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Toxin-Antitoxin systems eliminate defective cells and preserve symmetry in *Bacillus subtilis* biofilms

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Summary

Toxin-antitoxin modules are gene pairs encoding a toxin and its antitoxin, and are found on the chromosomes of many bacteria, including pathogens. Here, we characterize the specific contribution of the TxpA and YqcG toxins in elimination of defective cells from developing *Bacillus subtilis* biofilms. On nutrient limitation, defective cells accumulated in the biofilm breaking its symmetry. Deletion of the toxins resulted in accumulation of morphologically abnormal cells, and interfered with the proper development of the multicellular community. Dual physiological responses are of significance for TxpA and YqcG activation: nitrogen deprivation enhances the transcription of both TxpA and YqcG toxins, and simultaneously sensitizes the biofilm cells to their activity. Furthermore, we demonstrate that while both toxins when overexpressed affect the morphology of the developing biofilm, the toxin TxpA can act to lyse and dissolve pre-established *B. subtilis* biofilms.

Introduction

Toxin–antitoxin (TA) systems are specific genetic modules, and are ubiquitous in bacterial chromosomes and plasmids. Each module consists of a pair of genes that encodes for two components: a stable toxin and an unstable antitoxin that interferes with the lethal action of the toxin. Found first in *Escherichia coli* on low copy number plasmids, TA systems are responsible for what is called the post-segregational killing effect. When bacteria lose these plasmid(s) (or other extrachromosomal elements), the cured cells are selectively killed because the unstable antitoxin is degraded faster than the more stable toxin [reviewed in (Hayes, 2003; Engelberg-Kulka *et al.*, 2006; Hayes and Van Melderen, 2011). The cells are 'addicted' to the short-lived antitoxin product, because its *de novo* synthesis is essential for cell survival. TA systems, some of which are homologous to these extrachromosomal 'addiction modules', are widespread in prokaryote chromosomes, with species frequently possessing tens of plasmid and chromosomal TA loci (Hayes, 2003; Engelberg-Kulka *et al.*, 2006; Hayes and Van Melderen, 2011).

The complexes are categorized into five types based on genetic organisation, the nature of the antitoxin and the mechanism of action (Markovski and Wickner, 2013). TAs classified as type I and II systems are probably the most abundant and the most extensively studied (Hayes and Sauer, 2003; Fozo *et al.*, 2010). In these TA systems, the toxins are proteins directed against specific intracellular targets. The antitoxins are either small RNAs [Type I TA systems (Fozo *et al.*, 2010)] or proteins [Type II TA systems (Unterholzner *et al.*, 2013)] that inhibit toxin synthesis or alternatively, neutralize the toxin (Hayes, 2003).

As a result of TA systems activity in a subpopulation of the cells, key resources are conserved, ensuring the survival of individual cells and, consequently, of the bacterial population (Kolodkin-Gal *et al.*, 2007; Kolter, 2007; Moll and Engelberg-Kulka, 2012; Bayles, 2014). The toxin effects can be transient and reversible, permitting a set of dynamic, tunable responses that reflect environmental conditions. In *Bacillus subtilis* the EndoA toxin, encoded by the *ndoAI/ndoA* TA system (Park *et al.*, 2011), was shown to have an effect on cell counts during vegetative growth and to play a role in stress resistance and tolerance (Wu *et al.*, 2011).

A direct role for TA systems in controlling the transformation from planktonic to biofilm cells has long been debated: The MqsR toxin in *E. coli* was shown to be involved in the production of curli, the amyloid structural component of *E. coli* biofilms (Soo and Wood, 2013). In addition, deletion of the *mazEF* and *dinJ-yqfQ* TA systems in *E. coli* decreased biofilm formation (Kolodkin-Gal *et al.*, 2009). Last, the HipA-HipB TA system promoted biofilm formation

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in *Shewanella oneidensis* and in *E. coli*. presumably by the release of extracellular DNA (eDNA)(Zhao *et al.*, 2013). This direct role is reminiscent of the roles played by the holin−antiholin (murine hydrolase- cognate inhibitor) system during biofilm development (Sadykov and Bayles, 2012; Chen *et al.*, 2015).

The Gram-positive bacterium *B. subtilis* can form a structured biofilm colony on top of solid surfaces, where cells are encapsulated by an especially thick extracellular matrix (Branda *et al.*, 2001; Aguilar *et al.*, 2007). The main components of *B. subtilis* extracellular matrix are exopolysaccharides, synthesized by the *epsA-O* operon, TasA, a functional amyloid, encoded in the three-gene operon *tapA-sipW-tasA* (Branda *et al.*, 2006; Chu *et al.*, 2006), and BslA, which forms a hydrophobic coat over the biofilm (Mielich-Suss and Lopez, 2015).

In *B. subtilis*, the activation of the extracellular matrix genes is critical for biofilm formation. The master regulator Spo0A∼P governs matrix gene expression by controlling the activity of the master regulator SinR, a repressor of the *epsA-O* and *tapA-sipW-tasA* operons (Branda *et al.*, 2001; Chai *et al.*, 2010; Kearns *et al.*, 2005). Spo0A∼P also represses a second matrix gene repressor, AbrB. Like SinR, AbrB represses both the *tapA-sipW-tasA* and *eps A–O* operons. The two-component DegS–DegU system is an additional pathway that regulates the expression of the extracellular matrix operons, as well as BslA (Mielich-Suss and Lopez, 2015). During biofilm development, the extracellular toxins SDP (Sporulation Delaying Factor) and SKF (Sporulation Killing Factor) (Gonzalez-Pastor *et al.*, 2003; Ellermeier *et al.*, 2006) are secreted by extracellular matrix producer cells (Lopez *et al.*, 2009a,b; Rosenberg *et al.*, 2016).

Localized cell death is an additional factor recently discovered to participate in *B. subtilis* development (Asally *et al.*, 2012). During early biofilm development, cell death is triggered by mechanical forces and can enhance wrinkle formation.

In this work, we report on the involvement of two TA systems with a novel role in the process of biofilm morphogenesis and preservation of symmetry in developing colonies: *txpA-ratA*, a type I TA system and *yqcGF*, a type II TA system. These TA systems are located in an Horizontal Gene Transfer (HGT) region of the *skin* cryptic prophage (Canchaya *et al.*, 2003). TxpA toxin is a small toxic peptide that, in the absence of its cognate antitoxin, causes delayed lysis of colonies grown on a solid medium (Silvaggi *et al.*, 2005). The toxin YqcG is a toxin protein carrying C-terminal region that shares significant sequence identity with the toxic CdiA-CT effector domains (Hobbs *et al.*, 2010; Holberger *et al.*, 2012). These domains were characterized to participate in contact-dependent growth inhibition systems, which promote growth inhibition and cell death on direct contact (Aoki *et al.*, 2010). In a recent work, YqcG was also shown to act as a DNAse (Elbaz and Ben-Yehuda, 2015).

Both of these TA systems were previously reported to be under the direct or indirect control of the master regulator Spo0A (Molle *et al.*, 2003), which also regulates the expression of the extracellular matrix operons (Branda *et al.*, 2001; Chai *et al.*, 2008). The co-regulation of biofilm development and the expression of the toxins TxpA and YqcG, highlights the possibility that these toxins have a direct or indirect role in biofilm development.

Here, we demonstrate that *txpA-ratA* and *yqcGF* are activated similarly during nutrient starvation in biofilms. In agreement with this finding, we demonstrate that *txpA-ratA* and *yqcGF* specifically act to remove abnormal cells from the biofilm population during developmental processes.

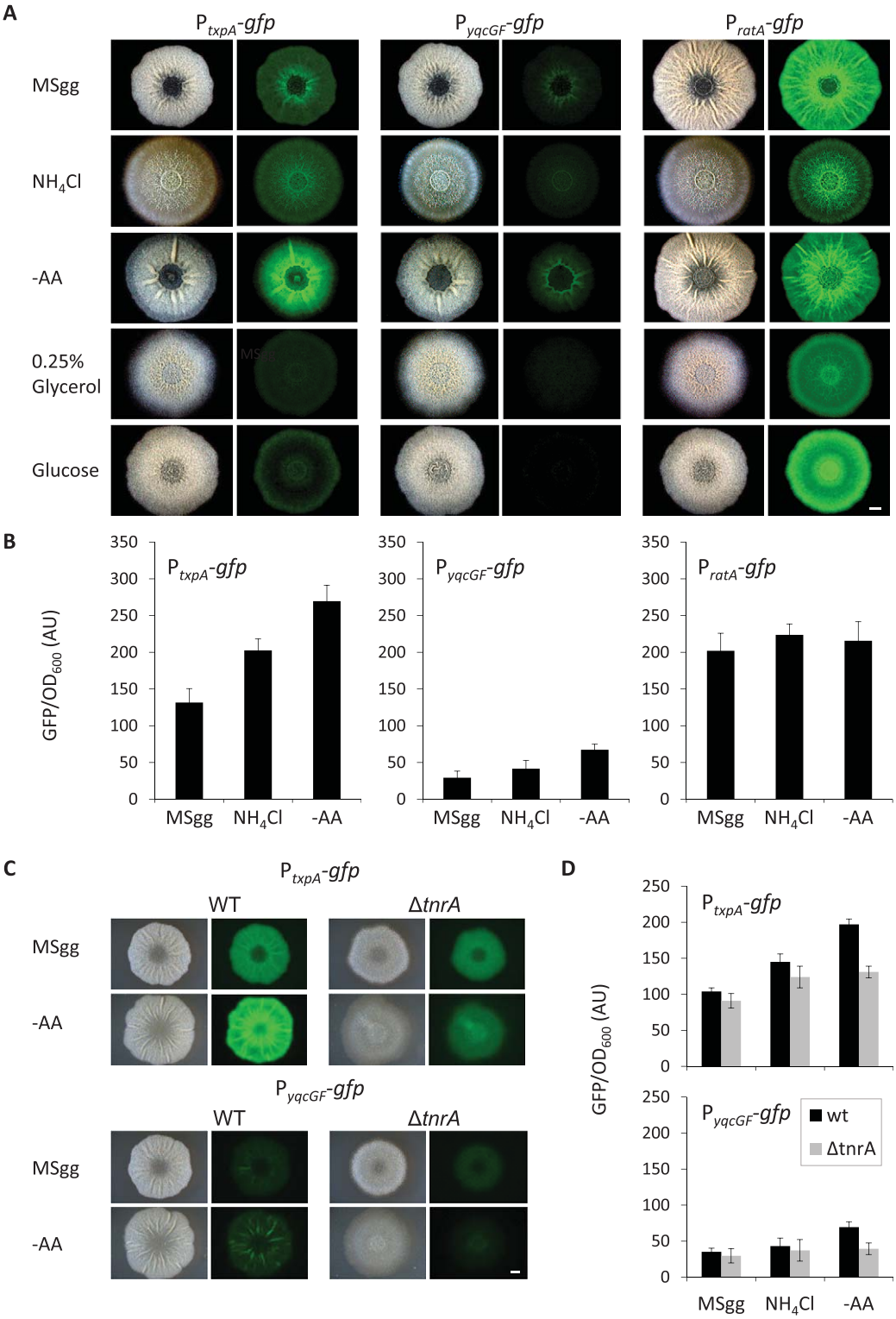
Results

The toxin genes yqcG and txpA are expressed in developing biofilms and enhanced under non-optimal nitrogen supply

We first set out to examine the expression patterns of the toxins within the developing colony. To this end, their promoters were fused to a gene encoding the green fluorescent protein (*gfp*) and monitored its expression using a stereo microscope. In addition, the fluorescence level of the colonies was measured using a microplate reader and normalized to the optical density. On standard biofilm medium (MSgg), *txpA* expression was pronounced and *yqcG* expression was significantly weaker (Fig. 1A and B).

The expression pattern of *txpA* toxin had clear spatial organisation within the biofilms (Fig. 1A and Supporting Information Figure S1), therefore, local nutrient limitation within the biofilm can account for the expression patterns. Thus, we monitored the expression of the toxins within biofilms grown on modified biofilm media with varying concentrations of nitrogen, amino acids and carbon sources. We found that alteration of the nitrogen source from glutamate towards a non-preferential nitrogen source, such as ammonium chloride (hereinafter 'NH_4Cl' medium), induced the expression of *txpA* (Fig. 1A and B). An induction of the expression of both toxins was observed when the biofilm medium was depleted from the non-essential amino acids (hereinafter '–AA' medium) (Fig. 1A and B). Alteration in the concentrations or source of the available carbon source did not induce the expression of *txpA* and *yqcG* (Fig. 1A and B), suggesting that the expression of the toxins is specifically up-regulated under conditions of nitrogen limitation.

The TA pair YqcG and YqcF is expressed from a common upstream promoter (Hobbs *et al.*, 2010). In contrast, RatA, the cognate antitoxin for the TxpA toxin, is expressed from a separate promoter and thus this TA system can also be activated by reducing the expression of the *ratA* promoter. Therefore, we investigated the



expression of *ratA*. *ratA* expression was more pronounced than the expression of its cognate toxin *txpA* on standard biofilm medium. Importantly, the induction of *txpA* under nitrogen starvation was specific to the toxin, as the promoter of *ratA*, remained largely unaltered (Fig. 1B), suggesting the TxpA-RatA TA system may be activated by fluctuations in the expression of the toxin, rather by a decrease in the antitoxin.

A transcription factor termed TnrA controls the expression of nitrogen-regulated genes in *B. subtilis*. This transcription

Fig. 1. Toxins and antitoxin systems *txpA-ratA* and *yqcGF* are expressed in *Bacillus subtilis* biofilms across environmental conditions.
A. Top view of biofilm morphology and GFP expression from the P_{txpA}-*gfp*, P_{yqcG}-*gfp* and P_{ratA}-*gfp* constructs following 48 h of development on different media: standard biofilm-inducing medium (MSgg), biofilm medium in which the nitrogen source was altered from glutamate to NH_4Cl in equivalent molar ratio ('NH_4Cl' medium), biofilm medium lacking tryptophan, phenylalanine and threonine ('-AA' medium), biofilm medium in which glycerol concentration was reduced to 0.25% ('0.25% glycerol' medium) and biofilm medium in which glycerol was altered to glucose in equivalent molar ratio ('glucose' medium). Images were taken with a stereo microscope using white light (left) or fluorescence (right). Scale bar corresponds to 2 mm.
B. Bar diagram representing the fluorescence levels for the P_{txpA}-*gfp*, P_{yqcG}-*gfp* and P_{ratA}-*gfp* reporters, across different conditions; standard biofilm inducing medium (MSgg), 'NH_4Cl' medium and '-AA' medium. Biofilms were harvested at 48 h, and mildly sonicated. Fluorescence and optical density for each sample were evaluated in the microplate reader (Synergy 2, BioTek). Bars show mean ± STD of three biological repeats. For P_{txpA}-*gfp*, P_{yqcG}-*gfp, P* value was calculated using a student's *t*-test. $P < 0.05$, for '-AA' medium compared with the expression of the same promoters on MSgg, $P < 0.1$, for 'NH_4Cl' medium compared with the expression of the same promoters on MSgg.
C. Top view of biofilm morphology and GFP expression following 48 h of development for the wild-type strains carrying the P_{txpA}-*gfp* reporter or P_{yqcG}-*gfp* reporter, and their *tnrA* mutant derivatives, grown on standard biofilm medium (MSgg) and '-AA' medium. Images were taken with a stereo microscope using white light (left) or fluorescence (right). Scale bar corresponds to 2 mm.
D. Bar diagram representing the fluorescence level for the wild-type and its *tnrA* mutant derivative carrying P_{txpA}-*gfp* (top) and P_{yqcG}-*gfp* (bottom) reporters, across different conditions; standard biofilm inducing media (MSgg), 'NH_4Cl' medium and '-AA' medium. Biofilms were harvested at 48 h, and mildly sonicated. Fluorescence and optical density for each sample were evaluated in a microplate reader (Synergy 2, BioTek). Bars show mean ± STD of three biological repeats.

regulator belongs to the MerR family and is active during nitrogen-limited growth (Wray *et al*., 1996). Most of the genes positively regulated by TnrA encode enzymes involved in ammonia uptake, nitrite and nitrate assimilation, while among the negatively regulated genes are the glutamate synthase and glutamine synthetase operons (Wray *et al*., 1996; Yoshida *et al*., 2003). TnrA function as a transcription factor is inhibited by a direct binding of glutamine synthetase in conditions of high nitrogen (Wray *et al*., 2001). Thus, TnrA is activated in response to a decrease in nitrogen levels.

As *txpA* and *yqcGF* transcription was induced under nitrogen limitation, we wondered whether they could be regulated by TnrA. Thus, we tested a mutant strain in *tnrA* for the expression *txpA* and *yqcGF*. In regular MSgg medium, the deletion of *tnrA* resulted in a severe delay in the formation of the biofilm wrinkles, but had little or no effect on the expression of the toxins' genes (Fig. 1C and D). In contrast, the induction of the expression of *txpA* and *yqcGF* in the '-AA' medium, was diminished in a *tnrA* mutant strain, indicating that the increased expression of the toxins under nitrogen limitation partially results from direct or indirect regulation by TnrA. Importantly, the effect of deleting TnrA on the expression of the TxpA and YqcG toxins was pronounced in the '–AA' medium, and modest in the 'NH_4Cl' medium (Fig. 1D). These results may indicate that TnrA is activated in response to the absence of amino acids in the growth media, and that non-essential amino acids are likely to be utilized as a nitrogen source.

Expression of yqcG and txpA toxins in biofilms is under the control of the master regulators: Spo0A, AbrB, DegU and SinR

As *txpA* and *yqcG* were induced during biofilm development, we asked whether the expression of the toxins is controlled by biofilm regulatory pathways. Importantly, a mutation in *tnrA* (Fig. 1C) had a little or no effect on the basal expression of the toxins on standard biofilm medium. Thus, it is plausible that in these conditions the observed expression of *txpA* and *yqcG* during biofilm development is coupled to the activation of master regulators of extracellular matrix production.

To investigate the possibility of transcriptional activation of the toxins by the known biofilm regulators (for a schematic model - see Fig. 2A), we studied the expression of *txpA* and *yqcG* reporters in the genetic background of mutants for biofilm master regulators *spo0A, abrB, sinR, sinI* and *degU*. The absence of Spo0A blocked the expression of both toxins, in agreement with previous observations regarding *yqcG* (Fig. 2B and C) (Molle *et al*., 2003). AbrB antagonizes the activity of Spo0A (Fig. 2A), and thus, if the toxins are activated directly or indirectly by Spo0A, expression will be enhanced in *abrB* mutants. Indeed, deletion of *abrB* significantly enhanced the expression of both *txpA* and *yqcG* throughout the biofilm (Fig. 2B).

If *yqcG* and *txpA* are regulated similarly to the matrix proteins, they should be activated in cells lacking the matrix repressor SinR (Kearns *et al*., 2005; Chai *et al*., 2008). Both *txpA* and *yqcG* were constantly expressed throughout *sinR* mutant colonies, suggesting that, in addition to Spo0A, SinR activity contributes to the activation of the toxins (Fig. 2B). SinR activity in repressing matrix proteins is antagonized by activation of SinI (Fig. 2A). Thus, we examined the expression of the toxins TxpA and YqcG in a *sinI* mutant background and found that expression was dramatically reduced (Fig. 2B and C), suggesting that SinR/SinI regulation plays an important role in activation of the toxins. To further assess the involvement of SinR/SinI pathway in the induction of *txpA* and *yqcG* and to rule out indirect effects of biofilm development, we measured promoter activity in shaking culture. As shown *txpA* and *yqcG* expression was decreased in the absence of SinI and

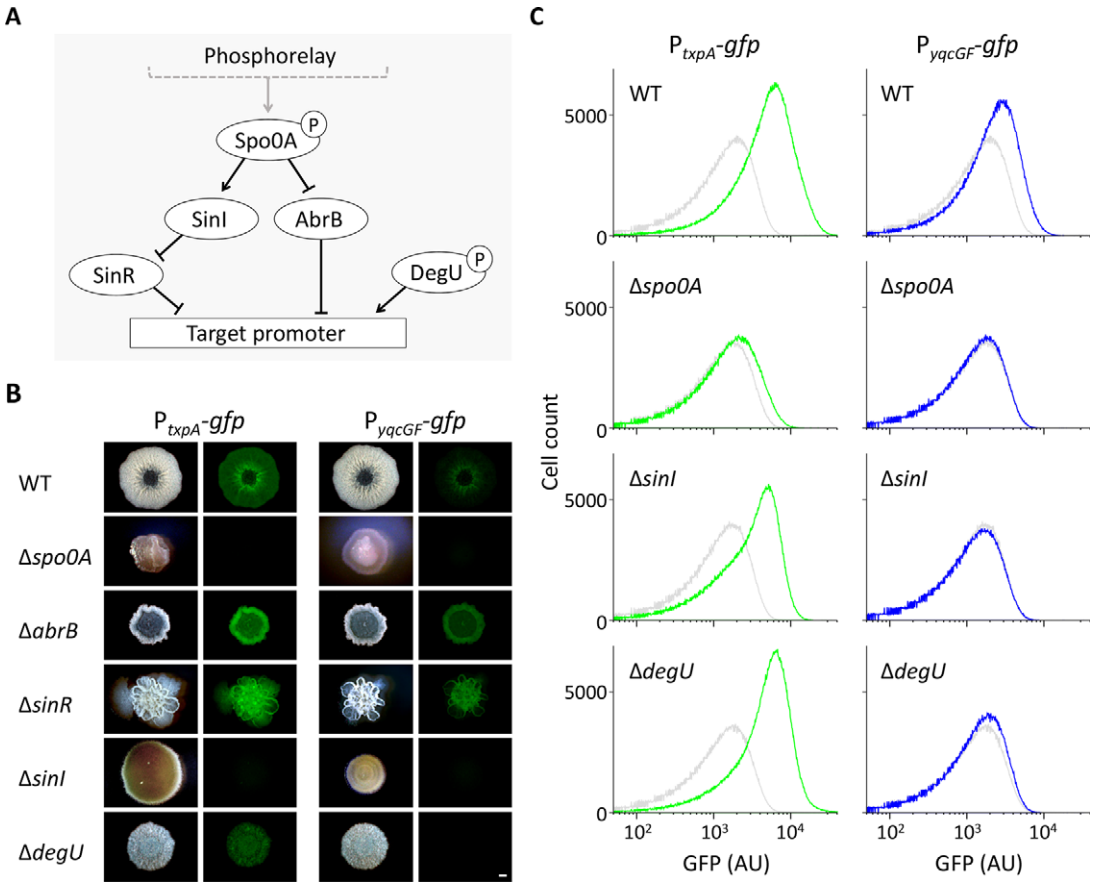


Fig. 2. Toxin genes are under the control of the biofilm master regulators.
A. A simplified scheme of the regulatory network that controls biofilm formation in *Bacillus subtilis*. Effects of biofilm master regulators are integrated to activate (arrows) or repress (T-bars) biofilm formation via activation of the matrix operons. Spo0A is phosphorylated by five histidine kinases, designated KinA-E (not shown). Spo0A phosphorylation then induces the SinI protein, which antagonizes the SinR repressor, which directly binds to and represses the matrix operons *epsA-O* and *tapA-sipW-tasA* (Target promoter). Spo0A∼P also represses a second repressor, AbrB, involved in the repression of *epsA-O* and *tapA-sipW-tasA*. An additional pathway that only regulates the expression of a small subset of matrix genes involves DegU∼P. The circuits involved in biofilm formation are described in detail in (Vlamakis *et al*., 2013).
B. Top view of biofilm morphology and GFP expression of the P_{txpA}-*gfp* and P_{yqcG}-*gfp* reporters, examined in a wild-type parental strain as well as in its mutant derivatives: Δ*spo0A,* Δ*abrB,* Δ*sinR,* Δ*sinI* and Δ*degU*. Images were taken with a stereo microscope using white light (left) or fluorescence (right). Scale bar corresponds to 2 mm.
C. Flow cytometry analysis of the P_{txpA}-*gfp* and P_{yqcG}-*gfp* reporters in various genetic backgrounds. A biofilm of a non-fluorescent strain of the same genetic background (gray) was compared to a strain containing the P_{txpA}-*gfp* reporter (green, left column), or the P_{yqcG}-*gfp* reporter (blue, right column).

Spo0A, and dramatically increased in the absence of SinR (Supporting Information Figures S2 and S3), further corroborating our results that these toxins are repressed by SinR.

We then asked whether the two-component DegS/DegU system, which participates in biofilm regulation, also plays a role in the induction of TxpA and YqcG during biofilm development. The expression of *yqcG* but not of *txpA* in a *degU* mutant was completely eliminated, (Fig. 2B and C).

Overall, these findings indicate that the expression of both TxpA and YqcG is consistent with that of genes participating in biofilm development and thus these toxins share an expression pattern with the matrix operons.

Overproduction of TxpA and YqcG results in cell death and hinders biofilm development

In order to further study the effect of YqcG and TxpA activity on the development of the biofilm, we overproduced the toxins by creating overexpression constructs of each toxin under the regulation of an Isopropyl β-d-1-thiogalactospyraniside (IPTG)-inducible promoter. Cells were cultured in liquid LB medium to mid-logarithmic phase and then diluted into a liquid biofilm medium with IPTG that would induce toxin expression, or without it. Overproduction of TxpA and YqcG in shaking liquid cultures, resulted in dramatic growth arrest within minutes from the initial induction (Supporting Information Figure

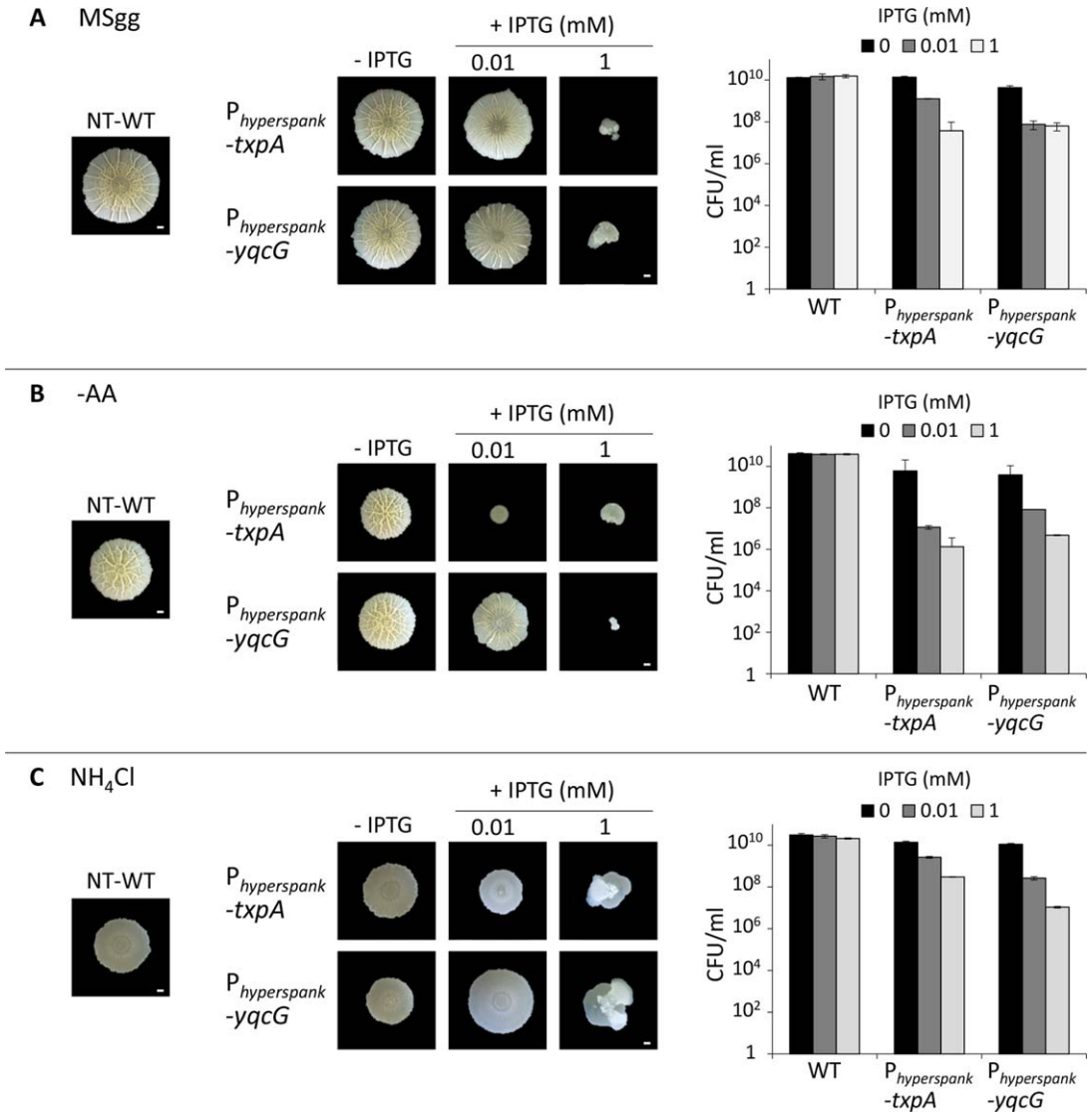


Fig. 3. Overproduction of *yqcG* and *txpA* results in altered toxicity depending on the growth medium.
A−C. Top view of biofilm morphology (middle column) and colony forming unit (CFU) counts (right column), of strains harboring the overexpression constructs of the toxins, $P_{hyperspank}$-*txpA* and $P_{hyperspank}$-*yqcG*, following 72 h of development with or without varying concentration of IPTG as indicated. An untreated wild-type (NT-WT) serves as a control (left column).
A. Standard biofilm inducing medium (MSgg).
B. '-AA' medium.
C. 'NH_4Cl' medium.

S4) and led to a two orders of magnitude drop in the number of culturable cells, as judged by the number of colony-forming units (CFU), which decreased from 10^7 to less than 10^5 (Data not shown).

yqcG and *txpA* expression is triggered by altering the nitrogen source to a less favourable source and by depleting the non-essential amino acids from the biofilm medium (Fig. 1). We asked whether these conditions may also affect the outcomes of the toxins' overexpression. To this end, we first compared the viability of strains overproducing each of the toxins to varying levels, achieved by varying the concentration of the IPTG from 1 mM to 0.01 Mm (Fig. 3). Cells were inoculated on either standard biofilm medium, on '-AA' medium, or on 'NH_4Cl' medium.

The various media had little effect on growth of the strains carrying the inducible overexpression construct of *yqcG* and *txpA* when IPTG was not added (Fig. 3). However, on addition of IPTG in concentration of 1 mM, a severe growth defect was observed in strains carrying the inducible overexpression constructs. Cells growth on the various biofilm media was completely arrested for 48 h (Supporting Information Figure S5), followed by a slow growth composed mostly of suppressor strains 72 h after inoculation (Fig. 3), carrying mutations on the overexpression

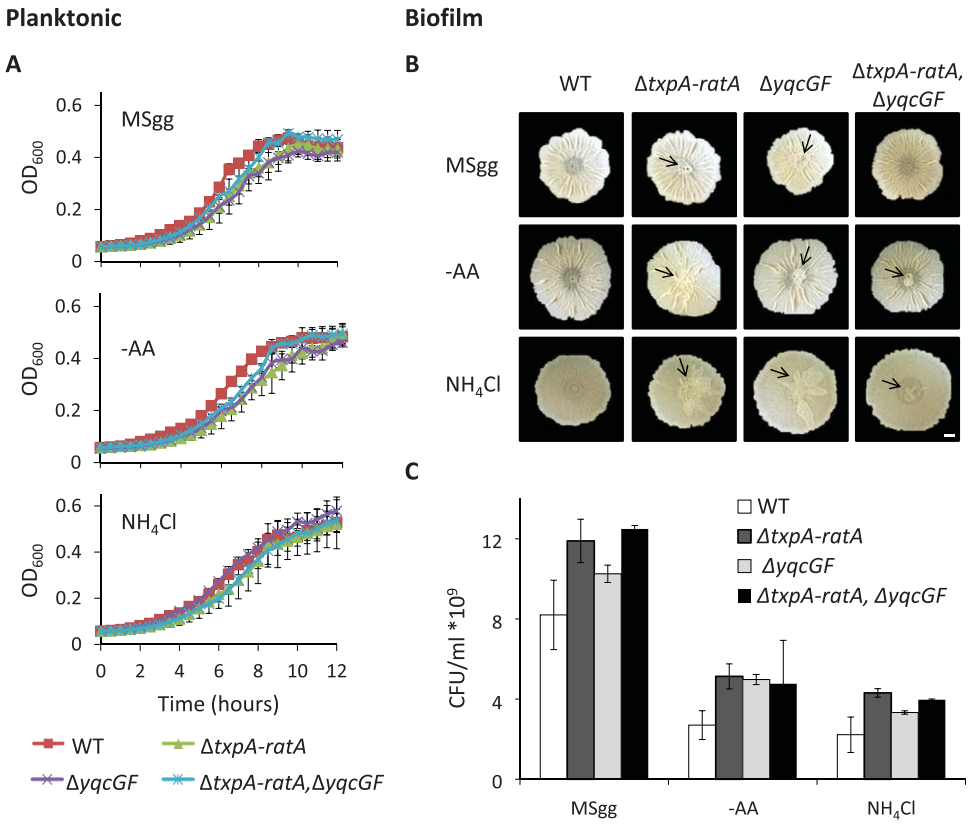


Fig. 4. Deletion of toxins and antitoxin systems *txpA-ratA* and *yqcG-yqcF* specifically alters biofilm formation.
A. Growth curves of liquid cultures of the following bacterial strains: WT, Δ*txpA-ratA* Δ*yqcGF*, and a double mutant for Δ*txpA-ratA*, Δ*yqcGF*. Cultures were grown in LB for 4 h and were then diluted (1:100) in either liquid standard biofilm medium (MSgg), '-AA' medium, or 'NH_4Cl' medium. Bacterial growth as OD_{600} was measured over time (hours). Each graph represents the mean ± STD of an independent representative experiment performed with seven independent technical repeats from at-least three independent experiments.
B. Top view images of biofilms following 72 h of development on the various biofilm media. Arrows indicate abnormal development.
C. Colony forming unit (CFU) count from developing bacterial biofilms. Biofilms such as in B were washed, diluted and plated on LB plates. Shown is mean and STD of three independent repeats, performed in triplicates.

constructs (data not shown). Importantly, the rise of suppressors between 48 and 72 h in the 1mM IPTG treatment influenced the CFU counts presented in Fig. 3 that were performed 72 h post inoculation, and it is highly feasible that the number of killed cells is underestimated.

Culturable cell number in the strain overexpressing TxpA was most dramatically reduced in the '-AA' medium (Fig. 3B). In addition, the drop in viable cell counts caused by the overexpression of YqcG was most severe in the 'NH_4Cl' medium (Fig. 3C).

When low inducer concentration (0.01 mM IPTG) was added to the standard 'MSgg' medium, the strain carrying the TxpA overexpressing construct, showed minor biofilm defects compared to the wild-type. Those biofilm defects were dramatically enhanced in the '-AA' and 'NH_4Cl' media (Fig. 3).

These results may suggest that the cells can be sensitized to the toxins activity in a manner that depends on the availability of nitrogen.

We then asked whether TxpA and YqcG are capable of promoting cell death, in addition to their capacity to induce severe growth arrest. To address this question, toxin expression was induced after cells were grown planktonically to the mid-logarithmic stage [$OD_{600} = 0.6$]. A dramatic reduction in the optical density was observed immediately after inducing TxpA expression, and a more modest reduction was demonstrated 2 h after inducing the production of YqcG (Supporting Information Figure S6). These results imply that TxpA is capable of induce of cell death and lysis of *B. subtilis* cells grown in cultures.

YqcG and TxpA have distinct effects on biofilm colony development

In a bacterial culture, the death or growth arrest of a defective cell subpopulation may provide extra nutrients for the cells that remain viable (Kolodkin-Gal *et al.*, 2007; Amitai *et al.*, 2009). As we found that *txpA* and *yqcG* are expressed during biofilm development, are under the regulation of the biofilm master regulators, and their

overexpression affects biofilm development, we then asked what would be the phenotypic outcome of their absence.

When grown planktonically (Fig. 4A and Supporting Information Figure S7), single mutants for each toxin and the double mutant had comparable growth rate to the wild-type (e.g., generation times in MSgg: wild-type, 1.64 ± 0.1 h; Δ*yqcGF*, 1.64 ± 0.04 h; Δ*txpA-ratA*, 1.59 ± 0.105 h; and ΔtxpA-ratA, Δ*yqcGF*, 1.67 ± 0.06 h). However, death or growth arrest of a defective cell subpopulation may become more influential in dense cell populations. If so, then the absence of TxpA and YqcG may have a more dramatic outcome during biofilm development.

Biofilm colonies of single mutants for each of the toxins, and the double mutant developed asymmetric masses and thick clusters in the centre of the biofilms (Fig. 4B). The physiology of the starter culture played a critical role in enhancing the macro-structure phenotype, as this phenotype was evident only when the biofilms were inoculated from starter cultures in the deep-stationary phase (Fig. 4B and Supporting Information Figure S8).

We then asked whether the effect of mutations in the *txpA-ratA* and *yqcGF* TA systems is reflected by an increase in the culturable cell counts in developing biofilms. We found that the total number of *B. subtilis* cells capable of forming colonies within the wild-type biofilms was compromised when compared to the mutant biofilms (Fig. 4C): *txpA* mutant showed a 30% increase in the culturable cell counts when grown on standard biofilm medium, *txpA* and *yqcG* mutants showed a 70% increase in in '-AA' medium, and an almost twofold increase in the viable cell counts when grown on 'NH_4Cl' medium (Fig. 4C).

Elimination of abnormal cells by TA systems may also occur via activation of additional TA systems. To evaluate this assumption, we deleted ten known and putative TA systems, predicted to be expressed during biofilm formation, as predicted from their promoter sequence (Supporting Information Table S3, Sberro *et al.*, 2013). Only *ndoA* toxin mutants had a subtle biofilm phenotype, reminiscent of TxpA and YqcG (Supporting Information Figure S9 and data not shown). Taken together, our results demonstrate that TxpA-RatA and YqcGF, out of the 10 we examined, are the primary TA systems responsible for eliminating defective biofilm cells during *B. subtilis* development.

YqcG and TxpA have distinct effects on the accumulation of abnormal cells during biofilm development

Following our observations that *txpA* and *yqcG* are robustly expressed in biofilms deprived of a preferential nitrogen source or amino acids, and that overproduction of the toxins has dramatic outcomes on bacterial development and viability, we turned to examine the effect of deletions of *yqcG and txpA* toxins at the single-cell level. We asked whether the loss of the toxin affects the morphology of the cells within the biofilms. As the expression of TxpA and YqcG was most pronounced in the centre of the biofilm, we examined the morphology of the cells in this area of colonies from a wild-type strain, and mutants for *txpA-ratA* or *yqcGF* TA systems. Single mutants accumulated cells exhibiting severe defects in cell shape and atypical nucleoid morphologies (Fig. 5). In addition, internal membrane invaginations were observed in many of the mutant cells, indicative of inappropriate cell divisions (Elbaz and Ben-Yehuda, 2010). Moreover, DAPI staining revealed a variety of nucleoid-free cells, which had lost their chromosomes following cell division (Fig. 5A). Increased exposure or DAPI staining further confirmed the presence of nucleoid-free cells (data not shown).

These phenotypes were even more dramatic among cells from colonies grown on '-AA' medium (Fig. 5B and Supporting Information Figures S10-11). The *txpA-ratA* mutant lost the rod shape characteristic of wild-type *B. subtilis* cells and instead, appeared as elongated spheres, which were significantly larger than normal. The spheroid-like morphology resembled the phenotype described for mutants defective in cell-wall synthesis (Bhavsar and Brown, 2006). In addition, we observed a higher frequency of internal membrane invaginations, compared with the frequencies observed in the TA mutants grown in standard biofilm medium. The *yqcGF* mutant demonstrated similar phenotypes but with fewer spheroid-like cells (Supporting Information Figure S10). Screened systematically, defective cells were also present in biofilms of the wild-type cells grown under starvation (Supporting Information Figure S11). However, the number of severely defective cells was significantly higher in the TA mutants. These results imply that TxpA and YqcG can act to remove defective cells from biofilms exposed to nitrogen starvation.

Interestingly, if TxpA and YqcG act to eliminate defective cells, their expression should increase in the abnormal cell masses accumulated in the centre of biofilms deleted for the TA systems. Indeed, when we examined the biofilms of the *txpA-ratA* mutant strain for *txpA* and *yqcGF* expression (Supporting Information Figure S12), cells located in the abnormal masses that developed in the mutant strain showed induction in the expression of the two TA toxins, and overall expression was higher when compared to the wild-type.

As TxpA and YqcG act intracellularily, we also compared the outcomes of their activation to the activation of the secreted cannibalism toxins SKF and SDP, produced in the biofilm environment (Lopez *et al.*, 2009b). We examined the phenotypes of biofilms formed by cells mutated in both cannibalism operons *sdpA-C* and *skfA-H* (hereby designated the *sdp* and *skf* operons). *sdp* and *skf* mutant colonies were often smaller on nitrogen starvation, but in contrast to *txpA-ratA* or *yqcGF* mutants had a symmetric centre comparable to the wild-type strain (Supporting Information Figure S13).

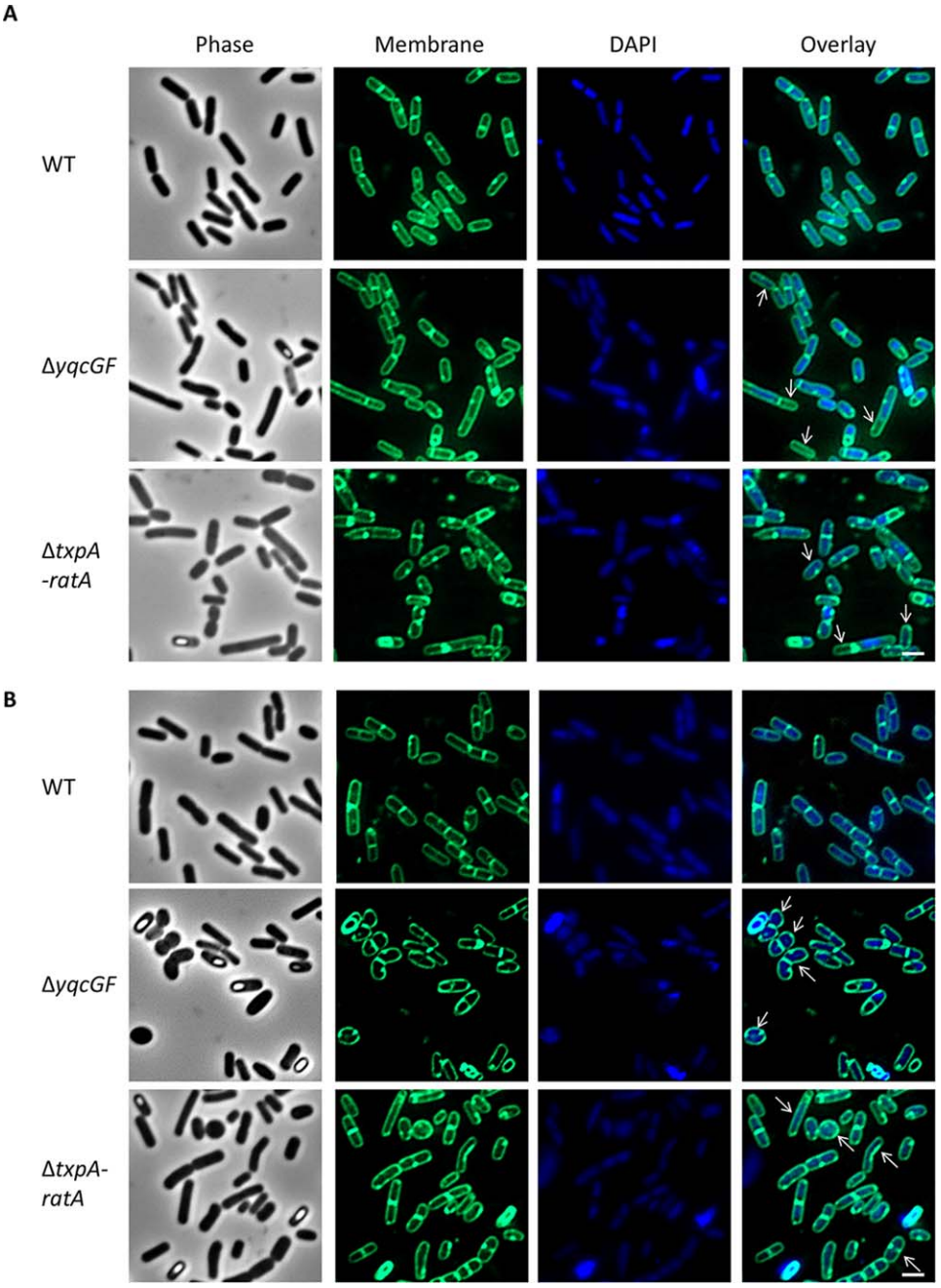


Fig. 5. *txpA-ratA* and *yqcGF* mutants accumulate abnormal cells in biofilms.
A-B. Phase-contrast and fluorescence images of cells from wild-type, Δ*yqcGF* and Δ*txpA-ratA*, taken from the centre of the biofilm. Cells were stained with membrane stain FM1-43 (green) and DAPI (blue). Scale bar corresponds to 2 μm. Arrows indicated deformed cells. Multiple criteria were used to determine cell abnormality in microscope images: cell size and shape (normal *B. subtilis* cell dimensions are 1μm width and 3–5 μm length), DNA presence and organisation, and membrane morphology (presence of puncta).
A. Cells taken from biofilms grown on biofilm-inducing medium (MSgg).
B. Cells taken from biofilms grown on '-AA' medium.

On the single-cell level, we did not detect increased accumulation of abnormal cells shaped as elongated spheres, enriched in internal membrane invaginations, or nucleoid-deficient cells. However, strikingly, we found that under all described conditions, *sdp* and *skf* mutants formed long, connected chains of sessile cells (Supporting Information Figure S14). These cells were maintained as chains throughout the development of biofilms in all tested growth media (Supporting Information Figure S14). These results indicate that the extracellular toxins SDP and SKF act as regulators of cell morphogenesis and chaining, while TA systems *txpA-ratA* and *yqcGF* play an alternative role of eliminating defective cells during *B. subtilis* development.

TxpA can lyse and disassemble pre-established biofilms

As we showed that TxpA and YqcG overexpression led to growth arrest and hampered biofilm development, we then asked whether overexpression of these genes may disperse or lyse pre-established biofilms. For this purpose, we allowed strains carrying the overexpression construct

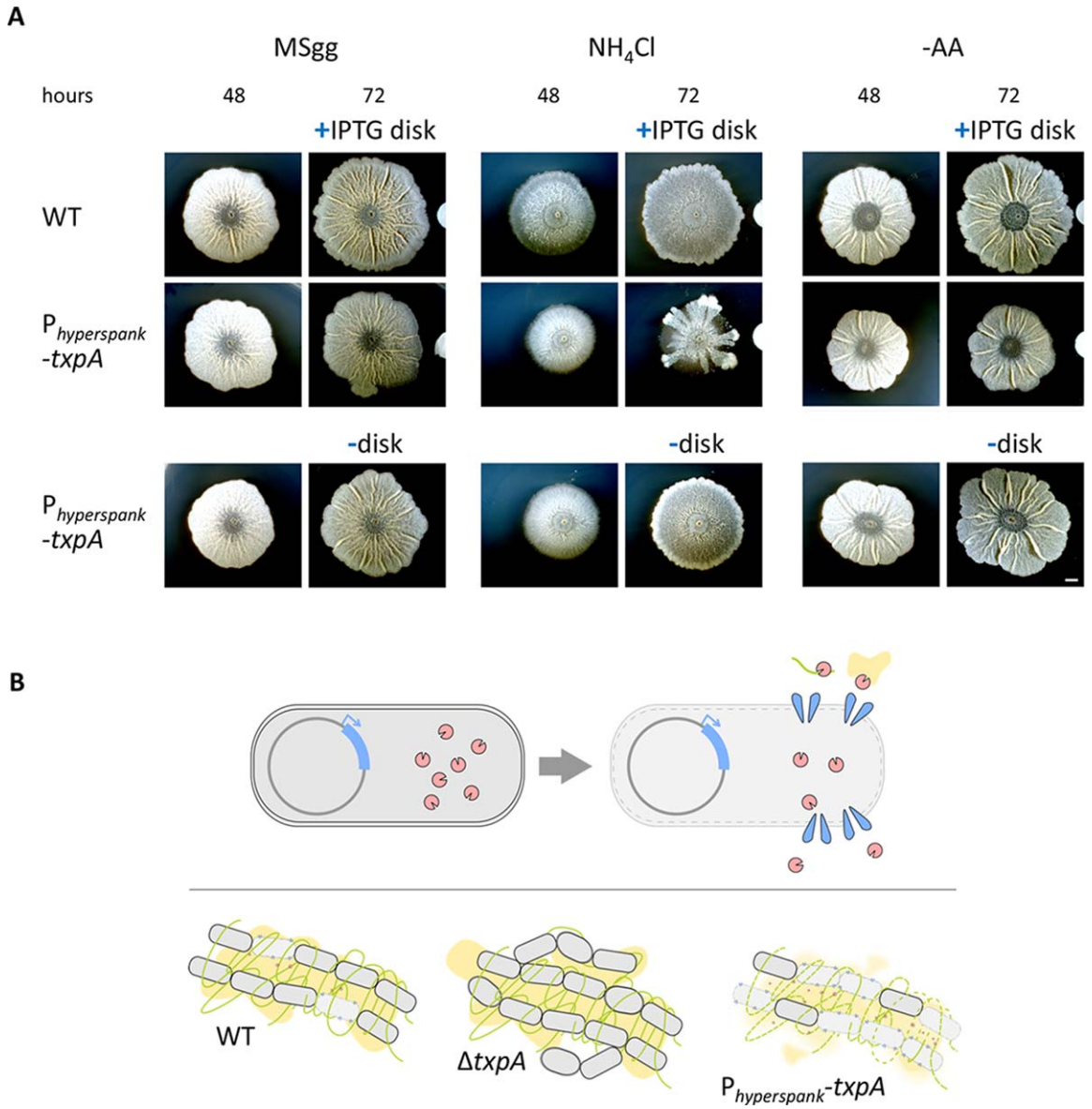


Fig. 6. Overproduction of TxpA can deform and lyse pre-established biofilms.
A. Top view of a wild-type, and $P_{hyperspank}$-*txpA* biofilms grown on various biofilm media before (left) or 24 h after placement of an IPTG disk (soaked for 1 h in 1M IPTG solution) on the periphery of developed colony (right). Images were taken with a stereo microscope. Scale bar corresponds to 2 mm.
B. A model describing the potential roles of the toxin TxpA in biofilms. *Upper*: Schematic representation of a single *B. subtilis* cell inside the biofilm. The *txpA* gene is depicted in blue, intracellular enzymes such as proteases, carbohydrases and endoribonucleases, are depicted in pink. Extracellular matrix components are depicted in yellow and green. Activation of *txpA* gene leads to membrane perforation, and the release of the intracellular enzymes that in turn catabolize the extracellular matrix. *Lower*: Schematic representation of biofilms of wild-type, deletion and over-expressing strains for the TxpA and YqcG toxins. In the deletion strain, there is accumulation of abnormal cells and structures while in the overexpressing strain biofilm is disrupted due to cell death and matrix decomposition.

to develop for 48 h, and then locally exposed them to IPTG at the edge of the colony by placement of IPTG-soaked paper disk (Fig. 6A). The outcomes of the overexpression of both toxins in pre-established biofilms differed dramatically. In all growth media, overexpression of YqcG resulted in only a minor inhibition of biofilm development (Supporting Information Figure S15). In contrast, when the toxin TxpA was expressed within pre-established biofilms on standard biofilm medium (MSgg), many of the wrinkles dissolved and the colony turned flatter. Quite strikingly, when biofilms were grown on 'NH_4Cl' medium, induction of TxpA resulted in dissolution of a significant portion of the

entire colony, both in proximity to the inducer, and throughout the biofilm. This dissolution of the pre-established biofilm colony is consistent with our finding in shaking cultures (Supporting Information Figure S6). Furthermore, it provides evidence that the activation of the toxin TxpA in a mature biofilm could both eliminate biofilm cells, and deform pre-established biofilms. Importantly, the ectopic induction of TxpA in the 'NH_4Cl' medium resulted in flatter and more transparent patches of the biofilm (Fig. 6A), that resemble the appearance of colonies of extracellular matrix mutant strains (Branda *et al*., 2004). Thus, the activation of the intracellular toxin may also result in the dissimilation of the extracellular material.

Discussion

Bacterial cells assemble into multicellular communities via aggregation. These ubiquitous multicellular communities are known as biofilms (Kolter and Greenberg, 2006). Biofilms have been well characterized owing to their prominent role in disease, and their increased antibiotic resistance compared with planktonic cells (Bryers, 2008). In a biofilm, complex signaling cascades lead to the formation of mutually-exclusive cell fates (Lopez *et al*., 2009a). The formation of different cell types allows the development of complex patterns.

Multiple studies demonstrated that the environmental and physiological conditions are not homogeneous throughout a biofilm (Rani *et al*., 2007; Stewart and Franklin, 2008; Liu *et al*., 2015). The metabolic activities of the cells, together with diffusional processes, result in nutrient concentration gradients (Liu *et al*., 2015). As the bacteria respond to these gradients, they adapt to the local chemical conditions, which can change over time as biofilms develop further. Therefore, cells that are growing in biofilms are not only physiologically distinct from planktonic cells, but also differ from one other, both spatially and temporally, as biofilm development proceeds.

In this study, we screened multiple TA systems to characterize their role in biofilm formation and identified two TA systems that are specifically associated with biofilm development: *txpA-ratA* and *yqcGF*. The *txpA-ratA* gene pair encodes a small RNA named RatA and a toxic peptide designated TxpA. RatA is an antisense RNA that binds to and blocks the accumulation of mRNA molecules of the TxpA toxin. This TA system was suggested to have a role in mother cell lysis during sporulation (Silvaggi *et al*., 2005). The second TA system found to play a role in biofilm formation is the *yqcGF* system. The toxin protein YqcG is partially homologues to the toxic CdiA-CT effector domains (Hobbs *et al*., 2010; Holberger *et al*., 2012) and was previously described to be under the control of the biofilm and sporulation master regulator Spo0A (Molle *et al*., 2003). We found that both TA systems are activated during biofilm formation, and their transcription is especially enhanced in localized regions within the biofilms (Fig. 1). Localized expression of the toxins could be an outcome of nutrient limitation, as alternating the nitrogen source towards a non-optimal nitrogen source, or depletion of amino acids from the growth media, further induced the promoter activity of the toxins. Nutrient starvation not only enhanced the transcription of both TxpA and YqcG toxins, but may also sensitize biofilm cells to their activity (Fig. 3).

Importantly, activation of both TxpA and YqcG in mature liquid cultures resulted in cell lysis, demonstrating that under the tested conditions, these toxins could mediate a certain degree of cell death. This finding is consistent with a previous demonstration of TxpA as a trigger of colony lysis in a domesticated strain of *B. subtilis* (Silvaggi *et al*., 2005).

Cell death has already been demonstrated to play an important role in the development of *Pseudomonas aeruginosa* and *Staphylococcal* biofilms, primarily due to release of eDNA, functioning as an extracellular matrix component (Sadykov and Bayles, 2012; Bayles, 2014). Importantly, mutants of *Staphylococcal* holin−antiholin system that triggers cell lysis and eDNA release had defects in biofilm architecture and significantly thinner biofilms (Rice *et al*., 2007). A recent work suggested that a similar phenomenon can occur in early *B. subtilis* biofilm development, as localized cell death was induced in *B. subtilis* colonies by mechanical forces, and promoted wrinkling (Asally *et al*., 2012).

In contrast to those examples, *txpA-ratA* and *yqcGF* mutants developed biofilms comparable in their thickness to the wild-type parental strain (Fig. 4). Thus, we sought to identify the biological function of the *txpA-ratA* and *yqcGF* modules. Detailed examination of cell morphology within mutant biofilms provided a possible clue. In the TA system mutant background, more cells with morphological defects accumulated at the centre of the biofilms, compared to the wild-type strain. As the nutritional stress increased, as in the case of biofilm media lacking amino acids, the TA mutants showed even a more dramatic change in population composition with more abnormal cells. The increase of abnormal cells resulted in a macroscopic change in colony morphology, leading to formation of asymmetric clusters in the centre of the colony. This result implies that the activated TA systems serve to eliminate or arrest growth of defective cells that are no longer required for the development of the community (See a model in Fig. 6B).

It has been previously shown that the subpopulation of cells in *B. subtilis* biofilms, which produces the extracellular matrix, co-expresses and secretes the extracellular cannibalism toxins SKF and SDP (Gonzalez-Pastor *et al*., 2003; Ellermeier *et al*., 2006; Lopez *et al*., 2009b). The effect of the cannibalism toxins on promoting biofilm development became evident only in cell-wall mutants sensitized to the

activity of such toxins (Lopez *et al*., 2009b). Here, we showed that a strain incapable of producing the secreted cannibalism toxins contained numerous intact elongated chains throughout its development, compared to the wild-type parental strain (Supporting Information Figure S13-14). This phenotype was distinct from the phenotypes of the intracellular toxins TxpA and YqcG that show accumulation of cells with spheroid-like shape and membrane invaginations.

Interestingly, both *txpA-ratA* and *yqcGF* are found in a 48-kb, phage-like element known as *skin*. The *skin* element interrupts the coding sequence of the mother cell-specific regulatory protein σ^K during sporulation (Canchaya *et al*., 2003). While it has been previously suggested that *skin* can be considered a selfish element that *B. subtilis* retains in a functional state to ensure spore formation, our results shed light on additional functions of the toxins before the commencement of sporulation. We suggest that the TAs carry a fitness advantage for the biofilm population as a whole, by decreasing the burden of cells that failed to adapt to a nutritional stress.

Quite strikingly, activation of the TxpA toxin in established biofilms resulted in disruption of the biofilm structure and lysis. This capacity can be attributed to the release of intracellular enzymes, such as proteases, carbohydrases and endoribonucleases, capable of degrading the biofilm extracellular matrix, following cell lysis (See a model in Fig. 6B). These results imply that efficient manipulation of peptide toxins similar to TxpA could serve to eliminate established biofilms, an immense need in relevant ecological, medical and biotechnological settings (Harmsen *et al*., 2010; Oppenheimer-Shaanan *et al*., 2013; Wood, 2014).

Materials and methods

Construction of strains

All experiments were performed with *B. subtilis* NCIB 3610 (Branda *et al*., 2001). Laboratory strains *B. subtilis* PY79 and *E. coli* DH5α were used for cloning purposes. Lists of strains and primers are provided in Supporting Information Table S1 and Table S2.

Transformation of *B. subtilis* PY79 with linearized plasmid or PCR products, was performed as previously described (Wilson and Bott, 1968).

Undomesticated *B. subtilis* strain NCIB 3610 is capable of integrating foreign DNA through transformation, although the average yield is up to twofolds lower than the yield of domesticated strains such as PY79 (Parashar *et al*., 2013). Thus, we transformed the linearized plasmid or PCR products into PY79, and used the genomic DNA from the PY79 mutant strains to transform NCIB 3610 (Bucher *et al*., 2015).

Deletion mutations were generated by long-flanking homology PCR mutagenesis (Wach, 1996). The deletion strains of NCIB 3610 were confirmed via PCR using inner and outer primers, and sequencing.

The plasmids pYC121 (Chai *et al*., 2008), which contains a functional GFP gene, and a chloramphenicol resistance gene, and pDR111 (Kolodkin-Gal *et al*., 2010), which contains the hyperspank promoter upstream to a cloning site, the *lacI* repressor gene, and a spectinomycin resistance gene, were used for the construction of GFP reporter strains and overexpression strains respectively. PCR fragments were amplified from NCIB 3610 chromosomal DNA, using primers with the suitable restriction sites for ligation into the plasmids. The ligated plasmids were then transformed into *E. coli* DH5α and ampicillin resistant colonies were selected and confirmed by sequencing. The GFP reporter or overexpression plasmids were then integrated into the neutral *amyE* locus of strain NCIB3610 by transformation, as described above, and selected for chloramphenicol or spectinomycin resistance respectively.

Growth media

The strains were routinely manipulated in LB broth (Difco) or MSgg medium (5 mM potassium phosphate, 100 mM MOPS pH 7, 2 mM $MgCl_2$, 50 μM $MnCl_2$, 50 μM $FeCl_3$ for liquid or 125 μM $FeCl_3$ for solid medium, 700 μM $CaCl_2$, 1 μM $ZnCl_2$, 2 μM thiamine, 0.5% glycerol, 0.5% glutamate, 50 μg ml^{-1} threonine, tryptophan and phenylalanine). The solid medium contained 1.5% bacto agar (Difco). Note that the iron concentration in the solid MSgg medium was 2.5-fold higher compared with the original recipe (Branda *et al*., 2001), as it improved the overall morphology development. Biofilm media were often modified as indicated in the legends for each figure.

Selective media were prepared with LB or LB-agar using antibiotics at the following final concentrations: 100 μg ml^{-1} ampicillin (AG Scientific), 10 μg ml^{-1} kanamycin (AG Scientific), 10 μg ml^{-1} chloramphenicol (Amresco), 10 μg ml^{-1} tetracycline (Amresco), 100 μg ml^{-1} spectinomycin (Tivan Biotech) and 1 μg ml^{-1} erythromycin (Amresco) + 25 μg ml^{-1} lincomycin (Sigma Aldrich) for MLS.

Biofilm development assay

A single *B. subtilis* colony, isolated on solid LB plates, was inoculated in 3 ml LB broth at 37˚C for 30 h unless indicated otherwise. Then, a 2 μL drop was spotted on MSgg solid medium. Plates were incubated at 30˚C for the time period indicated in the legend for each figure. Images and GFP signal intensity were obtained with a Stereo Discovery V20″ microscope with Objective Plan Apo S 0.5x FWD 134 mm or Apo S 1.0x FWD 60 mm (Zeiss) attached to an Axiocam camera. Data were analysed using Axiovision suite software (Zeiss).

Growth measurements

A single colony of *B. subtilis*, isolated on a solid LB plate, was inoculated in 3 ml LB broth and grown for 4 h to the mid-logarithmic growth phase. Cells were diluted 1:100 in 150 μl liquid MSgg medium per well of a 96-well microplate (Thermo Scientific). Cells were grown with agitation at 30°C for 16 h in a microplate reader (Synergy 2, BioTek), and the optical density at 600 nm (OD_{600}) was measured every 15 min.

Determination of live cell counts during growth and biofilm formation

To determine live cell counts of cells grown in shaking cultures or biofilms, cells were harvested either from shaking cultures or from biofilm grown for 72 h as indicated and thoroughly vortexed. For biofilm analysis: cells were mildly sonicated (BRANSON digital sonifier, Model 250, Microtip, amplitude 20%, pulse 3× 5 s). To determine the number of live cell counts, cells were serial diluted in phosphate-buffered saline (PBS) (Biological Industries, Israel), plated on LB-plates, and colony forming units (CFU) were counted after incubation at 23°C for 24 h.

Fluorescence microscopy

Briefly, biofilm cells were roughly separated by pipetting, centrifuged and re-suspended in 10 μl 1X PBS supplemented with 1 μg ml^{-1} of the fluorescent membrane stain FM1-43 (Molecular Probes; Invitrogen) and with 2 μg ml^{-1} of the DNA stain 4,6-diamidino-2-phenylindole (DAPI) (Sigma). Cells were then placed on poly-lysine-coated slides, visualized and photographed using an Axioplan2 microscope (Zeiss) equipped with a high-resolution microscopy Axiocam camera. Data were captured using Axiovision suite software.

Flow Cytometry

Flow cytometry used for mutant backgrounds, where the production of the extracellular matrix and growth rate differed dramatically from the wild-type. *Bacillus subtilis* biofilms were inoculated as described above, and incubated for the time period indicated in the legend for each figure. Biofilms were then scraped from the plate surface and separated into single cells by mild sonication, as previously described (Vlamakis *et al.*, 2008). Samples were measured using an LSR-II cytometer (Becton Dickinson, San Jose, CA, USA) operating a solid-state laser at 488 nm. GFP intensities were collected by 505 LP and 525/50 BP filters. For each sample, 10^6 events were recorded and analysed for GFP intensities. The auto florescence level was determined in each experiment by measuring a sample of a biofilm of a non-fluorescent strain of the same genetic background. Then, the distribution of GFP intensities was determined using a custom MATLAB code.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Bar diagrams representing cell counts using flow cytometry under different conditions for the P_{txpA}-*gfp* reporter, and comparing between the centre of the biofilm to its periphery.

Fig. S2. *txpA* promoter is under the control of the biofilm master regulators. GFP expression from *txpA* promoter normalized to optical density, of each of the indicated genetic backgrounds. Single colonies of the desired strains were grown to their mid-logarithmic phase of growth in LB. Starter cultures were diluted 1:100 in 150 μl liquid MSgg in each well of a 96-well optical-bottom plate (Thermo Scientific). The cultures were grown for 12 hours at 30°C with shaking in a microplate reader (Synergy 2, BioTek), and the optical density at 600 nm and fluorescence were measured every 15 min. For details about strain construction, see Materials and Methods. Mean and STD of at least five individual wells within one experiment are shown. Data shown are representatives from three individual experiments.

Fig. S3. *yqcGF* promoter is under the control of the biofilm master regulators. GFP expression from *yqcGF* promoter normalized to optical density, of each of the indicated genetic backgrounds. Single colonies of the desired strains were grown to their mid-logarithmic phase of growth. Starter cultures were diluted 1:100 in 150 μl liquid MSgg in each well of a 96-well optical-bottom plate (Thermo Scientific). The cultures were grown for 12 hours at 30°C with shaking in a microplate reader (Synergy 2, BioTek), and the optical density at 600 nm and fluorescence were measured every 15 min. For details about strain construction, see Materials and Methods. Mean and STD of at least five individual wells within one experiment are shown. Data shown are representatives from three individual experiments.

Fig. S4. Overproduction of *yqcG* and *txpA* results in toxicity to planktonically growing cells. Growth curve (OD600 vs. time (hours)) of strains carrying $P_{hyperspank}$-*txpA* and $P_{hyperspank}$-*yqcG* on MSgg with or without 0.1 mM IPTG (UI: uninduced).

Fig. S5. Overproduction of *yqcG* and *txpA* inhibits growth on top of a solid biofilm medium for 48 hours. Shown is a top view of biofilm colonies formed by either wild-type (NT-WT) or strains carrying $P_{hyperspank}$-*txpA* and $P_{hyperspank}$-*yqcG* on '-AA' medium, with 0.01 and 1 mM IPTG, or without it.

Fig. S6. Induction of the toxins results in cell lysis. Bacterial cultures were grown in biofilm inducing medium to an OD600 of 0.6, toxins were induced (by addition of 1mM IPTG) and OD600 was measured over time (hours). Each graph shows the mean ±STD of three biological repeats.

Fig. S7. Deletion of toxins and antitoxin systems *txpA-ratA* and *yqcGF* has little or no effect on planktonic growth in a biofilm media. Colony forming unit (CFU) counts of the following bacterial strains: WT, Δ*txpA-ratA*, Δ*yqcGF* and a double mutant for *txpA-ratA*, *yqcGF*, were grown in shaking liquid cultures in the indicated growth media. Samples of the cultures were diluted and plated at indicated times. Each graph represents the mean ± STD of three independent repeats performed in duplicates.

Fig. S8. Deletion of the toxins TxpA and YqcG has little effect on biofilm morphology when cells are inoculated from mid-logarithmic cultures. Top view images of biofilms following 72 hours of development on '-AA' medium.

Fig. S9. Deletion of the *ndoA* toxin has little effect on colony development. Top view images of biofilms grown for 72 hours at indicated media. Scale bar represents 2 mm

Fig. S10. *txpA-ratA* and *yqcGF* mutants accumulate abnormal cells in biofilms. Phase contrast and fluorescence images of cells from Δ*yqcGF*, Δ*txpA-ratA* biofilms were taken from the centre of the biofilm. Biofilms were grown on '-AA' medium. Cells were stained with membrane stain FM1-43 (green) and DAPI (blue). Scale bar corresponds to 2 μm. This experiment represents an independent repeat to the experiment shown in Figure 5B.

Fig. S11. *txpA-ratA* and *yqcGF* mutants accumulate abnormal cells in biofilms. Quantification of phase-contrast and fluorescence images of single cells imaged from wild-type, Δ*yqcGF* and Δ*txpA-ratA* biofilms, taken from the centre of the biofilm, as shown in Figure 5 and Figure S8. Biofilms were grown on '-AA' medium. At-least 1500 cells per strain, for three biological repeats, were counted. A cell was defined as 'Abnormal' if it manifested a severe deformation from the rod-shape cell morphology, lacked a DNA as judged by the DAPI stain, or had severe membrane deformations.

Fig. S12. TA systems *txpA-ratA* and *yqcGF* are induced in biofilms of *txpA-ratA* mutant. (A,B) Top view of biofilm morphology and GFP expression from the P_{txpA}-*gfp* (A) and P_{yqcGF}-*gfp* (B) reporters, in wild-type and *txpA-ratA* mutant strain, following 48 hours of development on standard biofilm-inducing medium (MSgg), and '-AA' medium. Scale bar corresponds to 2 mm. (C) Bar diagram representing the fluorescence level for the wild-type and its *txpA-ratA* mutant derivative carrying P_{txpA}-*gfp* and P_{yqcG}-*gfp* reporters, across different conditions; Biofilms were harvested at 48 h, and mildly sonicated. Fluorescence and optical density for each sample were evaluated in a microplate reader (Synergy 2, BioTek). Bars show mean ± STD of three biological repeats.

Fig. S13. Mutants in the secreted toxins' operons *sdp* and *skf* form smaller colonies with symmetric centres comparable to the wild-type parental strain. Top view images of

biofilms grown for 72 hours at indicated media. Scale bar represents 2 mm

Fig. S14. The accumulation of abnormally chained cells in a strain mutated in the *sdp* and *skf* cannibalism operons. Phase-contrast and fluorescence images of cells from a wild-type strain and Δ*sdpA-C*,*skfA-H*, taken from the centre of biofilms grown on a biofilm-inducing medium (MSgg) or '-AA' medium. Cells were stained with membrane stain FM1-43 (green) and DAPI (blue). Scale bar corresponds to 2 μm.

Fig. S15. The effect of overproduction of YqcG on pre-established biofilms Top view of a wild-type, and $P_{hyperspank}$-*yqcG* biofilms grown on various biofilm media before (left) or 24 hours after placement of an IPTG-soaked disk on the periphery of developed colony (right). Images were taken with a stereo microscope. Scale bar corresponds to 2 mm.

Table S1. Strains used for this study.

Table S2. Primers used for this study.

Table S3. Toxin- Antitoxin systems examined in *Bacillus subtilis* examined in this study.