Cell

A Self-Produced Trigger for Biofilm Disassembly that Targets Exopolysaccharide

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SUMMARY

Biofilms are structured communities of bacteria that are held together by an extracellular matrix consisting of protein and exopolysaccharide. Biofilms often have a limited lifespan, disassembling as nutrients become exhausted and waste products accu[illegible] D-amino acids were previously identified as a self-produced factor that mediates biofilm disasse[illegible]y by causing the release of the prot[illegible]mpon[illegible] of the matrix in *Bacillus subtilis*[illegible]ere[illegible] repo[illegible] that *B. subtilis* produces an[illegible]ditio[illegible] biofilm disassembly factor, norsper[illegible]idi[illegible]. Dynamic light scattering and scanning el[illegible]ron mic[illegible]copy exper-iments indicated that[illegible]spermidi[illegible] interacts directly and specifica[illegible]y w[illegible] exopolysaccharide. D-amino acids and[illegible]orspermidi[illegible]acted together to break down exi[illegible]g biofilms and[illegible]utants blocked in the produc[illegible]n of b[illegible]n factors formed long-lived biofilms. Norsp[illegible]ine, but[illegible]t closely related poly-amines, p[illegible]ente[illegible]iofil[illegible]ormation by *B. subtilis*, *Esch[illegible]ia[illegible]li*, and *[illegible]hylococcus aureus.*

INTR[illegible]

Many bact[illegible]form complex multicellular communities, known as biofilms, o[illegible]surfaces and interfaces (Bryers, 2008; O'Toole et al., 2000). A hallmark of biofilms is an extracellular matrix typically consisting of protein, exopolysaccharide, and sometimes DNA, that holds the cells together in the community. Biofilms are of high significance in agricultural, industrial, environmental, and clinical settings. For example, the soil bacterium *Bacillus subtilis* protects plants from a variety of pathogens by forming biofilms on the roots (Nagórska et al., 2007). Biofilms are inherently resistant to antimicrobial agents and are at the center of many persistent and chronic bacterial infections (Costerton et al., 1999). For example, biofilm formation plays a critical role in many device-related infections, infective endocarditis, urinary tract infections and a[illegible]e septic arthritis by pathogens such as[illegible]*lococcus aur[illegible]* and *Staphylococcus epidermidis* ([illegible]ers, 2008; Otto, 2008). Biofilms have natural life cycles; t[illegible]y form und[illegible]propitious conditions, and they disassemble a[illegible]ey age (K[illegible]tan and Watnick, 2009; Romero and Kolter, 20[illegible]).

Here[illegible]cus on the biofilm life cycle of *B. subtilis*, which [illegible]er laboratory conditions forms floating biofilms known as [illegible]at the air-liquid interface of standing cultures (Aguilar et al., 2007; Branda et al., 2001a; Lopez et al., 2009; Vlamakis et al., 2008). Cells in the pellicles are held together by an extracellular matrix consisting of exopolysaccharide and amyloid-like fibers largely composed of the protein TasA (Branda et al., 2006a; Branda et al., 2004; Romero et al., 2010). *B. subtilis* biofilms have a limited life span, maturing after 3 days of incubation in biofilm-inducing medium at 22°C but disassembling and releasing individual planktonic cells by 8 days (Kolodkin-Gal et al., 2010; Romero et al., 2011).

How do cells in the *B. subtilis* pellicle escape from the matrix and return to a planktonic existence during biofilm disassembly? We previously found that conditioned medium from an 8-day-old culture contains factors that prevent pellicle formation when added to a fresh culture. One such factor is a mixture of the D-amino acids D-Tyr, D-Leu, D-Trp, and D-Met. These amino acids are incorporated into the cell wall peptidoglycan where they trigger the release of the amyloid fibers from the cell (Cava et al., 2011; Kolodkin-Gal et al., 2010; Romero et al., 2011). Fiber release is mediated via an adaptor protein TapA, which forms D-amino acid-sensitive foci in the cell wall and is required for the formation of the fibers and their anchorage to the cell wall (Romero et al., 2011). D-amino acids were also found to inhibit biofilm formation by other bacteria, such as *S. aureus* and *Pseudomonas aeruginosa* (Hochbaum et al., 2011; Kolodkin-Gal et al., 2010).

Here we report the discovery of a second biofilm-disassembly factor present in conditioned medium from aging *B. subtilis* biofilms. The factor is the polyamine norspermidine, and evidence indicates that it directly interacts with the exopolysaccharide. The effect was specific in that other closely related polyamines, such as spermidine, had little biofilm-inhibiting activity. Mutants

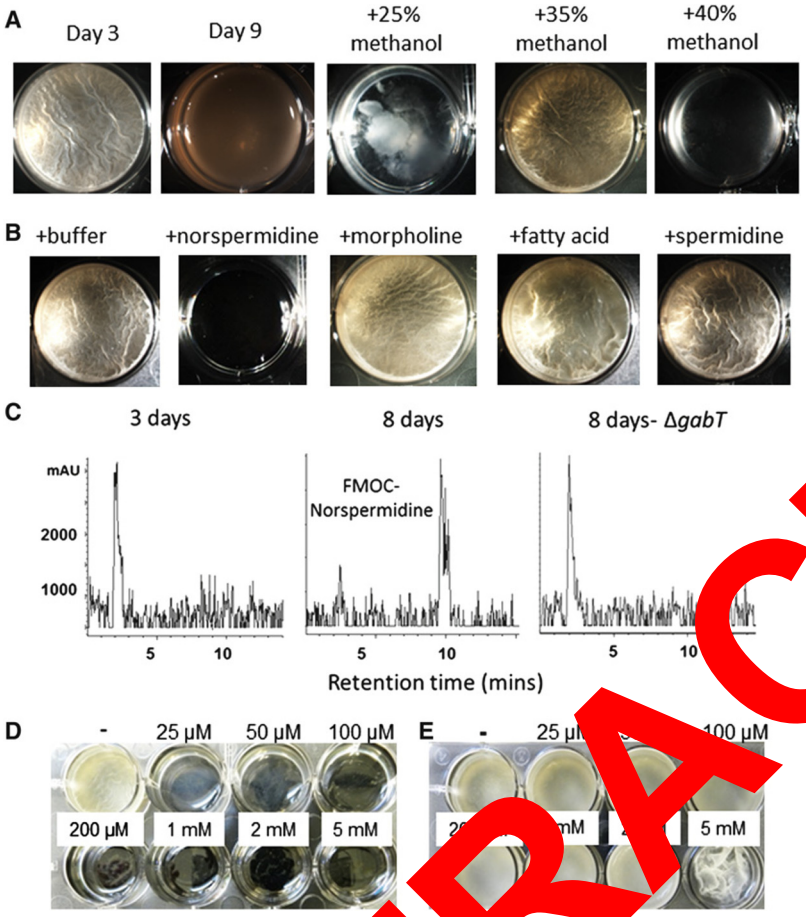

Figure 1. Identification of Norspermidine in Conditioned Medium from *B. subtilis* and Its Effect on Pellicle Formation

(A) Biofilm-inhibiting factors in conditioned medium. *B. subtilis* strain NCBI3610 was grown at 22°C in 12-well plates in liquid biofilm-inducing medium for 3 or 8 days. Conditioned medium (500 ml) from an 8-day-old culture was concentrated on the C-18 and eluted stepwise with methanol. Shown is growing cells in fresh medium to which has been added of the 25%, 35%, or 40% methanol s.

(B) Norspermidine inhibits film formati Cells of NCBI3610 were in fres dium c ining PBS buffer (contr norspermidine morpholine (100 μM), -purifie ty acid (μM), or spermidine (100 Bri images of the norspermidine-treat ell re cells near bottom of the well.

(tection o permi Pellicles were collected 3- and 8-day- es (100 ml) of the wild-type (N 10) and from -day-old culture (100 ml) of a *gabT* t (IKG623). After mild sonication of the pellicles, ce re separated from extracellular material. (Other expe ts showed that norspermidine is largely found in pellicles.) Norspermidine in the extracellular erial was derivatized with Fmoc-Cl, and the resulting c-norspermidine was detected with an Agilent LC/MS em. Fmoc-norspermidine was detectable in the old ellicle from wild-type cells but not in the young or mutant pellicles. See also Figure S1B.

(D and E) Quanitification of the biofilm-inhibiting activity of norspermidine and spermidine. Pellicle formation of strain NCBI3610 was tested in the presence of the indicated concentrations of norspermidine (D) or spermidine (E). See also Figure S1A.

blocked in the production of n D-a acids and norspermidine formed long-lived pe les, and D-a acids and norspermidine acted toget n breaking do existing, mature pellicles. Thus, *B. s is* pro es factors that act in a complementary manner t a co rom the protein and exopolysaccharide components e mat Finally, we report that norspermi s effe in ibiting biofilm formation by other b ria, i ding *S. s* and *Escherichia coli.*

RESU

Norsperm ls Self-Produced Biofilm-Inhibiting Factor

Building on earlier work indicating that aging *B. subtilis* pellicles produce two biofilm-inhibiting factors, we applied conditioned medium to a C-18 Sep-Pak column and collected fractions by using stepwise elution in 5% percent increments from 5% to 40% methanol. The 25% and 40% eluates contained compounds active in inhibiting biofilm formation, whereas the 35% eluate was inert (Figure 1A). As reported previously, the factor in the 40% eluate was a mixture of D-amino acids (Kolodkin-Gal et al., 2010). To identify the second biofilm-inhibiting factor, we carried out high-performance liquid chromatography (HPLC) on the 25% methanol eluate by using a phenyl-hexyl column. Inhibitory activity was recovered with an elution time of 40 min. Proton nucleic magnetic resonance (NMR) analysis of the active fraction revealed fatty acids, morpholine, and norspermidine. Further purification with a C-18 HPLC column identified the inhibitory agent as norspermidine, a finding confirmed with authentic norspermidine, which inhibited biofilm formation at 25 μM (Figure 1B and Figure S1A, available online). Pure morpholine and fatty acids detected by NMR were inactive (Figure 1B).

Norspermidine's tendency to form strong complexes with fatty acids, which explains its elution at 25% methanol from the C-18 Sep-Pak column, complicated its quantification. To circumvent this problem, we treated conditioned medium with 9-fluorenylmethyloxycarbonyl chloride (Fmoc-Cl), which protects the amino groups of norspermidine as carbamates and thereby prevents their interaction with other molecules (Molnár-Perl, 2003). Fmoc-norspermidine was detected and quantified by liquid chromotography/mass spectrometry (LC/MS) (Figure S1B). Using this procedure, we found that norspermidine was present at a concentration of 50–80 μM in 8-day-old, disassembling pellicles but at a concentration of less than 1 μM in a 3-day-old pellicle (Figure 1C).

Finally, the effect of norspermidine was specific in that a closely related polyamine, spermidine, which differs from norspermidine by the presence of an extra methylene group, was

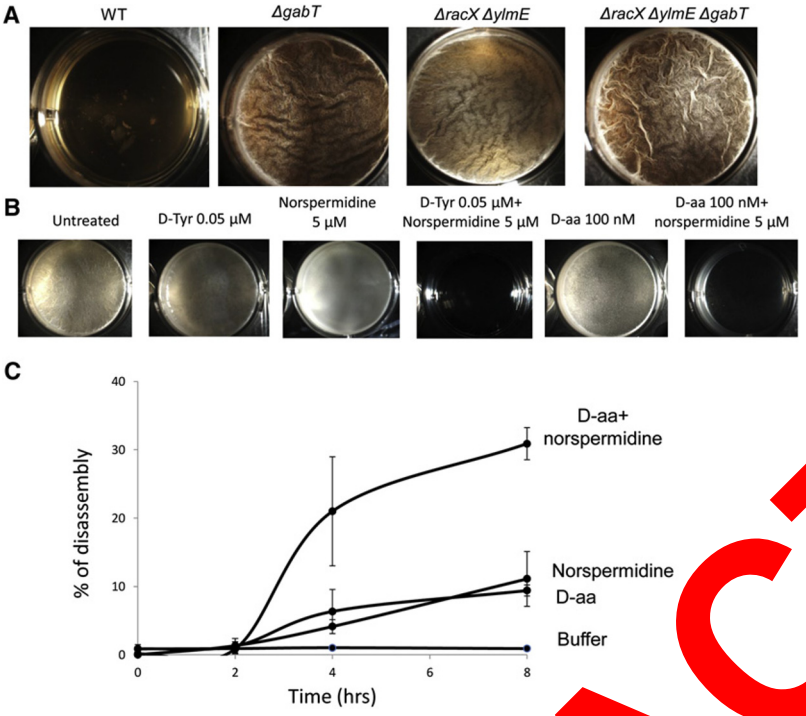

Figure 2. Norspermidine Acts Together with D-amino Acids

(A) Pellicle longevity. Shown are 7-day-old cultures of the wild-type (WT), a mutant (Δ*gbaT*) blocked in norspermidine production (IKG623), a double mutant (Δ*ylmE* Δ*racX*) blocked in D-amino acid production (IKG55), and a triple mutant (Δ*gbaT* Δ*ylmE* Δ*racX*) blocked in the production of both (IKG625). See also Figure S2.

(B) Preventing biofilm formation. Pellicles were grown for 3 days in medium containing as indicated D-tyrosine (D-Tyr), norspermidine, a mixture of D-tyrosine, D-methionine, D-leucine, and D-tryptophan (D-aa), and the indicated combinations of amino acids and norspermidine at the indicated concentrations.

(C) Quantifying pellicle breakdown. Shown on the surface of 3-day-old pellicles were placed droplets (50 μl) containing buffer (PBS), a mixture of D-tyrosine, D-methionine, D-leucine, and D-tryptophan each at final concentration of 5 μM, norspermidine at a final concentration of 50 μM, or both. As in (C), a combination of D-amino acids each at a concentration of only 2.5 μM and norspermidine at a concentration of 10 μM. After incubation for the indicated times, pellicle material and the medium were separated and each brought to a volume of 3 ml. After mild sonication, the OD_{600} was determined for each sample. The percentage of disassembly represents the OD_{600} of the medium as a percent of the sum of the OD_{600} of the medium and the OD_{600} of the pellicle. The results are an average of three independent experiments done in duplicate. The error bars indicate standard deviation.

inactive in inhibiting biofilm formation at concentrations up to 2 mM (Figures 1D and 1E).

A Mutant Blocked in Both D-Amino Acid and Norspermidine Production Forms Long-Lived Biofilms

To evaluate the contribution of norspermidine to biofilm disassembly genetically, we created a mutation blocking its production. Norspermidine is synthesized from aspartate-β-semialdehyde in a pathway involving the enzyme L-diaminobutyric acid transaminase (Kurihara et al., 2009). We constructed a mutant lacking the *B. subtilis* gene (*gabT*) encoding this enzyme and found that the mutant was blocked in norspermidine production (Figure 1C) and was partially impaired in biofilm disassembly (Figure 2A). The *gabT* mutant formed pellicles that remained relatively thick at a time (day 7) when the wild-type had undergone substantial disassembly. Nonetheless, the mutant pellicle had lost the wrinkly phenotype characteristic of young biofilms by day 7. We wondered whether the contribution of norspermidine to biofilm disassembly might be partially redundant with that of D-amino acids, which are produced by racemases encoded by *racX* and *ylmE*. Like a *gabT* mutant, a *racX ylmE* double mutant was partially impaired in biofilm disassembly. Strikingly, however, a *gabT racX ylmE* triple mutant formed robust pellicles that retained their wrinkly phenotype at a time (7 days) when the wild-type had substantially disassembled (Figure 2A). As a further test of the involvement of norspermidine in biofilm disassembly, we constructed a mutant lacking carboxynorspermidine decarboxylase, which catalyzes the last step in the biosynthetic pathway. As in the case of the *gabT* mutant, a mutant lacking the *B. subtilis* homolog (*yaaO*) of the decarboxylase gene was partially impaired in biofilm disassembly, and a *yaaO racX ylmE* triple mutant formed pellicles that remained intact at a time when the wild-type had disassembled (Figure S2A).

D-Amino Acids and Norspermidine Act Together in Preventing Biofilm Formation and Triggering Biofilm Disassembly

These findings suggest that norspermidine and D-amino acids act by different mechanisms to trigger biofilm disassembly. Consistent with this idea, combinations of norspermidine with D-Tyr or with a mixture of D-Met, D-Trp, D-Leu, and D-Tyr effectively prevented biofilm formation at concentrations that were ineffective in blocking biofilm formation when applied separately (Figure 2B). Similarly, a mixture of D-amino acids and norspermidine was more effective in causing the breakdown of an existing biofilm than were either D-amino acids or norspermidine alone (Figure 2C). Thus, D-amino acids and norspermidine act together in preventing biofilm formation and triggering the disassembly of mature biofilms.

Norspermidine Targets the Exopolysaccharide

How does norspermidine trigger biofilm disassembly? A clue came from the observation that the residual pellicle (wispy fragments of floating material with some structure) produced in the presence of norspermidine resembled pellicles seen for a mutant blocked in exopolysaccharide production but not those (thin, featureless pellicles) seen for a mutant blocked in amyloid-fiber production. More importantly, norspermidine had little effect on the residual pellicle produced by an exopolysaccharide mutant

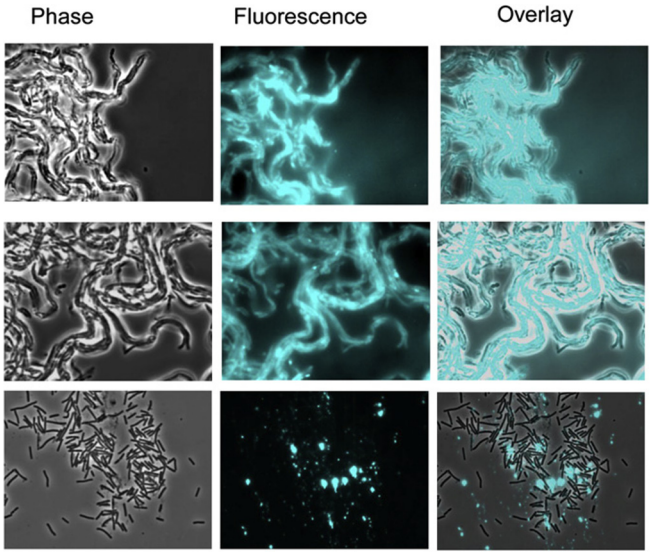

Figure 3. Norspermidine Disrupts Exopolysaccharide

Shown are phase contrast and fluorescence images of cells of the wild-type (WT; NCBI3610) harvested from pellicles grown in the presence or absence (untreated) of norspermidine (25 μM) or a high concentration of spermidine (1 mM). The cells were washed in PBS and stained for exopolysaccharide with a conjugate of concanavalin A with Texas r... ...ure S2B and Figure S3.

but abolished pellicle formation by the amyloid-fiber mutant. In other words, the effect of norspermidine was synergistic with that of a mutation blocking fiber formation but no... a mutant blocked in exopolysaccharide production (Figure ...3). These observations suggested that norspermidine was i... fering with the exopolysaccharide component of t... matrix.

To investigate this hypothesis, we visualize... ...saccha... ide by fluorescence microscopy by usin... conju...e of the carbohydrate-binding protein concanav... ...A wi... ...ed (Figure 3) (McSwain et al., 2005). As ...den... ...ecificity, ...e conjugate decorated wild-type ce... ...ut not ce... ...m a mutant (Δ*eps*) blocked in exopolysacc... ...production ...re S3A). Indeed, at an exposure at w... ...n co... ...navalin A staining with the wild-type strain gave ... extremely b... ...t fluorescent signal, little or no signal could ... detected for the ...s mutant, except at long exposures ... enhan... brightness (Figure S3A). Next, we investigated t... ffect... norspermidine and spermidine. Figure 3 shows tha... ...permidi... ...treatment disrupted the relatively u... ...cell-a... ...iate... ...attern of staining seen with untreat... ...ells, ...ulting i... ...ated patches of fluorescence. No s... effect ...as seen with cells treated with spermidine. Simply ...xi... ...ine, ...ine with concanavalin A did not quench th... ...ensity of fluorescence of the flurophore (data not shown). Her... ...he difference in the staining was evidently due to differential l... ...s of cell-associated exopolysaccharide. As a control, and in contrast to the results seen with concanavalin A, norspermidine had little or no effect on the protein component of the matrix as judged with a functional fusion of TasA to the fluorescent protein mCherry (Figure S3B) (Kolodkin-Gal et al., 2010). We infer that norspermidine disrupts the matrix and apparently does so by targeting exopolysaccharide.

Norspermidine but Not Spermidine Interacts with Exopolysaccharide

The expression of the operons *epsA-O* and *yqxM-sipW-tasA*, which specify the exopolysaccharide and protein components of the e... ...cellula... ...atrix, r... ...ectively, was not mea... ...bly ...paired by the addition of nor... ...rmidi... ...igure S... Also, cell growth ... not signi... ...tly ...hibited by norspermi... ...(Figure S4). ...herefore considered the pos... ...y that norspermidine was interacting with th... ...polysaccharide directly. To attempt to detect ... an interaction, we used dynamic light scattering, a standard procedure for ...asuring the average radius of polymers (Be... ...and Pec... ...1976; Orgad et al., 2011; Vinayahan et al., 2010).

Figure ... ...ows that purified exopolysaccharide exhibited ...erage radius of 585 ± 40 nm at pH 5.5, presumably repre... ...n effective radius for the interacting polymers. Strik... ...gly, treatment of the exopolysaccharide with norspermidine reduced the average radius substantially (175 ± 10 nm), whereas treatment with spermidine had only a small effect on the average radius (500 ± 20 nm). This indicates that the specificity of norspermidine resulted from a direct interaction with exopolysaccharide. The effect of norspermidine was seen over a range of exopolysaccharide concentrations (1–30 mg/ml) (Figure 4A and data not shown) and also at pH 7 (Figure 4A).

As an independent approach to detecting an interaction between norspermidine and exopolysaccharide, we carried out scanning electron microscopy. Purified exopolysaccharide was seen to be in the form of aggregates, which had an average diameter of ~570 nm (Figure 4B; data not shown). Strikingly, the addition of norspermidine reduced the diameter of the aggregates to ~85 nm (Figure 4B; data not shown). Once again, and, as a demonstration of specificity, spermidine had little effect on the size of the aggregates.

Use of a Panel of Small Molecules Reveals Features Important for Biofilm-Inhibitory Activity

To identify features of norspermidine important for its biofilm-disassembly activity, we tested a library of polyamines in our biofilm inhibition assay (see Figures 5A and 5B, Figure S5, and Table S2). In addition to norspermidine (1), we found that norspermine (2) exhibited biofilm-inhibitory activity against *B. subtilis*. These molecules have in common a motif consisting of three methylene groups flanked by two amino groups. The motif is present twice in (1) and three times in (2). Another polyamine, 1,3-diaminopropane (6), has only one copy of the motif and was significantly less active (>5 mM). Also relatively inactive (inhibition was only observed at concentrations above 2 mM)

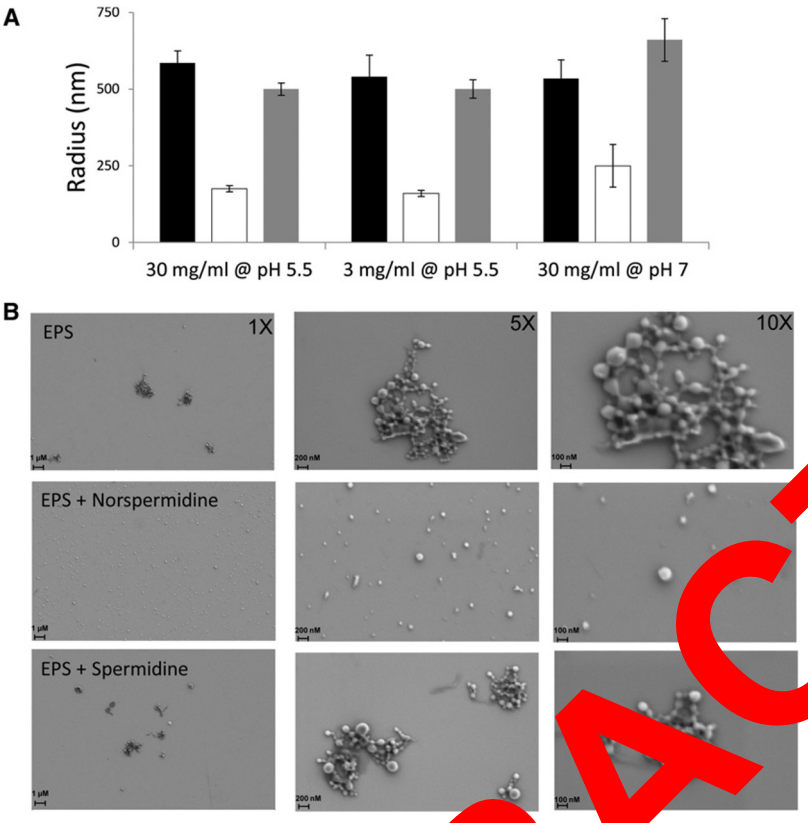

Figure 4. Norspermidine Interacts with Exopolysaccharide Polymers

(A) Dynamic light scattering. Listed are the average hydrodynamic radii of the exopolysaccharide as measured by dynamic light scattering. Exopolysaccharide was purified from pellicles. Light scattering was measured for exopolysaccharide alone as well as for exopolysaccharide that had been mixed with 0.75 [illegible]rspermidine or with 0.75 mM spermidine. Show[illegible] obtained in the absence of polyamine [illegible]ck) in the [illegible]nce of norspermidine (white) a[illegible] the presence [illegible]spermidine (gray) with exopo[illegible]ysacch[illegible] at the indi[illegible]ed concentrations and ph[illegible]or bars r[illegible]ent the [illegible]ndard deviation of poly[illegible]adii in a single [illegible]

(B) Sca[illegible]g elect[illegible]microsco[illegible] Purified exopolysaccha[illegible]s di[illegible]ved in DDW at a final concentration of [illegible] mg/[illegible] mixed w[illegible]either norspermidine or [illegible]midine (0.[illegible]M fina[illegible]ncentration). Samples were [illegible]pared as de[illegible]n Experimental Procedures. [illegible] are three diffe[illegible]t magnifications of representative fiel[illegible]wing exopolysaccharide alone (EPS) and exopolysa[illegible]ide that had been mixed with norspermidine (EPS + no[illegible]rmidine) or with spermidine (EPS + spermidine). See Figure S4 for controls showing little effect on [illegible]owth or *eps* transcription.

were spermidine (3), spermine (4), a[illegible] stres[illegible]hich each have a pair of amines separa[illegible] by [illegible]ethylenes, and cadaverine (19), which has a p[illegible]f amines [illegible]arated by five methylenes. Replacing some [illegible]f the amine[illegible]norspermidine with tertiary amines [illegible]9, 1[illegible] placing the secondary amine with an ether lin[illegible]e (10) or el[illegible]ating two or all of the amines (20, 11) resu[illegible] in molecules th[illegible]ere relatively inactive. Whereas rep[illegible]g the [illegible]minal amines with tertiary amines resulted in inacti[illegible]in [illegible]case (21) the presence of a tertiary amine at the middle [illegible]ion did [illegible]block activity. Importantly, the char[illegible]ach a[illegible] (at [illegible]e neutral pH of the medium) was a[illegible]impo[illegible]t for bi[illegible]nhibiting activity. Molecules that ha[illegible]utral a[illegible]e bonds instead of amines separated by three meth[illegible]es [illegible]only weakly active or inactive (Figure S5 and Tab[illegible]2).

We conc[illegible] that the structure and the charge of the polyamine are re[illegible]ed for biofilm-inhibiting activity. In particular, a motif consisting of three methylene groups flanked by two positively charged amino groups is favored for high biofilm-inhibiting activity. Reinforcing this hypothesis, three additional, synthetic polyamines exhibiting this motif (12, 13 and 14) were active (Figure S5).

Norspermidine but Not Spermidine Inhibits Biofilm Formation by *S. aureus* and *E. coli*

We wondered whether polyamines might prevent biofilm formation by other bacteria that produce an exopolysaccharide matrix. Indeed, the same molecules that inhibited biofilm formation by *B. subtilis* were effective in inhibiting biofilm formation (but not growth; data not shown) by *S. aureus* and *E. coli*, whereas those that were inactive with *B. subtilis* were not (Figures 6 and 7 and Figure S6). In testing *E. coli* we chose the biofilm-proficient strain MC4100 because a major component of exopolysaccharide is colanic acid (Danese et al., 2000; Price and Raivio, 2009). Colanic acid is a negatively charged polymer, and light scattering experiments indicated a direct interaction with norspermidine (data not shown). Reinforcing the idea that norspermidine was targeting the exopolysaccharide, fluorescence microscopy experiments analogous to those presented above for *B. subtilis* showed markedly diminished staining of exopolysaccharide when cells of *S. aureus* and *E. coli* were treated with norspermidine but not spermidine (data not shown).

Interestingly, and in contrast to the above results, norspermidine is reported to promote rather than inhibit biofilm formation by *Vibrio cholerae* (Lee et al., 2009). Evidence indicates that in *V. cholerae* norspermidine acts to de-repress biofilm formation via a signal transduction mechanism (Karatan et al., 2005). Nonetheless, and despite the *V. cholerae* exception, our results support the concept that biofilm formation can be prevented and existing biofilms disrupted by molecules that interact directly with exopolysaccharides.

DISCUSSION

B. subtilis forms architecturally complex biofilms (pellicles) at the air/liquid interface of standing cultures (Aguilar et al., 2007; Branda et al., 2001b). These floating communities are transient; they mature after 3 days in biofilm-inducing medium but then disassemble by 8 days, releasing individual planktonic bacteria

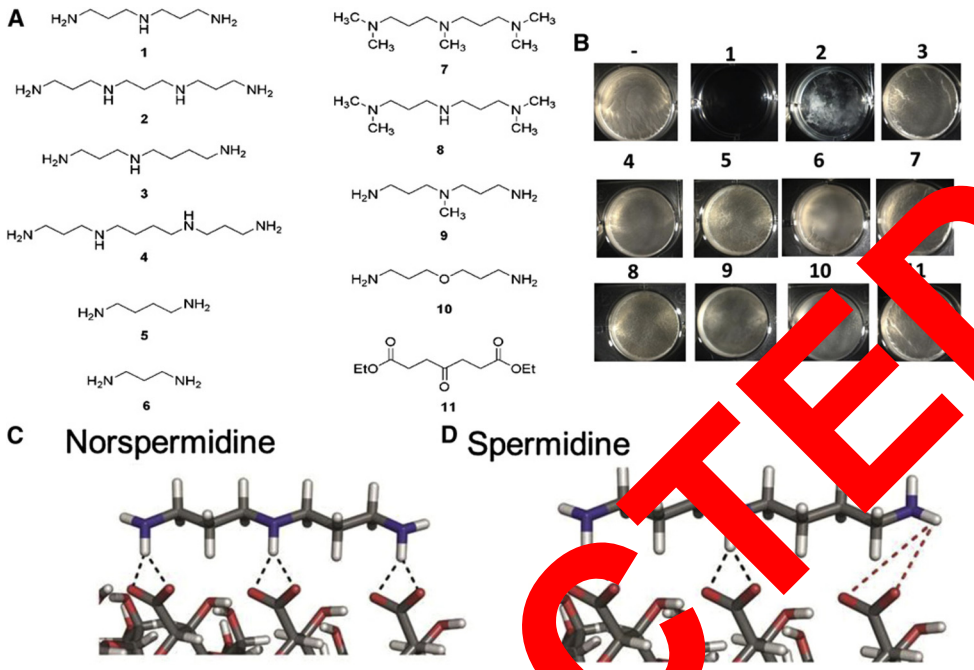

Figure 5. Structure Activity Relationship Study of Norspermi

(A) Shows compounds tested for biofilm-inhibiting activity.

(B) Shows the effect of the numbered compounds on pellicle formation by *B. subt* 3610). The compounds were tested at 200 μM.

(C and D) show the results of modeling the interaction of norspermidi nd rmidi with an acidic exopolysaccharide. Norspermidine binds via salt bridges between amino and carboxyl groups (dotted lines) lamp-like across the exopolysaccharide secondary structure of a disaccharide repeat. $[\alpha(1,6)\text{Glc-}\beta(1,3)\text{GlcA}]_n$. Whereas norspermidine aligns wit repeat, spacing of amino groups of spermidine does not match the symmetric pattern of anionic side groups, implying weaker affinity. S support information or modeling of plausible interactions with other charged and non-charged polysaccharide structures. For tests of additional cules s S5 and Table S2.

(Kolodkin-Gal et al., 2010). Cells i e biofilm a eld together by exopolysaccharide and amy e fibers larg onsisting of TasA (Branda et al., 2006b; randa l., 2004; Romero et al., 2010). The return of cells i he biofilm t planktonic state must therefore involve mech sms for their rele om the exopolysaccharide and pr n com ents of the matrix. One such mechanism is the p ucti ate in the life cycle of the D-amino acids D-Tyr, D-Leu, and D- , which are incorporated into the p ycan re trigger the release of the TasA fi (Kolo in-Gal e 2010). This release is mediated by a eptor p ein TapA, which forms D-amino acid-sensitive fo omero et al., 2011). Here we have reported t iscovery of a second biofilm-disassembly factor, norspermidi which is also produced late in the life cycle of the biofilm d is required for complete disassembly of *B. subtilis* biofilms. Importantly, mutants blocked in the production of both D-amino acids and norspermidine formed long-lived pellicles that retained their architectural complexity for extended periods of time. We do not fully know the mechanism(s) by which the production of norspermidine and D-amino acids is delayed until late in the biofilm life cycle but experiments based on the use of *lacZ* fused to genes involved in norspermidine (*gabT* and *yaaO*) and D-amino acid biosynthesis (the racemase genes *racX* and *ylmE*) indicate that regulation occurs at the level of gene transcription (L. Silverstein, Y. Chai, I.K.-G., unpublished results).

The biofilm-inhibiting effect of norspermidine was specific in that a closely related polyamine, spermidine (differing only by an extra methylene group), exhibited little activity. Interestingly, another polyamine, norspermine, was also active in biofilm inhibition, whereas its close relative spermine (once again having an extra methylene) was inactive. These results and the results of using a panel of seventeen additional compounds suggest that biofilm inhibition depends on a motif of two or three pairs of primary or secondary charged amines separated by three methylenes.

Several lines of evidence indicate that norspermidine acts in a complementary manner to D-amino acids by targeting the exopolysaccharide. First, norspermidine and D-amino acids acted cooperatively in inhibiting biofilm formation, suggesting that they function by different mechanisms. Second, pellicles formed in the presence of norspermidine resembled the wispy, fragmented material produced by an exopolysaccharide mutant but not the thin, flat, featureless pellicle of a mutant blocked in amyloid-fiber production. Third, fluorescence microscopy showed that norspermidine (but not spermidine) disrupted the normal uniform pattern of staining of exopolysaccharide but had little effect on the staining pattern of the protein component of the matrix. Finally, and most directly, light scattering and electron microscopy experiments revealed that norspermidine, but not spermidine, interacted with purified exopolysaccharide.

Remarkably, the biofilm-inhibiting effect of norspermidine and norspermine was not limited to *B. subtilis*. Both molecules

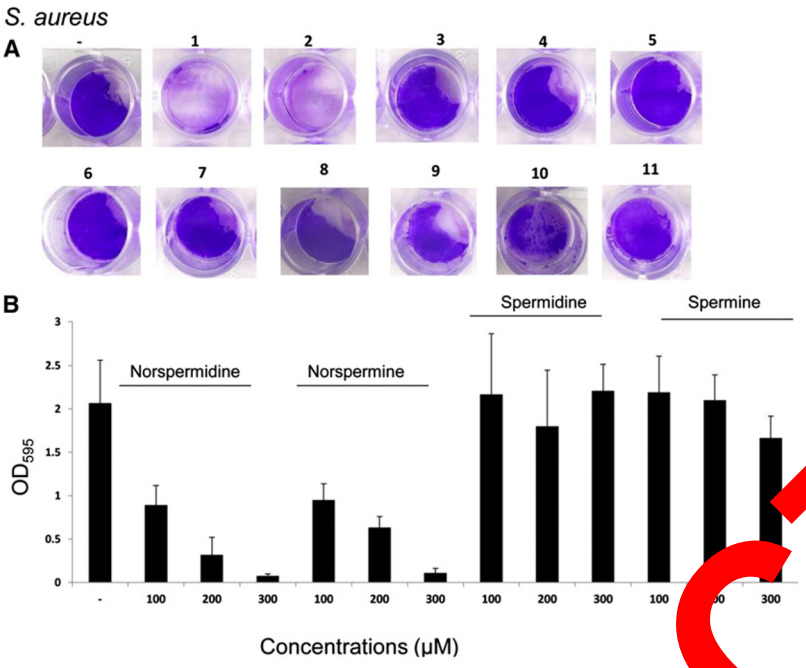

Figure 6. Norspermidine Inhibits Biofilm Formation by *S. aureus*

(A) The effect of the numbered compounds displayed in Figure 5A on the formation of submerged biofilms by *S. aureus* strain SCO1. The compounds were tested at 500 μM. Biofilm formation was visualized by CV staining of submerged biofilms.

(B) Quantification of the effects of norspermidine, norspermine, spermine, and spermidine as measured by CV staining (see Experimental Procedures). The results are an average of three independent experiments done in triplicate. The error bars indicate standard deviation. See also Figure S6.

inhibited the formation of submerged biofilms by *S. aureus* and *E. coli*. Indeed, the same pattern of molecules that were active or inactive in inhibiting biofilm formation by *B. subtilis* was observed for *S. aureus* and *E. coli*. It is therefore tempting to propose that the broad spectrum of norspermidine and spermidine reflects a common mechanism of targeting the exopolysaccharide. Indeed, this was supported by fluorescence microscopy with *S. aureus* and *E. coli* and light scattering experiments with purified exopolysaccharide from *E. coli*. However, we cannot exclude the possibility that norspermidine also targets DNA, which is, of course, negatively charged and is known to be a component of the matrix for certain bacteria, such as *S. aureus* (Branda et al., 2005).

What is the nature of interaction of norspermidine with exopolysaccharides? Exopolysaccharides often contain negatively charged residues (e.g., uronic acid) or neutral sugars with polar groups (e.g., poly-*N*-acetylglucosamine) (Kropec et al., 2005; Sutherland, 2001). Molecular modeling suggests that the amines in norspermidine, but not those in spermidine, are capable of interacting with such charged (Figures 5C and 5D) or polar groups (Figure S7) in secondary structure of the exopolysaccharide. We suggest that this interaction enhances the ability of the polymers to interact with each other or with other parts of the polymer chain. Indeed, the results of fluorescence

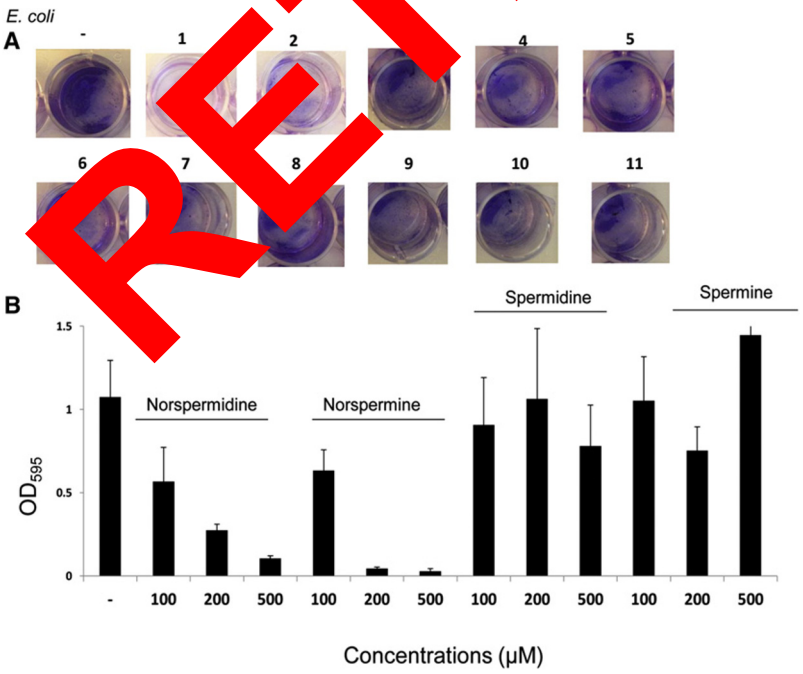

Figure 7. Norspermidine Inhibits Biofilm Formation by *E. coli*

(A) The effect of the numbered compounds shown in Figure 5A on submerged biofilm formation by *E. coli* strain MC4100. The compounds were tested at 500 μM. Biofilm formation was visualized by CV staining of submerged biofilms.

(B) shows quantification of the effects of norspermidine, norspermine, spermine, and spermidine as measured by CV staining (see Experimental Procedures). The results are an average of three independent experiments done in triplicate. The error bars indicate standard deviation. See also Figure S6.

microscopy (Figure 3), dynamic light scattering (Figure 4A), and scanning electron microscopy (Figure 4B) appear to indicate that the exopolysaccharide network collapses upon addition of norspermidine. We speculate that exopolysaccharide polymers form an interwoven meshwork in the matrix that helps hold cells together and that condensation of the polymers in response to norspermidine weakens the meshwork and causes release of polymers.

Given the apparent versatility of norspermidine and norspermine in inhibiting biofilm formation by a variety of bacteria, it is conceivable that these and other, tailor-made polyamines that bind with high affinity to specific exopolysaccharides might offer a general approach (in conjunction with D-amino acids) to preventing biofilm formation by medically and industrially important microorganisms. Indeed, in preliminary experiments we have succeeded in synthesizing polyamine-like molecules with enhanced potency in blocking biofilm formation by *S. aureus* that were designed on the basis of model building for optimal interaction with poly *N*-acetyl glucosamine (data not shown).

EXPERIMENTAL PROCEDURES

Colony and Pellicle Formation

For pellicle formation in liquid medium, cells were grown to exponential and 3 μl of culture were mixed with 3 ml of medium in a 12-well plate (Corning). Plates were incubated at 23°C for 3 days. Images of the pellicles were recorded similarly.

Submerged Biofilm Formation

For *S. aureus,* cells were grown in Luria-Bertani (LB) overnight and then diluted 1:1,000 in Tryptic Soy Broth (Sigma), applied with 3% NaCl and Plates were incubated at 37°C for 24 hr. For *E. coli*, cells were grown in LB overnight, then diluted 1:100 in M9 and supplied with 0.02% casamino acid solution and 0.5% glycerol. Plates were incubated in 30°C for 5 days.

Preparing Conditioned Medium

B. subtilis 3610 or its derivatives were grown in LB medium to exponential phase. A total of 1 ml of culture was then applied to 100 ml of MSgg medium and grown in a 500 liter fermentor at 23°C. Next, pellicles and conditioned medium were collected by centrifugation at 8,000 rpm for 15 min. The conditioned medium (supernatant fluid) was removed and filtered through a 0.22 μm filter. The filtrate was stored at 4°C. After purification the biofilm-inhibiting material was fractionated on a C-18 Sep Pak cartridge with a stepwise elution of 0% to 100% methanol with steps of 5%.

Separation, Identification, and Quantification of Norspermidine

See Supplemental Information for details.

Fluorescence Microscopy

Fluorescence microscopy was carried out with 3-day-old pellicles. For TasA-mcherry detection, cells were washed with PBS buffer and suspended in 50 μl of PBS buffer. For exopolysaccharide detection, pellicles were collected and washed with PBS. The cells were labeled by replacing the PBS with Texas red-concanavalin A (50 μM) and incubating with shaking in room temperature for an hour. The cells were rinsed again with PBS, placed on poly-L-lysine (Sigma) pretreated slides and then imaged with an Olympus workstation. Cells were imaged with various exposure times and images were taken under conditions in which concanavalin A staining was largely specific to exopolysaccharide (see Figure S3). Images were taken with an automated software program, SimplePCI.

Crystal Violet Staining

Crystal Violet (CV) staining was done as described previously except that the cells were grown in 12-well plates (O'Toole and Kolter, 1998). Wells were stained with 500 μl of 1.0% CV dye, rinsed twice with 2 ml doubly distilled water (DDW), and thoroughly dried. For quantification, 1 ml of 95% ethanol was added to each well. Plates were incubated for 1 hr at room temperature with shaking. CV solution was diluted and the optical density (OD) at 595 nm was measured with Ultraspec 2000 (Pharmacia Biotech).

Exopolysaccharide Purification

Pellicles were harvested in day 3, washed twice with PBS, and mildly sonicated. Cells were removed by centrifugation. The supernatant fluid was mixed with cold isopropanol in a 5:1 ratio and incubated at −20°C overnight. Samples were centrifuged at 8,000 rpm, 4°C for 10 min. Pellets were resuspended in a digestion mix of 0.1 M MgSO4, 0.1 mg/ml DNase, and 0.1 mg/ml RNase solution, mildly sonicated, and incubated for 4 hr at 37°C. Samples were extracted twice with phenol-chloroform. The aqueous fraction was dialyzed for 48 hr with Slide-A-Lyzer Dialysis Cassettes by Thermo Fisher, 3,500 MCWO. Samples were lyophilized.

Dynamic Light Scattering

See Supplemental Information for details.

Scanning Electron Microscopy

Exopolysaccharide was dissolved in DDW at a final concentration of 10 mg/ml and mixed with norspermidine or spermidine (0.75 mM final concentration). Samples were then spotted onto poly-L-lysine-coated Si surfaces and kept in a humidity chamber for 30 min. The Si pieces were then rinsed with DDW. To capture the native, hydrated, state of exopolysaccharide, we cryo-immobilized the samples. Images were obtained with a Zeiss Supra55 Field Emission scanning electron microscope. For further details see Extended Experimental Procedures.

Primers

See Table S1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two table and can be found with this article online at doi:10.1016/j.cell.2012.02.055.

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REFERENCES

Aguilar, C., Vlamakis, H., Losick, R., and Kolter, R. (2007). Thinking about *Bacillus subtilis* as a multicellular organism. Curr. Opin. Microbiol. *10*, 638–643.

Berne, B.J., and Pecora, R. (1976). Dynamic Light Scattering (New York: Wiley).

Branda, S.S., González-Pastor, J.E., Ben-Yehuda, S., Losick, R., and Kolter, R. (2001a). Fruiting body formation by *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA *98*, 11621–11626.

Branda, S.S., González-Pastor, J.E., Ben-Yehuda, S., Losick, R., and Kolter, R. (2001b). Fruiting body formation by Bacillus subtilis. Proc. Natl. Acad. Sci. USA *98*, 11621–11626.

Branda, S.S., González-Pastor, J.E., Dervyn, E., Ehrlich, S.D., Losick, R., and Kolter, R. (2004). Genes involved in formation of structured multicellular communities by Bacillus subtilis. J. Bacteriol. *186*, 3970–3979.

Branda, S.S., Vik, S., Friedman, L., and Kolter, R. (2005). Biofilms: the matrix revisited. Trends Microbiol. *13*, 20–26.

Branda, S.S., Chu, F., Kearns, D.B., Losick, R., and Kolter, R. (2006a). A major protein component of the *Bacillus subtilis* biofilm matrix. Mol. Microbiol. *59*, 1229–1238.

Branda, S.S., Chu, F., Kearns, D.B., Losick, R., and Kolter, R. (2006b). A major protein component of the Bacillus subtilis biofilm matrix. Mol. Microbiol. *59*, 1229–1238.

Bryers, J.D. (2008). Medical biofilms. Biotechnol. Bioeng. *100*, 1–18.

Cava, F., de Pedro, M.A., Lam, H., Davis, B.M., and Waldor, M.K. (2011). Distinct pathways for modification of the bacterial cell wall by non-canonical D-amino acids. EMBO J. *30*, 3442–3453.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. (1999). Bacterial biofilms: a common cause of persistent infections. Science *284*, 1318–1322.

Danese, P.N., Pratt, L.A., and Kolter, R. (2000). Exopolysaccharide production is required for development of Escherichia coli K-12 biofilm architecture. J. Bacteriol. *182*, 3593–3596.

Hochbaum, A.I., Kolodkin-Gal, I., Foulston, L., Kolter, R., Aizenber[illegible] Losick, R. (2011). Inhibitory effects of D-amino acids on Staphyl[illegible]ccus aureus biofilm development. J. Bacteriol. *193*, 5616–5622.

Karatan, E., and Watnick, P. (2009). Signals, regulatory n[illegible]orks, and [illegible] rials that build and break bacterial biofilms. Microb[illegible]iol. Rev. 310–347.

Karatan, E., Duncan, T.R., and Watnick, P.[illegible]05). Ns[illegible] predicte[illegible] polyamine sensor, mediates activation of *Vibrio* [illegible]on by norspermidine. J. Bacteriol. *187*, 743[illegible]43.

Kolodkin-Gal, I., Romero, D., Cao, S[illegible]rdy, J., Kolter[illegible]and Losick, R. (2010). D-amino acids trigger biofi[illegible]bly. Science [illegible]627–629.

Kropec, A., Maira-Litran, T., Jefferson, K.K., [illegible] M., Cramton, S.E., Götz, F., Goldmann, D.A., and Pier, [illegible](2005). Poly-N-[illegible]glucosamine production in Staphylococcus aur[illegible]s essential for virul[illegible]in murine models of systemic infection. In[illegible] Immun[illegible]6868–6876.

Lee, J., Sperandio, [illegible]antz[illegible], Longgood, J., Camilli, A., Phillips, M.A., and Michael, A.J. (200[illegible]lternative[illegible]yamine biosynthetic pathway is widespread in bacteria and essential for biofilm formation in Vibrio cholerae. J. Biol. Chem. *284*, 9899–9907.

Lopez, D., Vlamakis, H., and Kolter, R. (2009). Generation of multiple cell types in Bacillus subtilis. FEMS Microbiol. Rev. *33*, 152–163.

McSwain, B.S., Irvine, R.L., Hausner, M., and Wilderer, P.A. (2005). Composition and distribution of extracellular polymeric substances in aerobic flocs and granular sludge. Appl. Environ. Microbiol. *71*, 1051–1057.

Molnár-Perl, I. (2003). Quantitation of amino a[illegible]nes in the same matrix by high-performance liquid chromato[illegible]ny, eithe[illegible]ltaneously or separately. J. Chromatogr. A *987*, 291–30[illegible]

Nagórska, K., Bikowski, M., and [illegible]obucho[illegible] M. (2007[illegible]Multicellular behaviour and production of a [illegible]variety o[illegible]ic sub[illegible]ces support usage of Bacillus subtilis as a [illegible]erful biocontrol [illegible]a Biochim. Pol. *54*, 495–508.

O'Toole, G.A., and Kolter, R. ([illegible]. [illegible]llar and t[illegible]tching motility are necessary for Pseudomon[illegible]erugino[illegible]ilm dev[illegible]ment. Mol. Microbiol. *30*, 295–304.

O'Toole, G., K[illegible] and Kolter, R[illegible]). Biofilm formation as microbial developmen[illegible]nnu. R[illegible]icrobiol. *54*, 49–79.

Orgad, O., Oren, Y., Walke[illegible] and Herzberg, M. (2011). The role of alginate in P[illegible]nas aeruginosa [illegible]dherence, viscoelastic properties and cell a[illegible]ment. Biofouling *27*, 787–798.

[illegible] M. (2008). S[illegible]ylococcal biofilms. Curr. Top. Microbiol. Immunol. *322*, 2[illegible]28.

Pric[illegible] and [illegible]io, T.L. (2009). Characterization of the Cpx regulon in Escher[illegible]rain MC4100. J. Bacteriol. *191*, 1798–1815.

[illegible]mero, D., and Kolter, R. (2011). Will biofilm disassembly agents make it to [illegible]rends Microbiol. *19*, 304–306.

Romero, D., Aguilar, C., Losick, R., and Kolter, R. (2010). Amyloid fibers provide structural integrity to Bacillus subtilis biofilms. Proc. Natl. Acad. Sci. USA *107*, 2230–2234.

Romero, D., Vlamakis, H., Losick, R., and Kolter, R. (2011). An accessory protein required for anchoring and assembly of amyloid fibres in B. subtilis biofilms. Mol. Microbiol. *80*, 1155–1168.

Sutherland, I. (2001). Biofilm exopolysaccharides: a strong and sticky framework. Microbiology *147*, 3–9.

Vinayahan, T., Williams, P.A., and Phillips, G.O. (2010). Electrostatic interaction and complex formation between gum arabic and bovine serum albumin. Biomacromolecules *11*, 3367–3374.

Vlamakis, H., Aguilar, C., Losick, R., and Kolter, R. (2008). Control of cell fate by the formation of an architecturally complex bacterial community. Genes Dev. *22*, 945–953.