred polyglycerophosphate linker might lead to the poor sedimenting properties of these spores. This would not be the case with the *spsA-L* mutant, as not only the linkage point VioNAc in the rhamnose-related variant would be missing, but also the polyglycerolphosphate linker itself, due to the respective putative transferase SpsB missing as well. Therefore both variants would again be affected together.

Python script for screening the regulon SigE/K

```
# Import regular expression module.

import re

# Define input file.

inputfile = "SigE/K_regulon_SeqDepot.tsv"

# Define the Pfam domains of interest.

domains = ["Glyco_transf_9","Glyco_transf_20","Glyco_transf_28","Glyco_transf_6",
        "Glyco_tran_28_C","Glyco_transf_34","Glycos_transf_1","Glycos_transf_2",
        "Glycos_trans_3N","Gly_transf_sug","GT-D","Glyco_transf_4",
        "Glyco_transf_41","Glyco_trans_1_4","Glyco_transf_10","Glyco_transf_11",
        "Glyco_transf_15","Glyco_transf_43","Glyco_tran_WecB","Glyco_transf_21",
        "Glyco_transf_8C","Glyco_transf_92","Glyco_transf_25","Glyco_trans_4_2",
        "Glyco_trans_4_4","Glyco_trans_2_3","Glyco_trans_A_1","Glyco_trans_1_2",
        "PS_pyruv_trans"]

# Create loop that goes through each protein entry in SigE/K_regulon_SeqDepot.tsv
# and checks if any of the domains listed is present.

for entry in open (inputfile):
   if any(i in entry for i in domains): print entry
```

1. Bartels, J., López Castellanos, S., Radeck, J., and Mascher, T. (2018) Sporobeads: The Utilization of the *Bacillus subtilis* Endospore Crust as a Protein Display Platform, *ACS Synthetic Biology 7*, 452-461.
2. LMU-Munich. (2012) http://2012.igem.org/Team:LMU-Munich.
3. Roels, S., and Losick, R. (1995) Adjacent and divergently oriented operons under the control of the sporulation regulatory protein GerE in *Bacillus subtilis*, *Journal of Bacteriology 177*, 6263-6275.
4. Cangiano, G., Sirec, T., Panarella, C., Isticato, R., Baccigalupi, L., De Felice, M., and Ricca, E. (2014) The sps Gene Products Affect the Germination, Hydrophobicity, and Protein Adsorption of *Bacillus subtilis* Spores, *Applied and environmental microbiology 80*, 7293-7302.
5. Plata, G., Fuhrer, T., Hsiao, T. L., Sauer, U., and Vitkup, D. (2012) Global probabilistic annotation of metabolic networks enables enzyme discovery, *Nature chemical biology 8*, 848-854.
6. Knurr, J., Benedek, O., Heslop, J., Vinson, R. B., Boydston, J. A., McAndrew, J., Kearney, J. F., and Turnbough, C. L. (2003) Peptide Ligands That Bind Selectively to Spores of *Bacillus subtilis* and Closely Related Species, *Applied and environmental microbiology 69*, 6841-6847.
7. Arrieta-Ortiz, M. L., Hafemeister, C., Bate, A. R., Chu, T., Greenfield, A., Shuster, B., Barry, S. N., Gallitto, M., Liu, B., Kacmarczyk, T., Santoriello, F., Chen, J., Rodrigues, C. D., Sato, T., Rudner, D. Z., Driks, A., Bonneau, R., and Eichenberger, P. (2015) An experimentally supported model of the *Bacillus subtilis* global transcriptional regulatory network, *Molecular Systems Biology 11*.
8. Abe, K., Kawano, Y., Iwamoto, K., Arai, K., Maruyama, Y., Eichenberger, P., and Sato, T. (2014) Developmentally-Regulated E cision o t e rop age Reconstitutes a ene Re uired or pore En elope Maturation in *Bacillus subtilis*, *PLoS Genetics 10*, e1004636.
9. Eichenberger, P., Fujita, M., Jensen, S. T., Conlon, E. M., Rudner, D. Z., Wang, S. T., Ferguson, C., Haga, K., Sato, T., Liu, J. S., and Losick, R. (2004) The Program of Gene Transcription for a Single Differentiating Cell Type during Sporulation in *Bacillus subtilis*, *PLOS Biology 2*, e328.
10. Kiel, J. A. K. W., Boels, J. M., Beldman, G., and Venema, G. (1994) Glycogen in Bacillus subtilis: molecular characterization of an operon encoding enzymes involved in glycogen biosynthesis and degradation, *Molecular Microbiology 11*, 203-218.
11. Kodama, T., Takamatsu, H., Asai, K., Ogasawara, N., Sadaie, Y., and Watabe, K. (2000) Synthesis and Characterization of the Spore Proteins of *Bacillus subtilis* YdhD, YkuD, and YkvP, Which Carry a Motif Conserved among Cell Wall Binding Proteins, *The Journal of Biochemistry 128*, 655-663.
12. Buist, G., Steen, A., Kok, J., and Kuipers, O. P. (2008) LysM, a widely distributed protein motif for binding to (peptido)glycans, *Mol Microbiol 68*, 838-847.
13. Shuster, B., Khemmani, M., Abe, K., Huang, X., Nakaya, Y., Maryn, N., Buttar, S., Gonzalez, A. N., Driks, A., Sato, T., and Eichenberger, P. (2019) Contributions of crust proteins to spore surface properties in *Bacillus subtilis*, *Molecular Microbiology 111*, 825-843.
14. Imamura, D., Kuwana, R., Takamatsu, H., and Watabe, K. (2011) Proteins Involved in Formation of the Outermost Layer of *Bacillus subtilis* Spores, *Journal of Bacteriology 193*, 4075-4080.
15. McKenney, P. T., Driks, A., Eskandarian, H. A., Grabowski, P., Guberman, J., Wang, K. H., Gitai, Z., and Eichenberger, P. (2010) A Distance-Weighted Interaction Map Reveals a Previously Uncharacterized Layer of the *Bacillus subtilis* Spore Coat, *Current Biology 20*, 934-938.
16. Piacente, F., Marin, M., Molinaro, A., De Castro, C., Seltzer, V., Salis, A., Damonte, G., Bernardi, C., Claverie, J.-M., Abergel, C., and Tonetti, M. (2012) Giant DNA virus mimivirus encodes pathway for biosynthesis of unusual sugar 4-amino-4,6-dideoxy-D-glucose (Viosamine), *The Journal of biological chemistry 287*, 3009-3018.
17. Dong, S., McPherson, S. A., Tan, L., Chesnokova, O. N., Turnbough, C. L., and Pritchard, D. G. (2008) Anthrose Biosynthetic Operon of *Bacillus anthracis*, *Journal of Bacteriology 190*, 2350-2359.
18. Dong, S., McPherson, S. A., Wang, Y., Li, M., Wang, P., Turnbough, C. L., Jr., and Pritchard, D. G. (2010) Characterization of the enzymes encoded by the anthrose biosynthetic operon of *Bacillus anthracis*, *Journal of bacteriology 192*, 5053-5062.
19. Piacente, F., De Castro, C., Jeudy, S., Gaglianone, M., Laugieri, M. E., Notaro, A., Salis, A., Damonte, G., Abergel, C., and Tonetti, M. G. (2017) The rare sugar N-acetylated viosamine is a major component of Mimivirus fibers, *Journal of Biological Chemistry 292*, 7385-7394.

20. Pflüger, K., Baumann, S., Gottschalk, G., Lin, W., Santos, H., and Müller, V. (2003) Lysine-2,3-Aminomutase and -Lysine Acetyltransferase Genes of Methanogenic Archaea Are Salt Induced and Are Essential for the Biosynthesis of *N* -Acetyl- -Lysine and Growth at High Salinity, *Applied and environmental microbiology 69*, 6047-6055.
21. Zhang, D., de Souza, R. F., Anantharaman, V., Iyer, L. M., and Aravind, L. (2012) Polymorphic toxin systems: Comprehensive characterization of trafficking modes, processing, mechanisms of action, immunity and ecology using comparative genomics, *Biology Direct 7*, 18.
22. Hagiwara, K., Collet-Cassart, D., Kunihiko, K., and Vaerman, J.-P. (1988) Jacalin: isolation, characterization, and influence of various factors on its interaction with human IgA1, as assessed by precipitation and latex agglutination, *Molecular Immunology 25*, 69-83.
23. Soldo, B., Lazarevic, V., Pooley, H. M., and Karamata, D. (2002) Characterization of a *Bacillus subtilis* thermosensitive teichoic acid-deficient mutant: gene mnaA (yvyH) encodes the UDP-N-acetylglucosamine 2-epimerase, *Journal of bacteriology 184*, 4316-4320.
24. Brown, S., Santa Maria, J. P., Jr., and Walker, S. (2013) Wall teichoic acids of gram-positive bacteria, *Annual review of microbiology 67*, 313-336.
25. Wunschel, D., F. Fox, K., E. Black, G., and Fox, A. (1995) *Discrimination Among the B. Cereus Group, in Comparison to B. Subtilis, by Structural Carbohydrate Profiles and Ribosomal RNA Spacer Region PCR*, Vol. 17.

Supplemental Material for Manuscript I: Strategies for the improvement of the Sporobead display system

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Table of Contents:

Table S1: Plasmids created and used in this study

Plasmid[§]	Reference	Backbone	Integration Site	Antibiotic resistance	
				E. coli	*B. subtilis*
pSB1C3-[RFP]	Parts registry	-	-	Cm	-
pSB1C3-BpuL	Bartels et al. 2018[1]	pSB1C3	-	Cm	-
p1CSV-CgeA-N	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CgeA-C	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotZ-N	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotZ-C	Bartels et al. 2018[1]	pBS1C	*amyE*	Amp	Cm
p1CSV-CotY-N-GFP	Bartels et al. 2018[1]	p1CSV-CotY-N	*amyE*	Amp	Cm
p1CSV-CgeA-N-BpuL	Bartels et al. 2018[1]	p1CSV-CgeA-N	*amyE*	Amp	Cm
p1CSV-CotV-N-BpuL	Bartels et al. 2018[1]	p1CSV-CotV-N	*amyE*	Amp	Cm
p1CSV-CotW-N-BpuL	Bartels et al. 2018[1]	p1CSV-CotW-N	*amyE*	Amp	Cm
p1CSV-CotX-N-BpuL	Bartels et al. 2018[1]	p1CSV-CotX-N	*amyE*	Amp	Cm
p1CSV-CotY-N-BpuL	Bartels et al. 2018[1]	p1CSV-CotY-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BpuL	Bartels et al. 2018[1]	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotY-N-EcoL	Bartels et al. 2018[1]	p1CSV-CotY-N	*amyE*	Amp	Cm
p1CSV-CotY-C-EcoL	Bartels et al. 2018[1]	p1CSV-CotY-C	*amyE*	Amp	Cm
p1CSV-CotZ-N-EcoL	Bartels et al. 2018[1]	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-C-EcoL	Bartels et al. 2018[1]	p1CSV-CotZ-C	*amyE*	Amp	Cm
pSB1C3-BBa_K157009	Partsregistry[2]	pSB1C3	-	Amp	-
pSB1C3-BBa_K157013	Partsregistry[2]	pSB1C3	-	Amp	-
pSB1C3-BBa_K243004	Partsregistry[2]	pSB1C3	-	Amp	-
pSB1C3-BBa_K243005	Partsregistry[2]	pSB1C3	-	Amp	-
pSB1C3-BBa_K243006	Partsregistry[2]	pSB1C3	-	Amp	-
pSB1C3-BBa_K243029	Partsregistry[2]	pSB1C3	-	Amp	-
pSB1C3-BBa_K243030	Partsregistry[2]	pSB1C3	-	Amp	-
pSB1C3-BBa_K1351009 (L56)	Partsregistry[2]	pSB1C3	-	Amp	-
p1CSV-CotZ-N-BBa_K157009-[RFP]	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K157013-[RFP]	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K243004-[RFP]	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm

p1CSV-CotZ-N-BBa_K243005-[RFP]	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K243006-[RFP]	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K243029-[RFP]	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K243030-[RFP]	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-alpha-linker-[RFP]	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K1351009-[RFP]	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K157009-[RFP]	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K157013-[RFP]	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K243004-[RFP]	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K243005-[RFP]	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K243006-[RFP]	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K243029-[RFP]	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K243030-[RFP]	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-alpha-linker-[RFP]	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K1351009-[RFP]	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K157009-BpuL	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K157013-BpuL	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K243004-BpuL	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K243005-BpuL	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K243006-BpuL	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K243029-BpuL	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm

p1CSV-CotZ-N-BBa_K243030-BpuL	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-alpha-linker-BpuL	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-N-BBa_K1351009-BpuL	This study	p1CSV-CotZ-N	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K157009-BpuL	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K157013-BpuL	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K243004-BpuL	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K243005-BpuL	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K243006-BpuL	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K243029-BpuL	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K243030-BpuL	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-alpha-linker-BpuL	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CotZ-C-BBa_K1351009-BpuL	This study	p1CSV-CotZ-C	*amyE*	Amp	Cm
p1CSV-CgeA-N-alpha-linker-[RFP]	This study	p1CSV-CgeA-N	*amyE*	Amp	Cm
p1CSV- CgeA-N-BBa_K1351009-[RFP]	This study	p1CSV-CgeA-N	*amyE*	Amp	Cm
p1CSV-CgeA-C-alpha-linker-[RFP]	This study	p1CSV-CgeA-C	*amyE*	Amp	Cm
p1CSV- CgeA-C-BBa_K1351009-[RFP]	This study	p1CSV-CgeA-C	*amyE*	Amp	Cm
p1CSV-CgeA-N-alpha-linker-BpuL	This study	p1CSV-CgeA-N	*amyE*	Amp	Cm
p1CSV- CgeA-N-BBa_K1351009-BpuL	This study	p1CSV-CgeA-N	*amyE*	Amp	Cm
p1CSV-CgeA-C-alpha-linker-BpuL	This study	p1CSV-CgeA-C	*amyE*	Amp	Cm
p1CSV- CgeA-C-BBa_K1351009-BpuL	This study	p1CSV-CgeA-C	*amyE*	Amp	Cm

§ The Plasmids are annotated according to the backbone and the insert connected with a hyphen, which is also annotated in the according column. The Sporovectors (p1CSV-*crust gene*-N/C) are readily available from the BGSC. The denomination derives from a shortened code for the pBS1C (p1C) as the backbone, SV for Sporovector, followed by the crust protein coded in the Sporovector and the translational fusion site (N=N-terminal, C=C-terminal). They all confer ampicillin resistance in *E. coli*

and chloramphenicol resistance in *B. subtilis*. In *B. subtilis* they integrate into the genome at the *amyE*-locus, which can be verified by the inability to degrade starch on starch minimal plates.

Table S2: Strains created and used in this study

Strain	Reference	Genotyp[§]	Antibiotic resistance (*B. subtilis*)
TMB2131	Bartels et al. 2018[1]	W168, *cotA::mls*^{r}	MLS
TMB1886	iGEM LMU 2012[3]	*ΔcgeA*	none
TMB1891	iGEM LMU 2012[3]	*ΔcotZ*	none
TMB1942	iGEM LMU 2012[3]	*ΔcotZ ΔcgeA*	none
TMB2136	This study	W168, *cotA::mls*^{r}, *ΔcotZ*	MLS
TMB2132	This study	W168, *cotA::mls*^{r}, *ΔcgeA*	MLS
TMB2137	This study	W168, *cotA::mls*^{r}, *ΔcotZ ΔcgeA*	MLS
TMB4338	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotA-cotZ; cotA::mls*^{r}	Cm, MLS
TMB4314	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotZ-cotA; cotA::mls*^{r}	Cm, MLS
TMB5066	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotA-cgeA; cotA::mls*^{r}	Cm, MLS
TMB4306	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cgeA-cotA; cotA::mls*^{r}	Cm, MLS
TMB4982	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotA-cotV; cotA::mls*^{r}	Cm, MLS
TMB4543	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotA-cotW; cotA::mls*^{r}	Cm, MLS
TMB4974	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotA-cotX; cotA::mls*^{r}	Cm, MLS
TMB4547	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotA-cotY; cotA::mls*^{r}	Cm, MLS
TMB4998	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cueO-cotX; cotA::mls*^{r}	Cm, MLS
TMB5028	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cueO-cotY; cotA::mls*^{r}	Cm, MLS
TMB5030	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotY-cueO; cotA::mls*^{r}	Cm, MLS
TMB4322	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cueO-cotZ; cotA::mls*^{r}	Cm, MLS
TMB5026	Bartels et al. 2018[1]	W168, *amyE::*P_{cotYZ}*-cotZ-cueO; cotA::mls*^{r}	Cm, MLS
TMB5067	This study	W168, *amyE::*P_{cotYZ}*-cotA-cgeA; cotA::mls*^{r}*; ΔcotZ*	Cm, MLS
TMB4983	This study	W168, *amyE::*P_{cotYZ}*-cotA-cotV; cotA::mls*^{r}*; ΔcotZ*	Cm, MLS
TMB4544	This study	W168, *amyE::*P_{cotYZ}*-cotA-cotW; cotA::mls*^{r}*; ΔcotZ*	Cm, MLS
TMB4975	This study	W168, *amyE::*P_{cotYZ}*-cotA-cotX; cotA::mls*^{r}*; ΔcotZ*	Cm, MLS
TMB4548	This study	W168, *amyE::*P_{cotYZ}*-cotA-cotY; cotA::mls*^{r}*; ΔcotZ*	Cm, MLS
TMB4339	This study	W168, *amyE::*P_{cotYZ}*-cotA-cotZ; cotA::mls*^{r}*; ΔcotZ*	Cm, MLS
TMB2524	This study	W168, *amyE::*P_{cotYZ}*-gfp-cotY; ΔcotZ*	Cm
TMB4290	This study	W168, *amyE::*P_{cotYZ}*-cotA-cotZ; cotA::mls*^{r} *(old version, less active)*	Cm, MLS
TMB4291	This study	*ΔcotZ, amyE::*P_{cotYZ}*-cotA-cotZ; cotA::mls*^{r} *(old version, less active)*	Cm, MLS
TMB4353	This study	*ΔcotZ, amyE::*P_{cotYZ}*-cotA-cotZ; cotA::mls*^{r}	Cm, MLS
TMB4292	This study	*ΔcgeA, amyE::*P_{cotYZ}*-cotA-cotZ; cotA::mls*^{r} *(old version, less active)*	Cm, MLS
TMB4293	This study	*ΔcotZΔcgeA, amyE::*P_{cotYZ}*-cotA-cotZ; cotA::mls*^{r} *(old version, less active)*	Cm,MLS
TMB4339	This study	W168, *amyE::*P_{cotYZ}*-cotA-*BBa_K157009*-cotZ; cotA::mls*^{r}	Cm,MLS
TMB4340	This study	W168, *amyE::*P_{cotYZ}*-cotA-* BBa_K157013*-cotZ; cotA::mls*^{r}	Cm,MLS
TMB4341	This study	W168, *amyE::*P_{cotYZ}*-cotA-* BBa_K243004*-cotZ; cotA::mls*^{r}	Cm,MLS
TMB4342	This study	W168, *amyE::*P_{cotYZ}*-cotA-* BBa_K243005*-cotZ; cotA::mls*^{r}	Cm,MLS
TMB4343	This study	W168, *amyE::*P_{cotYZ}*-cotA-* BBa_K243006*-cotZ; cotA::mls*^{r}	Cm,MLS
TMB4344	This study	W168, *amyE::*P_{cotYZ}*-cotA-* BBa_K243029*-cotZ; cotA::mls*^{r}	Cm,MLS
TMB4345	This study	W168, *amyE::*P_{cotYZ}*-cotA-* BBa_K243030*-cotZ; cotA::mls*^{r}	Cm,MLS

TMB4440	This study	W168, *amyE::*P_{cotYZ}*-cotA-* alpha-linker-*cotZ; cotA::mls*^{r}	Cm,MLS
TMB4444	This study	W168, *amyE::*P_{cotYZ}*-cotA-* BBa_K1351009 (L56)-*cotZ; cotA::mls*^{r}	Cm,MLS
TMB4346	This study	W168, *amyE::*P_{cotYZ}*-cotZ-*BBa_K157009-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4347	This study	W168, *amyE::*P_{cotYZ}*-cotZ-* BBa_K157013-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4348	This study	W168, *amyE::*P_{cotYZ}*-cotZ-* BBa_K243004-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4349	This study	W168, *amyE::*P_{cotYZ}*-cotZ-* BBa_K243005-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4350	This study	W168, *amyE::*P_{cotYZ}*-cotZ-* BBa_K243006-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4351	This study	W168, *amyE::*P_{cotYZ}*-cotZ-* BBa_K243029-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4352	This study	W168, *amyE::*P_{cotYZ}*-cotZ-* BBa_K243030-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4442	This study	W168, *amyE::*P_{cotYZ}*-cotZ-* alpha-linker-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4446	This study	W168, *amyE::*P_{cotYZ}*-cotZ-* BBa_K1351009 (L56)-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4354	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotA-*BBa_K157009-*cotZ; cotA::mls*^{r}	Cm,MLS
TMB4355	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotA-* BBa_K157013-*cotZ; cotA::mls*^{r}	Cm,MLS
TMB4356	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotA-* BBa_K243004-*cotZ; cotA::mls*^{r}	Cm,MLS
TMB4357	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotA-* BBa_K243005-*cotZ; cotA::mls*^{r}	Cm,MLS
TMB4358	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotA-* BBa_K243006-*cotZ; cotA::mls*^{r}	Cm,MLS
TMB4359	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotA-* BBa_K243029-*cotZ; cotA::mls*^{r}	Cm,MLS
TMB4441	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotA-* BBa_K243030-*cotZ; cotA::mls*^{r}	Cm,MLS
TMB4445	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotA-* alpha-linker-*cotZ; cotA::mls*^{r}	Cm,MLS
TMB4360	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotA-* BBa_K1351009 (L56)-*cotZ; cotA::mls*^{r}	Cm,MLS
TMB4361	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotZ-*BBa_K157009-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4362	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotZ-* BBa_K157013-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4363	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotZ-* BBa_K243004-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4364	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotZ-* BBa_K243005-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4365	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotZ-* BBa_K243006-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4366	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotZ-* BBa_K243029-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4367	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotZ-* BBa_K243030-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4443	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotZ-* alpha-linker-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4447	This study	*ΔcotZ*, *amyE::*P_{cotYZ}*-cotZ-* BBa_K1351009 (L56)-*cotA; cotA::mls*^{r}	Cm,MLS

TMB4456	This study	W168, *amyE*::P_{cotYZ}-*cotA*- alpha-linker-*cgeA; cotA::mls*^{r}	Cm,MLS
TMB4460	This study	W168, *amyE*::P_{cotYZ}-*cotA*- BBa_K1351009 (L56)-*cgeA; cotA::mls*^{r}	Cm,MLS
TMB4458	This study	W168, *amyE*::P_{cotYZ}-*cgeA*- alpha-linker-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4462	This study	W168, *amyE*::P_{cotYZ}-*cgeA*- BBa_K1351009 (L56)-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4457	This study	*ΔcgeA*, *amyE*::P_{cotYZ}-*cotA*- alpha-linker-*cgeA; cotA::mls*^{r}	Cm,MLS
TMB4461	This study	*ΔcgeA*, *amyE*::P_{cotYZ}-*cotA*- BBa_K1351009 (L56)-*cgeA; cotA::mls*^{r}	Cm,MLS
TMB4459	This study	*ΔcgeA*, *amyE*::P_{cotYZ}-*cgeA*- alpha-linker-*cotA; cotA::mls*^{r}	Cm,MLS
TMB4463	This study	*ΔcgeA*, *amyE*::P_{cotYZ}-*cgeA*- BBa_K1351009 (L56)-*cotA; cotA::mls*^{r}	Cm,MLS

§ The strains all derive from the B. subtilis wildtype W168 or the mutant strains. The mutant strains derive from a genomic replacement of the respective genes with the mls^r in the wildtype W168 or the clean deletion derived from iGEM LMU-Munich 2012[3]. All of the other strains derived from the transformation of the respective Sporovectors containing GFP (gfp) or BpuL (cotA) integrated into the amyE-locus

Table S3: Primers used in this study

Primer number	Primername	Sequence (5‘ to 3‘)
TM2889	[RFP]-fwd	TGCCACCTGACGTCTAAG
TM3000	NgoMIV-[RFP]-rev	GATCGCCGGCTATAAACGCAGAAAGGCCC
TM2995	AgeI-[RFP]-fwd	GATCACCGGTGCAATACGCAAACCGCCTC
TM2890	[RFP]-rev	ATTACCGCCTTTGAGTGA
TM0139	Mls-fwd	CAGCGAACCATTTGAGGTGATAGGgatcctttaactctg gcaaccctc
TM0140	Mls-rev	CGATACAAATTCCTCGTAGGCGCTCGGgccgactgc gcaaaagacataatcg
TM3089	*cotA*-del-up-BglII	GATCAGATCTGTACAAGCCATTGTGGC
TM3165	*cotA*-up-rev-mls	CCTATCACCTCAAATGGTTCGCTGTTTCATCTGT CCTTATCTAAATTTTC
TM3166	*cotA*-do-fwd-mls	CGAGCGCCTACGAGGAATTTGTATCGCCCGACA AACTTGCCTCTAG
TM3090	*cotA*-del-do-SalI-rev	GATCGTCGACCCTTCTGTAGCGCCTGC
TM3513	AlphaLinker_ENP_SD_NgoM IV_precutEP_fwd	AATTCGCGGCCGCTTCTAGAG TAAGGAGG AA GCCGGC ggaggaggagaagcggcggcgaaaggaggagga ACCGGTTAATACTAGTAGCGGCCGCTGCA
TM3514	AlphaLinker_SNP_AgeI_prec utEP_rev	GCGGCCGCTACTAGTATTAACCGGT tcctcctcctttcgccgccgcttctcctcctcc GCCGGCTTCCTCCTTACTCTAGAAGCGGCCGCG

Table S4: Linker amino acid sequences

Linker	Length§	Amino acid sequence
BBa_K157009	17 aa	RPACKIPNDLKQKVMNH
BBa_K157013	15 aa	GGGGSGGGGSGGGGS
BBa_K243004	4 aa	GGSG
BBa_K243005	8 aa	GGSGGGSG
BBa_K243006	12 aa	GGSGGGSGGGSG
BBa_K243029	36 aa	GGSAGGSGSGSSGGSSGASGT GTAGGTGSGSGTGSG
BBa_K243030	36 aa	GGSGGGSEGGGSEGGGSEGGG SEGGGSEGGGSGGGS
Alpha-linker	11 aa	GGGEAAAKGGG
BBa_K1351009 (L56)	57 aa	MIDPAGSSPSTPEGPSTPSNPST PSPGPASSGSSSSSSSSSNASS GTTTTPDLPGSP

§ Length without the respective scar resulting from RFC25 cloning procedure adding an additional two amino acids (TG)

1. Bartels, J., López Castellanos, S., Radeck, J., and Mascher, T. (2018) Sporobeads: The Utilization of the Bacillus subtilis Endospore Crust as a Protein Display Platform, *ACS Synthetic Biology 7*, 452-461.
2. Knight, T. (2007) Draft Standard for Biobrick Biological Parts, http://hdl.handle.net/1721.1/45138.
3. LMU-Munich. (2012) http://2012.igem.org/Team:LMU-Munich.

14 Acknowledgements

The time spent on this doctor thesis was surely never easy, but the personal development as well as the clarity what the future might have in stock for me was worth the suffering. The best things in life always come with a price:

"Nothing in the world is worth having or worth doing unless it means effort, pain, difficulty…" Theodore Roosevelt

With this kept in mind I would like to thank my supervisor Prof. Dr. Thorsten Mascher for the good and the bad. Especially I would like to thank him, that he made it possible for me to work on a project, that was from the very beginning already quite far out of the reach of his scientific research interest and in time drifted further and further away. He never pulled the ripcord on this project and allowed me to freely grow personally and professionally on this project which was as he liked to call it "an island". For that I am truly grateful. In addition to this he also allowed the few months in Peru, Ecuador and Costa Rica, which I was able to take as a break in my first year of the thesis, even though this interfered with the plan of moving the complete lab to Dresden. Thorsten supported my plans in this regard, giving me additional space to personally grow with this life time experience. The start in Dresden did not come easy either, as always somehow myself being "an Island", I had to establish myself in a lab, which was far from working (at least with my throughput), with a supervisor jet setting between Munich and Dresden. But even though this did not fit into the plan Thorsten had as to how a lab move should work, he respected that this solution was personally best for me, just returning from my months abroad. In my last year in the lab I chose to have a child. He also supported this decision and understood the hardships of "morning sickness", which was for my first months rather a "whole day sickness" and never pushed me to work harder. Thank you for giving me the space and support needed for my journey.

A big thanks also goes to all the people in the lab, former and current, for making the lab such a fun place to be, for giving the right input at the right time, for sharing and caring for my sometimes VERY labware and equipment intensive experiments, for the elaborate coffee breaks I just sometimes so desperately needed. Special thanks goes to Annette and Susanne for always preparing the vast amounts of glassware I required, always being sympathetic

despite the overhead. Franzi, Dayane, Karen, Nina and Qian for the extracurricular time spent in the coffee room and the SLUB. Daniela and Diana for always having an open ear and helping hands for any problem I presented to them, even though it never was there responsibility. This made them to my personal “lab moms”, thank you for being there for me! And Zsolt for always having the time and the free hands to help with microscope problems or untightening the centrifuge (sorry, but as one of the few male members it seemed to always be you). Then a big thanks goes to members of the group Ansorge-Schuhmacher: Tarek and Eugen for the help with protein purification, Frances with the help on the alcohol dehydrogenase (which was a dead end) and Loui for the fruitful small cooperation project we had with the spores and the pickering solution.

One very special colleague and good friend that accompanied this journey from the very beginning was Jara Radeck. We studied together, partook in three iGEM competitions together (even though each time we said “never again”), worked on the same project in our bachelor thesis, worked in the same lab in the master thesis as well as the doctor thesis. It was a long ride, and we drifted apart both in time spent together as well as on the thematic level, but our personal and professional relation was always very fruitful, and I would not want to miss any minute of it! You always helped, challenged, motivated and inspired me to be my best and never stopped listening to my (sometimes) broken record of the hardships I had in the beginning of Dresden. Thank you for being there, challenging my view-points, listening, never judging and all the fun in our free time.

Then I would like to especially thank all my collaboration partners, which I compelled to help me with my project. Being an Island, I required a lot of outside help to accomplish the things presented to in this thesis. They gave the broader methodological spectrum for understanding the crust in a deeper fashion. Thank you Markus Günther, Anja Blüher and Petr Formánek for the possibility to take a deeper look into the surface and the structure of the crust with different electron microscopy methods. Thank you Markus Richter for running my HPLC samples, even if I came with yet another one, and another one, and just this one more mutant. The data won were very elucidating and the personal time spent discussing the method or just chit-chating were personally very rewarding for me.

Another person I would like to thank, is my bachelor student Sebastián López Castellanos. He succeeded in every single challenge I gave him, from transforming over hundreds of strains on one day to trying to make sense of data, that contained an error due to a peculiarity in one of the genotypes. I am at the same time highly impressed and truly sorry for everything I put you through. It only shows how exceedingly good you were, that both publications also carry your name! I hope that every lab experience in the future gets easier and more rewarding! Thank you for never giving up, it inspired me to do the same!

Then I would like to thank all my friends who gave me the distractions I sometimes desperately required and who showed to me, that there is more in this world than spores, cloning and microscopy.

In the end, the ones left to thank are those the closest to the good and the bad and the ugly: the family. I would like to thank you my beloved Husband Patrik Brehm, for being there for me even in the darkest of hours, never giving up hope and always supporting any decision I made, even if this meant more hardship. Thank you for fetching me from the lab in the middle of the night and picking me up when my cloning decided to completely never ever work at all, no matter how often I tried, until it suddenly did when a new batch of competent cells arrived. Then I would like to thank my father Hans-Werner Bartels for supporting me in every step of the way: when my project required a for the lab too expensive color camera, you came to the rescue. When my less-than-one-year old son made it hard to write on the Manuscript and the thesis, you came to the rescue. Thank you for being my personal thesis super-hero! Then I would like to thank my Mother Gabrielle Bartels and my Sister Laura Bartels for always having an open ear, even in times where it must have been nauseating to listen to my problems over and over again. My family was always my safe haven in the darkest times of my journey.

The very end is also the very beginning of something completely new: I would also like to thank my son Elias Brehm for not only being my natural deadline in this thesis (it could have never ended), giving me the energy and the joy to pull through with everything in life. You are truly my sunshine!

15 Curriculum Vitae

Personal information

Name: Julia Heidelinde Brehm (geborene Bartels)

Date of birth: 23. August 1990

Place of birth: München, Deutschland

Education and research experience

04/2014 – now	PhD Biology | Ludwig-Maximilians-Universität München (LMU) Supervised by Prof. Dr. Thorsten Mascher
10/2011 – 01/2014	M. Sc. Biochemistry | LMU | AG Mascher Very good (1,17). Majors: biochemistry, cell biology. Thesis: "Sporobeads: the utilization of *Bacillus subtilis* endospores for protein display". Very good (1,3)
10/2008 – 09/2011	B. Sc. Biology | LMU | AG Böttger Very good (1,39). Majors: biochemistry, microbiology, genetics, cell biology. Thesis: „ApoControl: Zwei neuartige Systeme zur Selektion transfizierter eukaryotischer Zellen mittels Apoptose". Very good (1,0)
09/2004 – 06/2008	**European Bacalaureat (equivalent to A-levels)| European School Munich** Very Good (1,5). Majors: Mathematics, Biologie, Chemistry.

Publications in peer-reviewed journals:

1. **Bartels J**, Blüher A, López Castellanos S, Formánek P, Günther M, Pinto D, Richter M, Mascher T (2019) The *Bacillus subtilis* endospore crust: protein interaction network, architecture and glycosylation state of a potential glycoprotein layer. Mol Microbiol. doi:10.1111/mmi.14381
2. **Bartels J**, López Castellanos S, Radeck J, Mascher T. (2018) Sporobeads: The Utilization of the *Bacillus subtilis* Endospore Crust as a Protein Display Platform. ACS Synth Biol. Feb 16;7(2):452-461. doi: 10.1021/acssynbio.7b00285. Epub 2018 Jan 19

3. Popp PF, Dotzler M, Radeck J, **Bartels J**, Mascher T. (2017) The Bacillus BioBrick Box 2.0: expanding the genetic toolbox for the standardized work with Bacillus subtilis. Sci Rep. Nov 8;7(1):15058. doi: 10.1038/s41598-017-15107-z.

4. Radeck J, Kraft K, **Bartels J**, Cikovic T, Dürr F, Emenegger J, Kelterborn S, Sauer C, Fritz G, Gebhard S, Mascher T (2013) The *Bacillus* BioBrick box: Generation and evaluation of essential genetic building blocks for standardized work with *Bacillus subtilis***. J Biol Eng**. **7(1)**:29. doi: 10.1186/1754-1611-7-29

Further publication:

1. Cikovic T, **Bartels J**, Dürr F, Emenegger J, Kelterborn S, Kraft K, Radeck J, and Mascher T (2013) Projekt Beadzillus. BIOspektrum. doi: 10.1007/s12268-013-0297-x

Presentations at scientific conferences:

Talks:

1. **Bartels J**, Mascher T (2018) The Bacillus subtilis endospore crust: protein interaction network, architecture and glycosylation state of a potential glycoprotein layer, 8th European Spores Conference, London, Großbritannien

2. **Bartels J**, Mascher T (2016) Utilization of the endospore crust for protein display in *Bacillus subtilis*, 7th European Spores Conference, London, Großbritannien

3. **Bartels J**, Radeck J, Cikovic T, Dürr F, Emenegger J, Kelterborn S, Mascher T (2014) Sporobeads: the utilization of *Bacillus subtilis* endospores for protein display, 6th European Spores Conference, London, Großbritannien

4. Bartels J*, **Radeck J*,** Kraft K, Cikovic T, Dürr F, Emenegger J, Kelterborn S, Mascher T (2013) Sporobeads: The utilization of *Bacillus subtilis* spores to display proteins. 8th CeBiTec Symposium, Bielefeld, Deutschland *shared talk

Posters:

1. **Bartels J**, Mascher T (2017) Sporobeads: The utilization of *Bacillus subtilis* spores to display proteins. The 7th International Meeting on Synthetic Biology (sb7.0), Singapore
2. **Bartels J**, Mascher T (2016) Sporobeads: The utilization of *Bacillus subtilis* spores to display proteins. Annual Conference of the VAAM, Jena, Deutschland

3. **Bartels J**, Mascher T (2016) Utilization of the endospore crust for protein display in *Bacillus subtilis* BACELL, Paris, Frankreich
4. Radeck J, Popp P, Dotzler M, **Bartels J**, Mascher T (2016) Expanding the genetic toolbox for *Bacillus subtilis*. BACELL, Paris, Frankreich
5. Radeck J, Kraft K, **Bartels J**, Cikovic T, Dürr F, Emenegger J, Kelterborn S, Sauer C, Fritz G, Gebhard S, Mascher T (2013) *Bacillus* BioBrick box (B4) - Generation and evaluation of essential genetic building blocks for the standardized work with *Bacillus subtilis*. BioBricks Foundation SB6.0, London, Großbritannien
6. **Bartels J**, Radeck J, Cikovic T, Dürr F, Emenegger J, Kelterborn S, Mascher T (2013) Sporobeads: the utilization of *Bacillus subtilis* endospores for protein display. Symposium Synthetische Biologie, Heidelberg, Deutschland
7. Radeck J, Kraft K, **Bartels J**, Cikovic T, Dürr F, Emenegger J, Kelterborn S, Sauer C, Fritz G, Gebhard S, Mascher T (2013) *Bacillus* BioBrick Box (B^4) - Generation and evaluation of essential genetic building blocks for the standardized work with *Bacillus subtilis*. Symposium Synthetische Biologie, Heidelberg, Deutschland

Contributions to the iGEM competition:

Member of the team LMU-Munich:

2010: ApoControl (apoptosis-based kill-switches for selection in human cell cultures)

2011: Bacterial heavy metal biosensors

2012: Beadzillus (Sporobeads: Using the crust of *B. subtilis* endospores for protein display)

Supervisor of the team LMU-Munich:

2014: BaKillus (enable B. *subtilis* to detect, attach to and kill *Staphylococcus aureus* and *Streptococcus pneumoniae*)

Contributions to teaching:

Supervision:

2015/2016: Supervision of a pupil with his “Projektarbeit” (Martin-Anderson-Nexö-Gymnasium)

2015/2016: Supervision of a bachelor student (Technische Universität Dresden)

2017/2018: Supervision of a master student (Teschnische Universität Dresden)

Coordination of practical course:

2016 (January): “M14-Molekulare Biotechnology” Dr. Diana Wolf (coordination, supervision and script), Julia Bartels (coordination, supervision and script) Susanne Krause (material and supervision) and Prof. Dr. Thorsten Mascher (scientific head and lecture)

2016 (December): “M23-Angewandte und Synthetische Mikrobiologie” Julia Bartels (coordination, supervision and script), Annett George (material and supervision) and Prof. Dr. Thorsten Mascher (scientific head and lecture)

2017 (November): “M23-Angewandte und Synthetische Mikrobiologie” Julia Bartels (coordination, supervision and script), Susanne Krause (material and supervision), Fynn Keßeler (supervision), Karen Stetter (supervision) and Prof. Dr. Thorsten Mascher (scientific head and lecture)

Honors and Awards.

04/2011 – 01/2014	Scholarship: Studienstiftung des deutschen Volkes
2012	· iGEM (the whole iGEM-Team LMU-Munich 2012): · 3rd runner up of 190 teams, “Best Wiki”-and “Best New Application”-Award, gold medal · Lehre@LMU: „LMU-Forscherpreis für exzellente Studierende“
2014	· iGEM (the whole iGEM-Team LMU-Munich 2014): · “Best-Wiki“-Award and gold medal