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Rhizobium common nod genes are required for biofilm formation

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Summary

In legume nitrogen-fixing symbioses, rhizobial nod genes are obligatory for initiating infection thread formation and root nodule development. Here we show that the common nod genes, nodD1ABC, whose products synthesize core Nod factor, a chitin-like oligomer, are also required for the establishment of the three-dimensional architecture of the biofilm of Sinorhizobium meliloti. Common nod gene mutants form a biofilm that is a monolayer. Moreover, adding Nod Factor antibody to S. meliloti cells inhibits biofilm formation, while chitinase treatment disrupts pre-formed biofilms. These results attest to the involvement of core Nod factor in rhizobial biofilm establishment. However, luteolin, the plant-derived inducer of S. meliloti’s nod genes, is not required for mature biofilm formation, although biofilm establishment is enhanced in the presence of this flavonoid inducer. Because biofilm formation is plant-inducer-independent and because all nodulating rhizobia, both alpha- and beta-proteobacteria have common nod genes, the role of core Nod factor in biofilm formation is likely to be an ancestral and evolutionarily conserved function of these genes.

Introduction

For more than 20 years, Rhizobium nod genes, and their product, Nod Factor (NF), have been recognized as essential for the development of nitrogen-fixing nodules on legume roots (Lerouge et al., 1990). Mutations within or deletions of entire nod genes result in a loss of the ability of rhizobial bacteria to induce nodules on the host, and therefore fix atmospheric nitrogen. NF is a β-linked N-acetylglucosamine oligomer, with a fatty acid chain attached to the terminal glucosamine and various substituents occurring on the chitin-like backbone. Although the precise mechanism whereby NF stimulates nodule formation remains uncertain, NF is known to trigger calcium spiking, cause root hair deformation and initiate nodule primordium formation (Geurts et al., 2005; Oldroyd and Downie, 2006).

The combination of the products from two different classes of nod genes, which are under the control of the lysR-like regulatory gene nodD, results in the synthesis of NF. NodD binds to the nod box region of the nod gene promoters; nodD1, one of three nodD genes in Sinorhizobium meliloti, is included among the common nod genes. The common nodulation genes (nodDABC) are found in all bacteria that nodulate legumes (with so far only one known exception; Giraud et al., 2007), including the beta-proteobacteria (beta-rhizobia), which also establish nodules on legume roots (Moulin et al., 2001). NodC is responsible for the biosynthesis of the N-acetylglucosamine trimeric to pentameric backbone, while NodB deacetylates a terminal glucosamine, leaving a free amino group, which is acylated by NodA. The second class of nod genes (in S. meliloti, nodEF, nodG, nodH, nodPQ, nodL) consists of the host-specific nodulation genes, whose products modify the N-acetylglucosamine backbone in ways that are unique to a particular Rhizobium species. Typically, the products of the host-specific nod genes are responsible for adding side group substituents and for determining the length and saturation of the fatty acid on the terminal glucosamine (Lerouge et al., 1990). These substituents confer specificity between a rhizobial species and the cognate host.

Sinorhizobium meliloti and Rhizobium leguminosarum bv. viciae, like many other bacteria, form biofilms on sterile inert substrates, including plastic, glass, sand and soil (Fujishige et al., 2006a). While screening symbiotic mutants for their effects on biofilm formation using microtitre plate and whole-root assays, we earlier found that R. leguminosarum bv. viciae deleted of pSym [an endogenous plasmid that carries both nod and nif (nitrogen fixation) genes] and S. meliloti pSymA-deletion mutants
exhibited significantly reduced biofilm formation (Fujishige et al., 2006b). This led to testing the effect on biofilm formation of individual and multiple nod gene mutations. Here we report that the products of the common nod genes have a hitherto unrecognized function – holding a rhizobial biofilm together.

Results

Effect of nod gene mutations on biofilm formation in vitro

Individual common nod gene mutants, the class of nod genes common to nodulating rhizobia, including the β-rhizobia, as well as mutants in the second class of nod genes, the host-specific nod genes, were tested for biofilming ability. We determined that S. meliloti mutants deleted of any one of the common nod genes or nodD1ABC exhibited significantly reduced (50–70%) biofilm formation not only in microtiter plate assays (Fig. 1A and B), but also on environmentally relevant substrates, including roots (Fig. 2A). Mutations in individual genes, nodA or nodC (Table 1), either in S. meliloti RCR2011 (Fig. 1A) or in the sequenced strain Rm1021 (Galibert et al., 2001; Fig. 1B), did not affect the growth of these bacteria compared with wild-type S. meliloti (data not shown), but did result in statistically significantly reduced biofilm formation. A similar result was observed for individual nodB mutants (data not shown). In contrast, host-specific nod mutants were not altered in their level of biofilm establishment compared with the wild-type strains in the RCR2011 (Fig. 1C) and Rm1021 genetic backgrounds (data not shown).

In S. meliloti, in addition to the three nodD genes, there is a nodD-like gene, syrM. SyrM and NodD3 together form a positive regulatory circuit (Swanson et al., 1993; Dusha et al., 1999a,b). In the microtiter plate assay, mutants of nodD1, nodD2, nodD1D2D3 and syrM developed reduced biofilms, whereas the nodD3 mutant was unaffected (Fig. 1D). The difference between syrM and nodD3 may lie in SyrM’s involvement in succinoglycan (EPSI) biosynthesis (Swanson et al., 1993); we previously reported that S. meliloti EPSI mutants exhibit reduced biofilm formation (Fujishige et al., 2006a). The nodD1 gene is activated by plant-produced flavonoids, such as...
luteolin (Mulligan and Long, 1985), whereas nodD2 is activated by plant-produced betaines (Phillips et al., 1992). No change in biofilm formation was observed when betaine was added to the culture medium (data not shown), whereas luteolin addition enhanced biofilm establishment (see later section).

Confocal imaging of wild-type green fluorescent protein (GFP)-labelled rhizobia showed that mature biofilms consist of towers and ridges (Fig. 2D and E). This biofilm morphology contrasted with that produced by Nod− mutant bacteria, which only established a monolayer with few bacteria attached to one another (Fig. 2F and G). When viewed under transmission electron microscopy (TEM), wild-type S. meliloti biofilms revealed extensive cell-to-cell contacts (Fig. 3A), whereas the nod mutations remained as single cells or occasionally as doublets (Fig. 3B).

### Table 1. Strains and plasmids used in this study.

<table>
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<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>RCR2011</td>
<td>Wild-type derivative of SU47</td>
<td>Jean Dénarié</td>
</tr>
<tr>
<td>Rm1021</td>
<td>Wild-type Sm′ derivative of 2011</td>
<td>Meade et al. (1982)</td>
</tr>
<tr>
<td>Rm1021 (pRm57)</td>
<td>Rm1021 nodC::lacZ</td>
<td>Mulligan and Long (1985)</td>
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<td>GMI3253</td>
<td>Rm1021 ΔnodA null</td>
<td>Jean Dénarié</td>
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<tr>
<td>GMI5612</td>
<td>Rm1021 nodC::Tn5</td>
<td>Ethan R. Signer</td>
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<td>SL4</td>
<td>Rm1021 ΔnodD1ABC</td>
<td>Sharon R. Long</td>
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<td>GMI5383</td>
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<td>GMI357</td>
<td>RCR2011 ΔnodD1ABC</td>
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<td>Miller et al. (2000)</td>
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<td>Lerouge et al. (1990)</td>
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<tr>
<td>pRmJ30</td>
<td>8.7 kb EcoRI fragment carrying nodD1ABCUI</td>
<td>Jacobs et al. (1985)</td>
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**Effect of nod gene mutations on biofilm formation**

We extended the findings from the microtitre plate assay by examining biofilm formation on roots of white sweet-clover (Melilotus alba Desr.). Confocal laser scanning micrographs of roots inoculated with Rm1021 showed distinct microcolonies along the root surface, which remained adherent after extensive washing (arrows, Fig. 2A). In contrast, few nodC mutant cells remained attached after washing (arrow, Fig. 2B). The number of colony-forming units (cfu) per gram of root tissue demonstrated a significant reduction (> 50%) in attachment ability of both nodC and nodD1D2D3 mutants compared with the wild-type control Rm1021 (Fig. 2C).

**Luteolin is not required for biofilm formation**

We hypothesized that the common nod genes are expressed in the biofilm independently of plant-derived inducers because biofilms developed in the microtitre plate wells without adding luteolin (Fig. 1). To test this hypothesis specifically, a nodA–gfp transcription fusion was introduced into wild-type Rm1021 cells, which were inoculated onto sand particles. Using this transcriptional fusion, attached single cells and small microcolonies were visualized by their fluorescence 4 h after the initiation of
A and B. Confocal images showing the attachment of (A) wild-type *S. meliloti* (Rm1021) and (B) a *nodC* mutant (Rm5612) to roots of *Melilotus alba* Desr. 72 h post inoculation. Bar, 250 μm.

C. Colony-forming unit (cfu) counts from roots 48 h post inoculation with either wild-type (Rm1021) or *nod* mutant bacteria (*nodD1D2D3* and *nodC*).

D. Confocal imaging (top and side views) of a wild-type *gfp*-expressing RCR2011 strain 72 h post inoculation shows biofilms composed of ridges (arrows) and towers (arrowheads). Bar, 10 μm.

E. Confocal imaging (top and side views) of a wild-type *gfp*-expressing RCR2011 strain 5 days post inoculation. Bar, 100 μm.

F. Very few ∆*nodD1ABC* mutant cells (top and side views) remain attached to each other or to the glass coverslip 72 h post inoculation. Bar, 10 μm.

G. After 5 days, the ∆*nodD1ABC* mutant bacteria remain in a monolayer. Bar, 100 μm.

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the experiment (Fig. 4A). By 24 h after the start of the experiment without the luteolin inducer, large GFP-positive colonies were observed on the sand particles (Fig. 4B).

Wild-type S. meliloti biofilm formation was enhanced by adding 1 μM luteolin to the culture medium, whereas the nodC mutant showed no difference in biofilm establishment in the presence or absence of luteolin (Fig. 4C). Luteolin induced an almost twofold increase in β-galactosidase activity in biofilm S. meliloti carrying a nodC-lacZ transcriptional fusion (Mulligan and Long, 1985) over the control, which was treated with the solvent methanol (Fig. 4D). As expected, planktonic cells also showed increased β-galactosidase activity in the presence of luteolin.

Because biofilm formation correlated with augmented NF production brought about by luteolin addition, we examined the effect of NF overproduction by introducing the plasmid pRMJ30, which carries the common nod genes, or the plasmid pGMI149, which contains both common and host-specific nod genes, or pLAFR1, the vector control, into the RCR2011 genetic background (Table 1). Each nod gene containing plasmid enhanced biofilm formation in the absence of luteolin by approximately 25% (Fig. 5A). A similar result was found for the Rm1021 wild-type strain (data not shown). No enhancement was observed for strains containing the vector pLAFR1 (data not shown). Interestingly, we detected no statistical difference between strains carrying only the common nod genes versus those carrying a plasmid with the full complement of nod genes, indicating that the contribution of host-specific nod genes to biofilm formation is minimal (see also Fig. 1C). When the plasmid carrying nodD1ABC was introduced into a mutant deleted of these genes, biofilm restoration was restored (Fig. 5B).

As a further gain-of-function test, we introduced a series of nod plasmids into another member of the Rhizobiaceae, Agrobacterium tumefaciens strain A348, which generates tumours on plant tissues (Garfinkel et al., 1981). In all cases, there was a clear enhancement of biofilm formation, even when only nodD1ABC was introduced (Fig. 5C). This finding shows that the minimalist or core NF contributes to the enhancement of biofilm formation in A. tumefaciens as it does in S. meliloti.

Core NF facilitates cell-to-cell adhesion

Based on the morphology of the wild-type versus nod mutant biofilms (Figs 2D–G and 3), we hypothesized that core NF causes the rhizobial cells to adhere to one another. Cell-to-cell adhesion would allow the rhizobial cells to remain closely attached to roots until an adequate population accumulated to produce a sufficient localized concentration of the host-specific signalling NF, which is required for root hair calcium spiking and deformation. We examined biofilms formed by mixing a GFP-labelled nodC mutant strain 1:1 with wild-type Rm1021 labelled with DsRed (Fig. 4E). Interestingly, the nodC mutant strain was excluded from the biofilm, suggesting that the lack of NF kept the mutant from integrating into the Rm1021 biofilm. A similar response was observed when a nodC-gfp mutant was mixed with a DsRed-labelled exoY mutant (see Fig. S1).

An in silico investigation revealed an overall similarity of 37% between Staphylococcus epidermidis IcaA and S. meliloti NodC on the protein level (data not shown). IcaA and other proteins encoded by the ica gene cluster synthesize a long chain of N-acetylglucosamines known as polysaccharide intercellular adhesin (PIA), which is essential for maintaining S. epidermidis biofilm adherence (Heilmann et al., 1996; Götz, 2002). PIA is detected in fibrous material surrounding the bacterial cells within the S. epidermidis biofilm (Vuong et al., 2004). However, we detected no cross reaction between PIA and S. meliloti or between PIA and NF (data not shown).
We utilized an *S. meliloti* NF-specific antibody (Timmers *et al.*, 1998) conjugated to colloidal gold in TEM studies against biofilms (Lévesque *et al.*, 2004), but found no labelling of any definite structures on the bacterial cell surface. However, the gold-labelled antibody was detected in both the external milieu and on the cell membrane (Fig. S2). Because NF is only three to five *N*-acetylglucosamines long, detecting a fibrous component analogous to PIA, which is a long chain of 100–120 *N*-acetylglucosamine residues, is unlikely. On the other hand, we observed that wild-type *S. meliloti* biofilm formation was reduced in the microtiter plate assay with antibody dilutions ranging from 1:10 000 to 1:100 (Fig. 5D). The NF antibody at dilutions of 1:10 000 (Fig. 5E) and 1:1000 (data not shown) similarly reduced biofilm formation of *A. tumefaciens* A348 carrying pRmJ30, but no effect on A348 biofilms was detected at any concentration. This result strongly suggests that a molecule recognized by NF antibody is present on the surface of or within the biofilm matrix of agrobacteria expressing the common *nod* genes.

Fig. 4. Expression of *nod* genes in response to adherence to a surface and to the flavonoid inducer luteolin.
A. Rm1021 with a *nodA*-gfp transcriptional fusion fluoresced as single cells and as microcolonies attached to sand particles as early as 4 h after inoculation. Bar, 40 μm.
B. After 24 h, the microcolonies were significantly larger. Bar, 40 μm.
C. The microtiter plate assays show an increase in Rm1021 biofilms when 1 μM luteolin was added to RDM, but no change in the *nodC* mutant biofilms occurred between the luteolin-treated and untreated samples.
D. Luteolin induced an almost twofold increase in β-galactosidase activity over the control in biofilm *S. meliloti* Rm1021 carrying a *nodC–lacZ* transcriptional fusion. The planktonic cells as expected also showed an increase in β-galactosidase activity.
E. Mixed biofilm of Rm5612-gfp (*nodC*:Tn5) and Rm1021-DsRed as viewed with epifluorescence. The green *Nod*+ cells remained on the top of the biofilm. Bar, 20 μm.
**Sinorhizobium meliloti** biofilms were next treated with chitinase, which caused the dispersal of a pre-formed 24-h-old Rm1021 biofilm, more than twofold over the control (Fig. 6A). Numerous cells were released from the biofilm after 90 min of chitinase treatment, resulting in large areas of the surface that were bacteria-free. After 180 min of chitinase treatment, the biofilm had completely dispersed. These data show that the structure of the biofilm is broken down by chitinase, which is consistent with NF composition.

Core NF is similar in structure to chitosan, which has been reported to promote *Escherichia coli* CSH57 adhesion by making the microbial surface more hydrophobic, thereby enhancing biofilm formation (Goldberg et al., 1990). Two different assays demonstrated that the wild-type strains are more hydrophobic than the Nod- mutan strains. For example, in the salt aggregation test (Honda et al., 1983), both Rm1021 (Fig. 6B) and RCR2011 (Fig. 6C) aggregated at a lower concentration of ammonium sulphate than the Nod- mutants. Treatment with 10 μM luteolin enhanced aggregation of the wild-type strains, but no increase in aggregation was observed for the nodulation-defective *S. meliloti* mutant (Fig. 6B and C).

**Discussion**

Taken together, the data presented herein indicate that NF is critical for establishing a mature rhizobial biofilm. This is a new function for NF, and is distinctly different from the established role as a morphogen for inducing legume nodule development. The involvement of core NF in biofilm establishment has been hitherto unrecognized in part because of the prior emphasis on the signalling functions of rhizobial NF (Ardourel et al., 1994). We propose that the biofilm function may reflect an earlier evolutionary development, as this property is encoded by genes common to all nodulating rhizobia, including *Burkholderia* and *Cupriavidus* strains, the so-called...
β-rhizobia (Moulin et al., 2001), whereas the host-specific nod genes needed for the signalling function vary depending on the rhizobial species. In addition, plant-derived activators, such as luteolin, are not required for S. meliloti biofilm formation, further supporting the hypothesis that this function is more ancestral or primitive. Apparently, some change in rhizobial behaviour, brought about by contact to either abiotic surfaces or to roots, leads to the expression of the common nod genes. Supporting this is the fact that S. meliloti containing nodC–lacZ fusions, when tested in microtiter plates for β-galactosidase activity, turned blue even without luteolin addition (data not shown). Ongoing research may identify the factors important for manifesting this change.

Lending support to the idea that core NF may play a structural role, perhaps by associating with the bacterial cell surface, is the fact that we could not rescue the monolayer biofilms established by Nod− rhizobia by adding purified NF (data not shown). Similarly, NF addition does not restore a wild-type phenotype to Nod+ S. meliloti (Hirsch et al., 1993), although it triggers the beginnings of nodule development on alfalfa, which is very sensitive to NF application (Truchet et al., 1991). This lack of rescue may be characteristic of rhizobia that nodulate...
indeterminate nodule-forming legumes, because purified NF does not rescue the aberrant phenotype of a *R. leguminosarum* bv. *viciae nod*EnoD double mutant either (Walker and Downie, 2000). On the other hand, adding NF rescues the *Nod* phenotype of mutants of *Rhizobium NGR234* and *Bradyrhizobium japonicum*, which nodulate determinate nodule-forming legumes (Relic et al., 1993). These results suggest that for rhizobia interacting with indeterminate nodule-forming plants, NF must be associated in some way with the rhizobial cell surface to complement both the biofilm and nodulation phenotypes of *Nod* mutants. If NF is not localized to the cell surface, as is the case for exogenous NF, it is incompatible with cell-to-cell contact and subsequent rhizobial invasion.

Core NF’s importance has been previously thought of only in terms of its being a backbone for host-specific determinants. However, core NF is critical for root colonization and biofilm formation in that it holds the rhizobia together until a threshold population density is achieved and sufficient host-specific signalling NF is synthesized to act as a morphogen. It may also protect non-spore forming prokaryotes, such as rhizobia from desiccation, especially in the absence of a host legume, by facilitating the adherence of cells together in a biofilm on soil particles or on non-host roots. Moreover, core NF’s involvement in biofilm formation may shield attached rhizobia from host defence reactions, in a manner similar to PIA, which inhibits the defence mechanisms of human innate immunity (Vuong et al., 2004).

Interestingly, molecules similar to core NF are involved in non-signalling functions in other bacteria. For example, *N*-acetylglucosamines are reported to act as adhesins not only in *Staphylococcus* species (Heilmann et al., 1996; Götz, 2002), but also in *Caulobacter crescentus* (Merker and Smit, 1988; Ong et al., 1990). In the latter, *N*-acetylglucosamines are localized to the holdfast of the stalked cells, promoting cell-to-cell adhesion (rosette formation) and adherence to abiotic surfaces. Based on the fact that *N*-acetylglucosamines function as adhesins in a number of bacteria and on our results showing the importance of core NF for biofilm formation, we propose that core NF plays a similar role in *S. meliloti*.

The realization that core NF has dual functions, both as a structural component of the biofilm and independently as a precursor to host-specific morphogens, implies the likely existence of two different sets of control mechanisms, one luteolin-dependent and the other luteolin-independent, which regulate NF production. Also, based on our data, it is very likely that NodD1 probably regulates the expression of other genes, exclusive of *nod*, which are essential for biofilm formation. A fruitful area of future research will be to tease these systems apart, and thus arrive at an understanding of the factors that separately regulate production of the structural and morphogenic components.

### Experimental procedures

#### Strains and plasmids

Bacterial strains and plasmids used for the biofilm analysis are listed in Table 1. Triparental matings were performed as described (Figurski and Helinski, 1979).

#### Construction of the *nodA-gfp* transcriptional fusion

A 400 bp Eagl–Sacl fragment of the *S. meliloti nodA* promoter was ligated into the multicloning site of the broad host-range promoter-GFP vector, pPROBE-AT (Miller et al., 2000). This fragment includes the *nod* box, to which the NodD transcriptional activators bind, and 81 bp of the *nodA* gene. The resulting plasmid, pNF2, was transformed into the chemically competent *E. coli* strain, DH5α (Sambrook and Russell, 2001). Plasmid pNF2 was conjugated into *S. meliloti* strain RCR2011 by triparental mating, using DH5α (pRK2013) as the helper plasmid (Figurski and Helinski, 1979).

#### Biofilm preparation

Biofilms were established as described (Fujishige et al., 2006a) and grown for 24 h before staining with crystal violet. Each data point is the average of at least 18 wells. Error bars indicate the standard deviation from the mean. Root biofilms were prepared (Fujishige et al., 2006c) and harvested 48–72 h post inoculation with either wild-type or *nod* mutant bacteria. The strains constitutively expressed GFP. Attached cells were quantified by counting clus (Fujishige et al., 2006c). Error bars indicate the standard deviation from the mean.

#### β-Galactosidase activity

Biofilms were grown for 48 h in 96 well plates as previously described (Fujishige et al., 2006a). Biofilm and planktonic cells carrying a *nodC–lacZ* transcriptional fusion (Mulligan and Long, 1985) were grown in supplemented Rhizobium defined medium (RDM) culture medium (Fujishige et al., 2006a) with added solvent (methanol) or 10 μM luteolin dissolved in methanol. β-Galactosidase-specific activity of biofilm and planktonic cells was measured as described in Stanley et al. (2003). To reprise, 80 μl of planktonic bacteria were removed carefully from each well. The biofilms were gently rinsed three times with sterile RDM to remove the planktonic cells, and the biofilm cells were scraped from the wells into RDM. The wells were vigorously washed to remove all bacteria (wells were subsequently stained with crystal violet, as described above, to verify that bacteria were thoroughly removed). Biofilm cells were dispersed by extensive vortexing. To quantify cell number, the *OD*₅₉₅ of the separate preparations of planktonic and biofilm cells was measured. The planktonic or biofilm cells were mixed with 100 μl of Z-buffer (60 mM Na₂HPO₄, 4 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄ and 50 mM β-mercaptoethanol). To each of these samples, 1 μl of SDS (1% w/v) and 2 μl of chloroform were added. Reactions were pre-incubated at 28°C for 5 min, and then 20 μl of ONPG (4 mg ml⁻¹) were added to each sample. Reactions were stopped by the addition of 50 μl of Na₂CO₃. Samples were centrifuged for 2 min at 8000 g. The *OD*₅₉₅ of the supernatants were measured, and Miller units were calculated as follows:

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Transmission electron microscopy

Twenty-four-hour-old biofilm cells were scraped off glass coverslips and resuspended in PBS, pH 7.4. A drop of each suspension was placed on a carbon-coated grid, and the bacteria were allowed to settle. The cells on the grid were negatively stained with 2% uranyl acetate for 35-60 s, and subsequently examined under a JEOL-100CX transmission electron microscope.

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Supplementary material

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