

Cannibalism enhances biofilm development in *Bacillus subtilis*

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Summary

Cannibalism is a mechanism to delay sporulation in *Bacillus subtilis*. Cannibal cells express the *skf* and *sdp* toxin systems to lyse a fraction of their sensitive siblings. The lysed cells release nutrients that serve to feed the community, effectively delaying spore formation. Here we provide evidence that the subpopulation of cells that differentiates into cannibals is the same subpopulation that produces the extracellular matrix that holds cells together in biofilms. Cannibalism and matrix formation are both triggered in response to the signalling molecule surfactin. Nutrients released by the cannibalized cells are preferentially used by matrix-producing cells, as they are the only cells expressing resistance to the Skf and Sdp toxins. As a result this subpopulation increases in number and matrix production is enhanced when cannibalism toxins are produced. The cannibal/matrix-producing subpopulation is also generated in response to antimicrobials produced by other microorganisms and may thus constitute a defense mechanism to protect *B. subtilis* from the action of antibiotics in natural settings.

Introduction

The ability to differentiate into many distinct cell types is a hallmark of the soil-dwelling, Gram-positive bacterium *Bacillus subtilis*. For example, in nutrient-limiting conditions a subpopulation of cells differentiates into spores (Errington, 2003; Piggot and Hilbert, 2004). Spore development is energy intensive and, once committed, cells

may not exit this state for prolonged periods. Thus, this bacterium has evolved mechanisms to delay entry into sporulation as long as possible. It accomplishes this by having the capacity to direct a subpopulation of cells down a differentiation pathway that gives rise to so-called cannibals (Gonzalez-Pastor *et al.*, 2003; Ellermeier *et al.*, 2006; Claverys and Havarstein, 2007). Cannibal cells are resistant to two toxins, Skf and Sdp, that they secrete to kill a fraction of their siblings. As a result, cannibals overcome nutritional limitation and delay the onset of sporulation.

In addition to spores and cannibals, *B. subtilis* can undergo several other developmental processes. For instance, within multicellular aggregates known as biofilms a subpopulation of cells differentiates to produce an extracellular matrix that encases the community (Branda *et al.*, 2006; Chu *et al.*, 2006; Chai *et al.*, 2008; Vlamakis *et al.*, 2008). This specialized subpopulation is termed matrix-producing because it highly expresses the *epsA–O* (henceforth *eps*) and *yqxM–sipW–tasA* (henceforth *yqxM*) operons that encode the machinery to produce an exopolysaccharide (Eps) and the protein TasA respectively. Eps and TasA are the two main components of the extracellular matrix.

The three developmental pathways described above for *B. subtilis*, sporulation, cannibalism and matrix production, are strongly interconnected. They are activated by the same master regulatory protein, Spo0A (Gonzalez-Pastor *et al.*, 2003; Branda *et al.*, 2004; Fujita *et al.*, 2005; Kearns *et al.*, 2005; Ellermeier *et al.*, 2006). However, Spo0A does not regulate these three pathways identically. For instance, the subpopulation of sporulating cells, which derives from the subpopulation of matrix-producing cells, shows different spatiotemporal distribution within the biofilm when compared with the matrix producers (Vlamakis *et al.*, 2008).

The differential expression of Spo0A-regulated genes is thought to be due to the fact that different promoters respond to different concentrations of phosphorylated Spo0A (Spo0A–P) in the cell (Fujita *et al.*, 2005; Veening *et al.*, 2008). Spo0A can be phosphorylated, either directly or indirectly, by the action of five different kinases (KinA–E) (LeDeaux *et al.*, 1995; Jiang *et al.*, 2000). Presumably depending on the ability of those kinases to

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phosphorylate Spo0A, different levels of phosphorylation can be reached. Lower levels of Spo0A-P suffice for the expression of genes involved in matrix production and cannibalism, whereas higher levels of Spo0A-P are necessary to trigger sporulation (Fujita *et al.*, 2005). Depending on the ability of each kinase to sense different signals, the three differentiation pathways may be activated in distinct subpopulations of cells. Despite many efforts, the nature of most of the signals sensed by the kinases remains a mystery.

We recently identified the lipopeptide surfactin as a 'quorum-sensing' molecule produced by *B. subtilis* that appears to activate the membrane histidine kinase KinC with the consequent phosphorylation of Spo0A (Lopez *et al.*, 2009). We presume this results in low levels of Spo0A-P because KinC activation leads to transcription of *sinI*, a gene whose promoter requires low levels of Spo0A-P for activation (Fujita *et al.*, 2005). In addition, we have demonstrated that *sinI* expression displays bimodality, i.e. only a subpopulation of the cells expresses *sinI* after KinC activation by surfactin (Chai *et al.*, 2008; Lopez *et al.*, 2009). In this subpopulation, the antirepressor SinI antagonizes the repressor SinR, allowing expression of the *eps* and *yqxM* operons leading to matrix production (Kearns *et al.*, 2005).

Cannibalism and matrix production are both activated by low levels of Spo0A-P (Fujita *et al.*, 2005). Low levels of Spo0A-P also directly induce the expression of the *skfA-H* operon and indirectly, by repressing the repressor AbrB, the expression of the *sdpABC* operon. These operons encode the two cannibalism toxins, Skf and Sdp (Gonzalez-Pastor *et al.*, 2003; Fujita *et al.*, 2005). Given that both processes are co-ordinated by low levels of Spo0A-P, we postulated that matrix production and cannibalism could be triggered by the same signalling molecule, surfactin.

In this article, we show that surfactin triggers both matrix and cannibal toxin production in the same subpopulation of cells. This subpopulation secretes cannibalism toxins into the milieu, concomitant with expression of the immunity machinery used to resist the action of these toxins. At the same time, these cells produce the extracellular matrix that encases them in the biofilm. Surrounded by the toxins, only matrix producers are favoured to grow, as they are able to use the nutrients released by their killed siblings. This phenomenon increases the number of matrix-producing cells within the population. In turn, with the increase in the relative number of matrix-producing cells, these communities are able to produce more extracellular matrix. We propose that this behaviour is a mechanism to eliminate cell types that are no longer required for the development of the community and to promote the subpopulation of matrix producers.

Results

Surfactin induces matrix production and cannibalism in the same subpopulation

To test the hypothesis that matrix and cannibal toxin production occur in the same subpopulation we fused specific promoters to genes encoding different fluorescent proteins and monitored their expression in single cells using flow cytometry. Extending earlier work by Hobbs (2006), we first tested whether the genes for Skf synthesis were induced by surfactin in a KinC-dependent manner, as is the case for the matrix operon *yqxM* (Lopez *et al.*, 2009).

We followed Skf gene expression using the reporter P_{skfA} -*yfp* in the wild-type strain grown in LB medium, where surfactin production is not detectable (Lopez *et al.*, 2009). Under these conditions, we did not observe any reporter activation (Fig. 1, blue trace). The addition of purified surfactin to the medium resulted in a subpopulation of cells highly expressing the reporter (Fig. 1, red trace). The *skfA*-expressing subpopulation was not observed in cultures of the $\Delta kinC$ mutant when surfactin was added (Fig. 1, green trace). These results indicate that the expression of cannibalism genes is indeed triggered by the same signalling molecule that triggers matrix production.

It therefore seemed feasible that the fraction of cells sensing surfactin and thus, differentiating into matrix producers, could also differentiate into cannibals. We

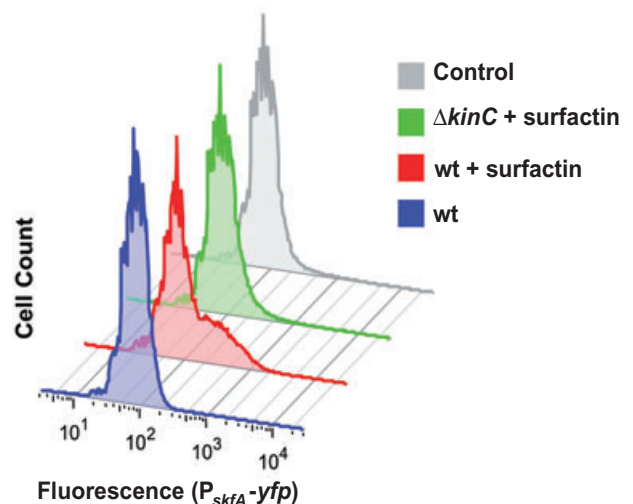


Fig. 1. Activation of cannibalism by the quorum-sensing molecule surfactin. Flow cytometry monitoring the expression of the reporter P_{skfA} -*yfp* in LB cultures treated with surfactin after 8 h of incubation. The addition of surfactin induced higher expression of the reporter in a subpopulation of cells, as evidenced by the shoulder observed in the expression profile of treated cultures. This subpopulation was not observed when surfactin was added in the absence of the membrane kinase KinC. Fluorescence is shown in arbitrary units.

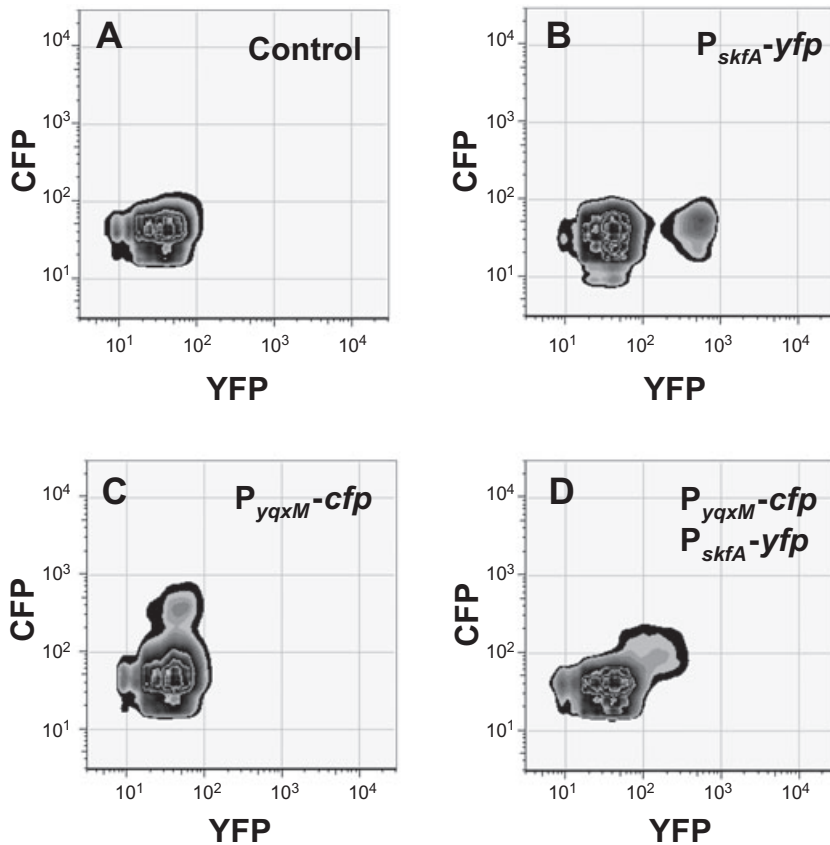


Fig. 2. Subpopulations of cannibals and matrix producers are the same cells. Flow cytometry monitoring the subpopulations of cannibals (P_{skfA} -*yfp*) and matrix producers (P_{yqxM} -*cfp*). Fluorescence intensity for the YFP channel is presented on the x-axis and for the CFP channel is presented on the y-axis. Fluorescence is measured in arbitrary units. A. Wild-type strain expressing no fluorescence protein was used as the negative control. B. Strain harbouring only the P_{skfA} -*yfp* (cannibalism) reporter. C. Strain harbouring only the P_{yqxM} -*cfp* (matrix) reporter. D. Double-labelled strain P_{skfA} -*yfp*, P_{yqxM} -*cfp*.

monitored the subpopulation of matrix producers and cannibals using the reporters P_{yqxM} -*Cfp* and P_{skfA} -*yfp* in single- and double-labelled strains to simultaneously measure YFP- and CFP-producing cells in the same strain using flow cytometry. We plotted the results in graphs where CFP fluorescence is on the y-axis and YFP fluorescence is on the x-axis. The number of cells expressing different levels of fluorescence was then plotted on a z-axis perpendicular to the plane of the x- and y-axes, as shown by the isolines. Figure 2A presents the control of background fluorescence for both CFP and YFP in a strain harbouring no fluorescent protein genes. We first distinguished each subpopulation of cells (matrix producers and cannibals) in each fluorescence channel using single-labelled strains as controls (P_{skfA} -*yfp* and P_{yqxM} -*cfp* in Fig. 2B and C respectively). The double-labelled strain P_{skfA} -*yfp*, P_{yqxM} -*cfp* in Fig. 2D showed a single subpopulation of fluorescent cells, and these cells were expressing both YFP and CFP. This finding indicates both cell differentiation pathways are co-ordinately activated in the same subpopulation.

Mutations that increase cannibalism

Given that the same cells express both cannibalism and matrix production genes, we were compelled to study the

influence of cannibalism toxins on biofilm morphology. To this end, we deleted genes predicted to have a role either in the production of, or in the sensitivity to, cannibalism toxins. Then, we analysed biofilm development in those mutant strains.

We first attempted to obtain mutants with an increase in the expression of the *skf* and *sdp* operons. Through literature searches, we identified two regulators proposed to negatively regulate the expression of several antimicrobials, including cannibalism toxins. These regulators were identified as AbrB (Antibiotic regulator of *B. subtilis*) and Abh (AbrB homologue) (Trowsdale *et al.*, 1978; 1979; Zuber and Losick, 1987; Yao and Strauch, 2005; Bobay *et al.*, 2006). Mutants lacking either of these two regulators have increased expression of antimicrobial genes and more specifically, the cannibalism toxins (Strauch *et al.*, 2007). In the Δ *abrB* mutant, biofilm development was altered independent of its effect associated with cannibalism expression (Fig. S1). This was expected as we had previously shown that AbrB directly regulates the expression of matrix genes (Chu *et al.*, 2008). Thus, we did not pursue the Δ *abrB* mutant further. Unlike AbrB, the Abh regulator is believed to regulate the expression of genes involved in matrix production indirectly (Strauch *et al.*, 2007), a point to which we return below. To determine if the Δ *abh* mutant has increased expression of the

cannibalism operons in the strain used in our study, we made *lacZ* transcriptional reporter fusions to the two cannibalism operons *skf* and *sdp* and measured the level of β -galactosidase activity in the Δabh mutant compared with the wild type. Deletion of *abh* caused a remarkable increase in expression of both *skf* and *sdp* operons (Fig. 3A). Thus, *abh* indeed functions to negatively regulate cannibalism and the Δabh mutant overexpresses both the *skf* and *sdp* operons.

In addition to identifying mutants that overproduce the cannibalism toxins, we also searched for mutants that might be more sensitive to the action of these toxins. We once again selected targets based on previous publications. We focused our search on structures located

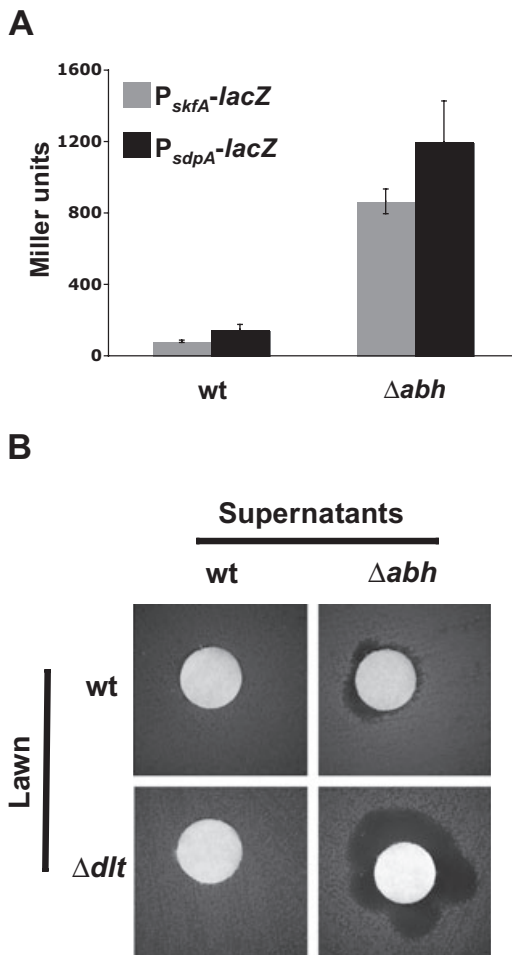


Fig. 3. The Δabh Δdlt mutant overexpresses cannibalism genes. A. β -Galactosidase assay of the transcriptional gene expression of the cannibalism operons (*skf* and *sdp*) in the absence of their repressor Abh. Assays were performed on extracts of cells grown in MSgg medium for 72 h. B. Antibioassay of the supernatant produced by wild type or the Δabh mutant in a disc over a lawn of wild type or the Δdlt mutant strain. Lawns and extracts were obtained from cells grown in MSgg medium. Assay was performed on MSgg medium for 24 h prior to imaging.

in the cell envelope of *B. subtilis*, known to provide resistance to the action of antimicrobial peptides. The *dltAB-CDE* operon (henceforth *dlt*) encodes the protein machinery responsible for the D-alanine esterification of cell wall teichoic acids (Perego *et al.*, 1995). The products of the *dlt* operon introduce positive charges in the cell envelope, making it a repulsive barrier against some peptide toxins (Perego *et al.*, 1995; Cao and Helmann, 2004; Hyyrylainen *et al.*, 2007). We reasoned that a Δdlt mutant lacking the protective barrier in the cell envelope would be more sensitive to the action of the cannibalism toxins. To test the sensitivity of a Δdlt mutant, a classical antibiogram experiment was performed using a lawn of the Δdlt mutant overlaid with a disc containing filter-sterilized supernatant of the Δabh mutant, which overproduces antimicrobials, as explained above. In this assay, the Δdlt mutant had a larger zone of clearance than the wild type in response to the Δabh supernatant (Fig. 3B). These results indicate that the Δdlt mutant is more sensitive to the action of antimicrobials than the wild type, although the wild-type supernatant did not visibly affect viability of the Δdlt mutant (Fig. 3B). However, the Δabh mutant does indeed produce more antimicrobials as can be observed in the ability of its supernatant to generate a killing zone when spotted over the wild-type lawn (Fig. 3B).

Our next step was to generate a strain lacking both the *dlt* and *abh* genes. As shown in the following section, the Δdlt Δabh mutant does not appear to have a growth defect compared with wild type, yet it displays a stronger response to cannibalism. It highly produces the cannibalism toxins and is at the same time more sensitive to their action. We used this mutant to study the influence of hypercannibalism in biofilm development.

Hypercannibalism influences biofilm development

To study the effect of the Skf and Sdp toxins on biofilm development in greater detail, colony morphology of the hypercannibal mutant (Δdlt Δabh) was investigated. To do this, a spot of liquid culture was placed on the solid defined medium MSgg (Branda *et al.*, 2001). After 3 days of incubation, biofilm colonies exhibit several morphological features indicative of the differentiation of distinct cell subpopulations (Fig. 4A). For example, the production of the extracellular matrix in wild-type cells results in the formation of wrinkles on the surface of the colony, which is a feature that can be correlated with the presence of matrix-producing cells (Branda *et al.*, 2004). Similarly, the raising of aerial structures on the surface of the biofilm is indicative of the presence of a subpopulation of sporulating cells, since the spores localized in the apical area of these structures (Branda *et al.*, 2001) (Fig. 4B).

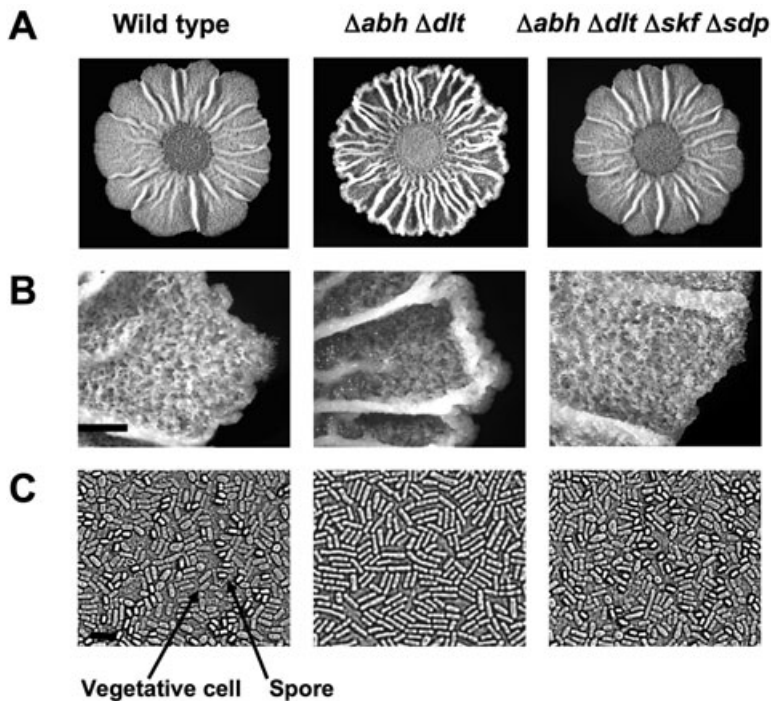


Fig. 4. The hypercannibal $\Delta dlt \Delta abh$ mutant overproduced extracellular matrix and sporulating cells are absent.

A. Colony pictures of the strains grown on MSgg medium for 72 h.

B. Magnified images detailing the occurrence of aerial structures where spores localize on the surface of the biofilm. Scale bar is 1 mm.

C. Microscopy pictures used to determine the presence of spores in each biofilm.

Representative spores and vegetative cells are denoted using arrows. Scale bar is 3 μm .

We compared the morphology of the biofilm from the hypercannibal mutant ($\Delta dlt \Delta abh$) with wild-type cells grown on MSgg medium. Strikingly, the hypercannibal mutant displayed increased wrinkling compared with wild type, suggesting an increase in the production of extracellular matrix in these colonies (Fig. 4A). In addition, analysis at higher magnification revealed that the hypercannibal mutant lacked the aerial structures (noticeable in between the large wrinkles) where spores preferentially are localized (Fig. 4B). Microscopic analysis showed an absence of the subpopulation of sporulating cells in the hypercannibal mutant (Fig. 4C). However, quantification of the number of viable spores over time showed that sporulation was not abolished in the hypercannibal mutant but rather delayed. After 6 days of biofilm development, the number of spores observed in the hypercannibal mutant was comparable to the wild type (Fig. 5A). These results further validate the proposal that cannibalism serves to delay sporulation. The original observation of Gonzalez-Pastor *et al.* (2003) was that mutants unable to make the toxins sporulated early. This result is recapitulated in Fig. 5A (diamonds, $\Delta skf \Delta sdg$). Now we show a complementary strain, which overproduces the cannibalism toxins thus sporulation is further delayed (Fig. 5A, triangles, $\Delta dlt \Delta abh$).

Both developmental alterations, hyperwrinkling and sporulation delay, might be explained by the action of the cannibalism toxins on the cell population. Since matrix producers secrete the cannibalism toxins and express the resistance machinery to these, an increase in toxin production should also increase the fraction of matrix produc-

ers in the population. This is because the non-matrix-producing cells are sensitive to the toxins and thus killed, ultimately serving as food for the toxin-resistant matrix producers. Thus, a greater proportion of matrix producers constitute the cell community, leading to higher production of extracellular matrix. Additionally, since cells sensitive to the toxins lyse, the process of sporulation is delayed, presumably due to the nutrients released. To test this hypothesis, we used flow cytometry to quantify the subpopulation of matrix producers in the hypercannibal mutant in comparison with the wild type. Matrix-producing cells were monitored in both the hypercannibal mutant ($\Delta dlt \Delta abh$) and wild type by measuring expression of the transcriptional reporter $P_{yqzM}\text{-yfp}$. Results indicated that the subpopulation of matrix producers was increased in the hypercannibal mutant in comparison to the wild type (Fig. 5B). Interestingly, we did not observe any increase in the intensity of the signal but rather the number of cells expressing the reporter was greater.

To distinguish whether the increase in matrix producers in the hypercannibal mutant is exclusively related to cannibalism or if other effects associated with the mutations were involved, we deleted both *skf* and *sdg* operons in the hypercannibal background. The quadruple mutant ($\Delta dlt \Delta abh \Delta skf \Delta sdg$) showed a complete abrogation of the hypercannibalism phenotype. The level of colony wrinkling was comparable to the wild type indicating that matrix was not overexpressed, and the aerial structures were restored and harboured spores (Fig. 4). Measuring expression of the transcriptional reporter $P_{yqzM}\text{-yfp}$ in the

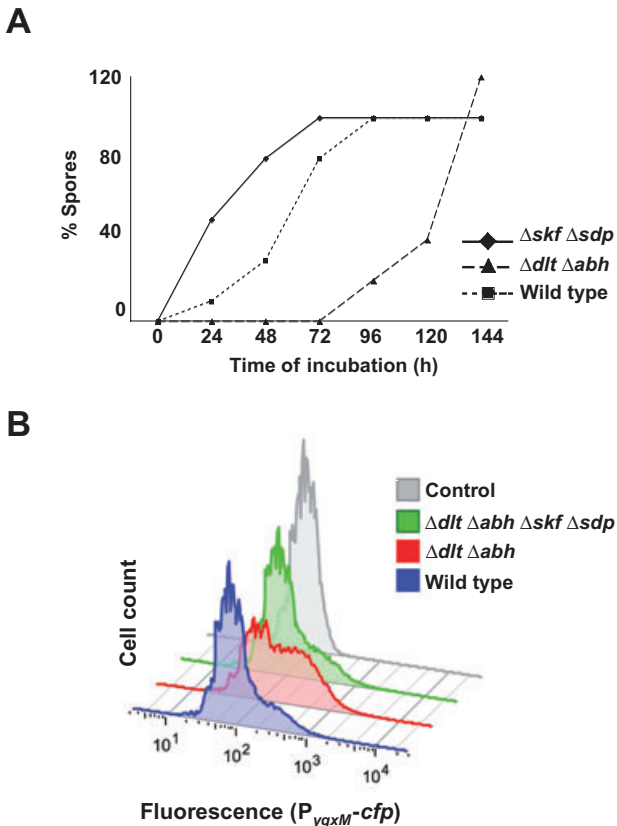


Fig. 5. A. Sporulation is delayed in the hypercannibal $\Delta dlt \Delta abh$ mutant. Quantification of vegetative colony-forming units (cfu) after heat treatment over time in different mutants. Samples were grown as colonies on MSgg and incubated at 30°C for the indicated time prior to analysis. Per cent of spores is compared with the number of wild-type spores at day six of the assay. B. Hypercannibalism increased the subpopulation of matrix-producing cells. Flow cytometry monitoring matrix producers using the reporter P_{yqxM} -*cfp*. The $\Delta dlt \Delta abh$ mutant showed a twofold increase in cells differentiated as matrix producers compared with the wild type.

quadruple mutant demonstrated that the subpopulation of matrix producers was restored to levels comparable to the wild type (Fig. 5B). These experiments indicated that the cannibalism genes are the principal factors inducing developmental changes in the hypercannibal mutant. We also conclude that the previously reported effect of Δabh on transcription from the *yqxM* promoter (Strauch *et al.*, 2007) is an indirect consequence of its effect on *skf* and *sdp* expression.

Hypercannibalism depends on surfactin signalling

The quorum-sensing molecule surfactin activates both matrix production and cannibalism in the same subpopulation of cells via activation of the membrane kinase KinC (see Figs 1 and 2) (Lopez *et al.*, 2009). It therefore follows that the morphological defect associated with hypercannibalism depends on the ability of cells to sense surfactin as a signal molecule. We used the hypercannibal mutant as a background strain and inhibited the production of surfactin by deleting the *srf* operon (Δsrf). The colony morphologies of the Δsrf and $\Delta dlt \Delta abh \Delta srf$ mutants were indistinguishable, suggesting that the absence of surfactin also inhibits the expression of the cannibalism toxins and decreases matrix production (Fig. 6). Furthermore, the addition of purified surfactin to the Δsrf and $\Delta dlt \Delta abh \Delta srf$ mutants restored the previous morphology described for the wild type and the hypercannibal mutant (Fig. 6). Consistent with a requirement for surfactin signalling, elimination of the surfactin-sensing kinase KinC in $\Delta kinC$ and $\Delta dlt \Delta abh \Delta kinC$ mutants also led to a loss of wrinkled colony morphology, which is consistent with a decrease in matrix production. Again, the $\Delta kinC$ and the $\Delta dlt \Delta abh \Delta kinC$ mutants were phenotypically indistinguishable, suggesting that the

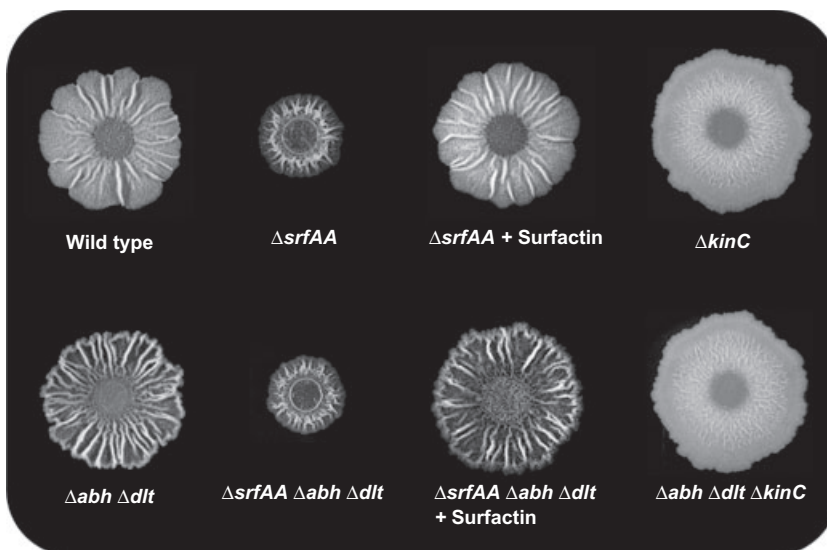


Fig. 6. The signalling molecule surfactin and the histidine kinase KinC are essential for the hypercannibalism phenotype. Effect of the Δsrf and the $\Delta kinC$ mutations on wild-type and hypercannibalism $\Delta dlt \Delta abh$ mutant biofilms. Colonies were grown on MSgg at 30°C and photographed after 72 h.

cannibalism genes are not expressed when the surfactin-signalling pathway is silenced.

Effect of other antimicrobial peptides

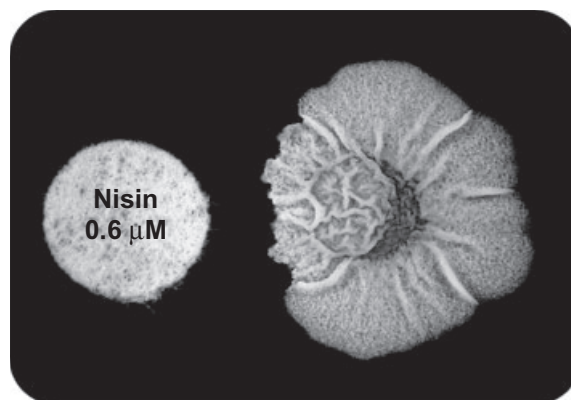
The subpopulation of matrix producers is resistant to the action of the cannibalism toxins due to concomitant expression of the immunity machinery required to detoxify the toxins (Gonzalez-Pastor *et al.*, 2003). Interestingly, the protein machineries described in *B. subtilis* for the detoxification of antimicrobials work promiscuously for other toxins produced by different bacteria (Lewis, 1999; Butcher and Helmann, 2006). We speculate this promiscuity could be used to detoxify a large number of toxins secreted by competitors. To investigate this, we asked if another antimicrobial peptide could mimic the cannibalism effect in *B. subtilis*.

Because the molecular structures of the cannibalism toxins have not yet been elucidated, we looked for a natural product that might have similar interactions with *B. subtilis* as does Skf. We focused on nisin, produced by *Lactococcus lactis* (Mattick and Hirsch, 1944). Nisin is a peptide that exhibits antimicrobial activity towards other Gram-positive bacteria, including *B. subtilis* (Buchman *et al.*, 1988). Like Skf, nisin is also repelled by the barrier that the *dlt* operon provides to the cell wall of *B. subtilis* (Cao and Helmann, 2004). We assayed the effect of nisin on the biofilm of *B. subtilis* by simulating the secretion of nisin from a neighbouring colony using an antibiogram disc impregnated with 0.6 μM of purified antibiotic. This concentration did not affect growth. After 3 days of incubation near the artificial *hostile neighbour*, the biofilm of wild-type *B. subtilis* displayed a remarkable increase in wrinkles in the area closest to the disc, suggesting an increase in the production of extracellular matrix in this region of the colony. Moreover, the region affected by nisin was also characterized by the absence of spores (Fig. 7A and B). The increase in matrix production and decrease in sporulation observed in the nisin-treated cells mimic the hypercannibal effect observed in the $\Delta dlt \Delta abh$ mutant (see Fig. 4). We propose that other antimicrobial peptides, including nisin, similar to the cannibalism toxins, caused the same developmental effect cannibalism exerts on the biofilms of *B. subtilis*.

Discussion

We have presented evidence that cannibalism is involved in the development of biofilm communities in *B. subtilis*. The extracellular matrix of the biofilm and the Skf and Sdp cannibalism toxins are produced by the same subpopulation of cells. This subpopulation also produces the resistance machinery for the toxins. When Skf and Sdp are secreted and massive cell killing ensues the matrix produc-

A



B

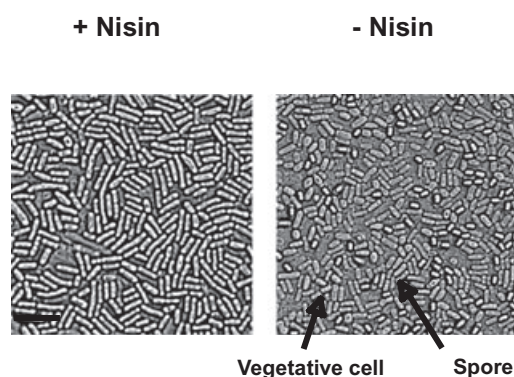


Fig. 7. Other peptide antimicrobials mimic the effect of cannibalism.

A. Disc impregnated with nisin (0.6 μM) set near the growth area of *B. subtilis*. Image was taken after 72 h of incubation on a MSgg plate at 30°C.

B. Microscopy images detailing the occurrence of spores in the region affected and non-affected with nisin. Scale bar is 3 μm . Representative spores and vegetative cells are denoted with arrows.

ers are able to thrive using the nutrients released by lysed siblings that are sensitive to the toxins. In turn, release of nutrients promotes growth of the subpopulation of matrix producers, which increases the production of extracellular matrix and causes a temporary inhibition of sporulation.

Cannibalism functions as a mechanism of programmed cell death (PCD) to modulate diverse developmental processes in bacteria (Engelberg-Kulka *et al.*, 2006). For example, PCD participates in the formation of fruiting bodies in *Myxococcus xanthus*, in which a portion of cells are killed prior to differentiation of myxospores (Nariya and Inouye, 2008). In contrast, cannibalism in *B. subtilis* delays sporulation (Gonzalez-Pastor *et al.*, 2003) and inhibits the formation of the aerial structures where spores localize. In *Streptococcus pneumoniae*, PCD is driven by a subpopulation of competent cells that lyse the non-competent siblings present in the same niche (Guiral

et al., 2005; Claverys *et al.*, 2007). In contrast to what is observed in *M. xanthus* and *B. subtilis*, the fratricide process in *S. pneumoniae* is triggered by excess of nutrients rather than starvation. This argues for other additional functions, aside from nutrient release, associated with PCD in bacteria.

Although the secretion of toxins promotes different developmental processes in these distinct model organisms, they might have a common feature that defines the functionality of PCD in bacteria. The antimicrobial action of the toxins promotes the survival of a sustainable subpopulation while eliminating other subpopulations no longer required to deal with external aggressions, such as nutrient limitation, pH changes, presence of antibiotics or the immune response (Lewis, 2000; Claverys and Havarstein, 2007). Based on this idea, we propose an additional role for cannibalism in *B. subtilis*. This would be to promote the growth of the subpopulation of matrix producers and boost the production of extracellular matrix. Other cell types will be killed by the action of the Skf and Sdp toxins. However, the remarkable resistance of spores to external damages protects them from the action of these cannibalism toxins (Setlow, 2006; Wang *et al.*, 2006). Yet, sporulation is itself delayed by the release of nutrients from the killed cells.

Co-ordinated expression of cannibalism and matrix production in a subpopulation of cells is triggered by the quorum-sensing molecule surfactin. This could result in a fitness advantage in natural habitats, where producing an extracellular matrix would provide protection at the same time as an effective arsenal of toxins to kill neighbouring bacteria that may be competing for the same resources. Consistent with this, it has been reported that *B. subtilis* preferentially uses the Skf and Sdp toxins to kill other bacteria rather than itself in mixed cultures (Lin *et al.*, 2001; Nandy *et al.*, 2007).

The chances that other soil bacteria will trigger cannibalism in *B. subtilis* increase with the ability of other soil microorganisms to produce natural products that mimic the action of the signalling molecule surfactin. The membrane kinase KinC senses leakage of potassium caused by the action of surfactin in the cell membrane, rather than the structure of the molecule itself. This mechanism of activation allows cells to promiscuously sense the presence of other small molecules with physiological effects similar to those of surfactin. These natural products include the structurally unrelated compounds nystatin, amphotericin, valinomycin and gramicidin which can all induce the quorum-sensing pathway and cause differentiation of the subpopulation of matrix producers (Lopez *et al.*, 2009). Additionally, other antimicrobial peptides secreted by soil bacteria could mimic the effect of the cannibalism toxins, due to the ability of the resistance machinery to work non-specifically for several similar mol-

ecules (Butcher and Helmann, 2006). This ability to sense small molecules produced by a range of diverse soil microorganisms suggests a broad mechanism that *B. subtilis* uses to respond to surrounding bacterial communities by altering its development.

Experimental procedures

Strains, media and culture conditions

Bacillus subtilis strain NCIB3610 was used as the wild type. For biofilm formation experiments 3 µl of a cell suspension was spotted onto defined MSgg medium supplemented with 1.5% Bacto agar and incubated at 30°C for 3 days (Branda *et al.*, 2001). For Fig. 1, 20 µM purified surfactin (Sigma) was added to LB broth prior to inoculation and cells were incubated for 8 h prior to harvesting for flow cytometry. For Fig. 6, surfactin was added by spotting 3 µl of 10 mM purified surfactin onto the surface of the MSgg agar prior to inoculation.

Strains, reporter construction and measurements

Strains used and generated in this work are listed in Table S1. Deletion mutants $\Delta abh::km$, $\Delta dlt::tet$, $\Delta dlt::km$, $\Delta skf::cm$, $\Delta sdp::spc$ were generated using long flanking homology PCR (Wach, 1996) (using the primers listed in Table S2). Transcriptional fusions to *lacZ* were cloned in pDG1663 vector using primers listed in Table S2 (Guerout-Fleury *et al.*, 1996) and inserted into the *amyE* locus by natural competence. All constructions were transferred to NCIB3610 and mutant strains when required by SPP1 phage transduction (Yasbin and Young, 1974). β -Galactosidase assays and spore counting measurements were performed according to the literature (Kearns *et al.*, 2005; Vlamakis *et al.*, 2008).

Image capture and analysis

Colonies were photographed using a Zeiss Stemi SV6 Stereoscope connected to a colour AxioCam[®]. Microscopy images were taken on a Nikon Eclipse TE2000-U microscope equipped with an X-cite 120 illumination system, using a Hamamatsu digital camera model ORCA-ER. Fluorescence signal was detected with a Ex436/500 filter. Image processing was performed using MetaMorph[®] Software and Photoshop[®].

Flow cytometry

Biofilms were harvested, re-suspended in 1 ml of PBS buffer and dispersed with three pulses of mild sonication. Cells were fixed in 4% paraformaldehyde for 7 min, washed with PBS and re-suspended in GTE buffer (50 mM Glucose, 10 mM EDTA pH 8, 20 mM Tris-HCl pH 8). Mild sonication of the samples was required to obtain single cells (Branda *et al.*, 2006).

For flow cytometric analysis, cells were diluted 1:100 in PBS and measured on a BD LSR II flow cytometer (BD Biosciences) using a solid state laser. For YFP fluorescence, we used a laser excitation of 488 nm coupled with 530/30 and

505LP sequential filters. For CFP fluorescence, we used laser excitation at 405 nm coupled with 408/40 and 460LP sequential filters. The photomultiplier voltage was set between 400 and 500 V. Every sample was analysed measuring 50 000 events using FACS Diva (BD Biosciences) software to capture the data. Further analysis was performed using FlowJo 8.7.2 software (<http://www.flowjo.com>).

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