Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*

Zhiqiang Qin,^{1†} Yuanzhu Ou,^{1†} Liang Yang,² Yuli Zhu,¹
Tim Tolker-Nielsen,² Soeren Molin² and Di Qu¹

Correspondence

Soeren Molin
sm@biocentrum.dtu.dk

¹Key Laboratory of Medical Molecular Virology of Ministry of Education and Public Health, Institutes of Biomedical Sciences and Medical Microbiology, Shanghai Medical School of Fudan University Box 228, Yi Xue Yuan Road 138#, Shanghai 200032, P. R. China

²BioCentrum-DTU, Building 301, Technical University of Denmark, DK-2800 Lyngby, Denmark

Staphylococcus epidermidis has become a serious nosocomial pathogen frequently causing infections associated with implanted foreign materials. Biofilm formation is considered a major factor determining *S. epidermidis* pathogenicity in such device-associated infections. Here, evidence is presented that extracellular DNA is important for the initial phase of biofilm development by *S. epidermidis* on polystyrene or glass surfaces under static or hydrodynamic conditions. Comparative PCR amplification from *S. epidermidis* chromosomal and extracellular DNA indicated that the extracellular DNA is similar to chromosomal DNA. Experiments involving the *S. epidermidis* wild-type and an isogenic *atlE* mutant indicated that most of the extracellular DNA in *S. epidermidis* cultures and biofilms is generated through activity of the autolysin AtlE. The presented results suggest that extracellular DNA is generated in *S. epidermidis* populations through AtlE-mediated lysis of a subpopulation of the bacteria, and that the extracellular DNA promotes biofilm formation of the remaining population.

Received 14 January 2007

Revised 7 February 2007

Accepted 9 February 2007

INTRODUCTION

Bacteria in natural environments predominantly live in biofilms, i.e. surface-attached microbial communities embedded in a self-produced extracellular matrix (Costerton *et al.*, 1995). The extracellular polymeric substance (EPS) matrix, which can constitute up to 90% of the biofilm biomass (Flemming *et al.*, 2000), is a complex mixture of exopolysaccharides, proteins, DNA and other macromolecules (Sutherland, 2001). The EPS matrix plays a structure-stabilizing role in biofilms, and it provides protection against some antibiotics and host-defence activities (Mah & O'Toole, 2001; Stewart & Costerton, 2001).

In recent years, coagulase-negative *Staphylococcus epidermidis* has become a serious nosocomial pathogen, frequently causing infections associated with implanted foreign materials (Rupp & Archer, 1994). Biofilm formation is a major factor determining *S. epidermidis* pathogenicity in such device-associated infections (Rupp *et al.*, 2001). *S. epidermidis* biofilm formation has been described as a two-step process (Gotz *et al.*, 2000). The first stage involves attachment of cells to a surface (initial attachment

phase). The second stage includes cell–cell aggregation and the formation of a multilayered architecture (accumulative phase). Much attention has been focused on the polysaccharide intercellular adhesion (PIA) component of the EPS matrix of *S. epidermidis*, which is considered a major cell-to-cell interconnecting compound during biofilm formation (Heilmann *et al.*, 1996a; Mack, 1999). However, it is possible that other matrix components may be important for biofilm development of *S. epidermidis*, such as extracellular DNA, which has been shown to be important for biofilm formation of *Pseudomonas aeruginosa*, *Streptococcus intermedius* and *Streptococcus mutans* (Whitchurch *et al.*, 2002; Nemoto *et al.*, 2003; Petersen *et al.*, 2004, 2005; Allesen-Holm *et al.*, 2006). In *P. aeruginosa* biofilms, extracellular DNA functions as a cell–cell interconnecting compound and structural component, and its production has been shown to be regulated via quorum sensing (Allesen-Holm *et al.*, 2006).

In this study, we show that extracellular DNA is present in cultures and biofilms of *S. epidermidis* strains under different growth conditions. The extracellular DNA is found as a major component required for initial bacterial attachment to surfaces, as well as for the subsequent early phase of biofilm development by *S. epidermidis*. Moreover, evidence is presented that release of extracellular DNA from *S. epidermidis* is mainly caused by the activity of the autolysin AtlE.

†These authors contributed equally to this work.

Abbreviations: CLSM, confocal laser scanning microscopy; DDAO, 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one); EPS, extracellular polymeric substance; PI, propidium iodide.

Table 1. Bacterial strains, plasmids and primers used in the present studyAbbreviations: *erm*, erythromycin; ts, temperature sensitive.

Strains and plasmids	Description	Source or reference
Strains		
Se886	Biofilm-positive clinical isolate	This study
Se698	Biofilm-positive clinical isolate	This study
Se340	Biofilm-positive clinical isolate	This study
Se527	Biofilm-positive clinical isolate	This study
SeRP62A	Biofilm-positive laboratory strain	Christensen <i>et al.</i> (1982)
Se1457	Biofilm-positive laboratory strain	Mack <i>et al.</i> (1992)
Se1457 Δ <i>atlE</i>	<i>atlE</i> : : <i>erm</i> derivative of Se1457	This study
Se1457 Δ <i>atlE</i> (pRC21)	<i>atlE</i> complementary strain	This study
Se1457 Δ <i>atlE</i> (pRB473)	<i>atlE</i> mutant carrying the control vector	This study
Plasmids		
pBT2	ts <i>Escherichia coli</i> / <i>Staphylococcus</i> shuttle vector	Bruckner (1997)
pBT <i>atlE</i>	ts vector for allelic gene replacement of <i>atlE</i> in <i>S. epidermidis</i>	This study
pRC21	Plasmid pRB473 carrying full fragment of <i>atlE</i> gene	Heilmann <i>et al.</i> (1997)
Primers		
	Restriction	Sequence (5'–3')*
Primers used for allelic gene replacement		
up <i>atlE</i> R1	<i>Kpn</i> I	CGGGGTACCTACTTACCGACTTCACCAA
up <i>atlE</i> L1	<i>Xba</i> I	GCTCTAGA TAACAGAACAGGATGGATTG
down <i>atlE</i> R1	<i>Hind</i> III	CCCAAGCTTAGAATTAACCTTTGGTGTATAT
down <i>atlE</i> L1	<i>Nhe</i> I	CTAGCTAGCATTACAAATTACCG TCTATGG
Primers used for identification of extracellular DNA derivation		
<i>trpSR</i> 1		ATGGAACCTTTATTCTCAGGAATTCAGCC
<i>trpSL</i> 1		TTATCTTTTTTCGACCTAAGCCCATTGC
<i>srrAR</i> 1		ATGACTA ACGAAATTT
<i>srrAL</i> 1		CTATTTAGTCGGTTCA
<i>sigBR</i> 1		CTGTCTTGCAGCTTCTTGTAAT
<i>sigBL</i> 1		ATGGCGAAAAGATCGAA ATCAG
<i>isaAR</i> 1		AAAAAACAGTTATCGCTTCTA
<i>isaAL</i> 1		CTTTATAAGCGGCATTGATT
<i>yycFR</i> 1		ATGACTAACGAAATTT
<i>yycFL</i> 1		CTATTTAGTCGGTTCA

*Underlined type shows the restriction site.

METHODS

Bacterial strains, plasmids and growth media. All the bacterial strains and plasmids used in this study are listed in Table 1, of which Se886, Se698, Se340 and Se527 are clinical strains isolated from different patients with implanted catheter infection in Zhongshan Hospital (Shanghai, China). Tryptic soy broth (TSB; Oxoid) medium containing 0.25 % glucose was used for biofilm formation in the static chamber system. AB medium (Clark & Maaløe, 1967) supplemented with 0.3 mM glucose and 3 % TSB was used for biofilm cultivation in the flow-chamber system. Biofilms and batch cultures were grown at 37 °C, unless otherwise indicated. DNase I (Sigma) was used at a concentration of 2 mg ml⁻¹ in medium supplemented with MgCl₂ (5 mM). SYTO 9 and propidium iodide (PI) (Live_Dead reagents, Molecular Probes) were used at a concentration of 1 µM for staining live or dead bacteria in biofilms. DDAO [7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)] (Molecular Probes) was used at a concentration of 1 µM to stain extracellular DNA in biofilms. Antibiotics were used at the following concentrations: erythromycin, 10 µg ml⁻¹; chloramphenicol, 10 µg ml⁻¹; ampicillin, 100 µg ml⁻¹.

Bacterial initial attachment assays. Initial cell attachment was tested as described elsewhere (Heilmann *et al.*, 1996b). Briefly, cell suspensions from mid-exponential phase were diluted to OD₆₀₀=0.1 in PBS, and then incubated in wells (1 ml per well) of cover-glass cell-culture chambers (Nunc) for 30 min at 37 °C, after which attached cells were identified by microscopy.

Construction of the *S. epidermidis* 1457 Δ *atlE* mutant. The *atlE* gene in *S. epidermidis* 1457 was inactivated via homologous recombination using the temperature-sensitive shuttle vector pBT2, as described elsewhere (Bruckner, 1997; Vuong *et al.*, 2000). An *Xba*I/*Hind*III-digested erythromycin-resistance cassette (*erm*) from plasmid pEC1 was inserted into the pBT2 plasmid, upon which PCR-amplified regions flanking the *atlE* gene were cloned into the plasmid to construct the plasmid pBT2*ΔatlE*. Sequences of the primers are listed in Table 1. Proper cloning of the *erm* fragment and *atlE* flanking regions in plasmid pBT2*ΔatlE* was verified by direct sequencing. *S. epidermidis* 1457 was transformed by electroporation with plasmid pBT*ΔatlE*, and a procedure promoting allelic displacement of the *atlE* gene was performed as described elsewhere (Bruckner, 1997).

Proper allelic displacement of the *atlE* gene was verified by direct sequencing.

Measurement of extracellular DNA in microtitre plate cultures. Overnight cultures grown in TSB medium containing 0.25 % glucose were diluted to $OD_{600}=0.001$ in AB medium supplemented with 0.5 % glucose, 0.05 mM PI and 10 % TSB. The diluted cultures were transferred to wells of polystyrene microtitre plates (150 μ l per well) and incubated for 24 h at 37 °C, upon which PI absorbance was measured at 480 nm and cell density was measured by OD_{600} using a Wallac microtitre plate reader.

Extraction of *S. epidermidis* genomic DNA and extracellular DNA, and PCR. Bacteria were grown to late-exponential phase ($OD_{600}=2.0$) in 5 ml TSB medium at 37 °C, and were then harvested by centrifugation. Bacterial pellets were resuspended in 500 μ l buffer containing 25 % sucrose, 10 mM Tris/HCl (pH 7.5) and 0.2 mg lysostaphin (Sigma-Aldrich). After incubation at 37 °C for 30 min, bacterial genomic DNA was extracted with phenol/chloroform-isoamyl alcohol, precipitated by ethanol and resuspended in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). Culture samples from late-exponential phase were centrifuged and the supernatant was filtered (0.2 μ m pore-size), after which 500 μ l filtered supernatant was transferred to a new Eppendorf tube. The extracellular DNA was precipitated with ethanol and resuspended in TE buffer. Primers for amplification of *trpS* (SERP0575, 569 129–570 118), *srrA* (SERP1055, 1 101 714–1102439), *sigB* (SERP1677, 1 722 805–1 723 575), *isaA* (SERP2138, 2 164 774–2 165 481) and *yycF* (SERP2534, 2 591 085–2 591 786) were designed based on the *S. epidermidis* strain RP62A genomic sequence (GenBank accession no. CP000029) from the National Center for Biotechnology Information (NCBI) genome database (<http://www.ncbi.nlm.nih.gov>). All primer sequences are listed in Table 1. All PCR reactions were performed using *Taq* DNA polymerase (TaKaRa). The PCR reaction was performed on a T3 Thermocycler (Biometra): 45 s at 95 °C, 45 s at 52 °C and 1 min at 72 °C for 28 cycles.

Cultivation of *S. epidermidis* biofilms

In polystyrene microtitre plates. Biofilm cultivation in polystyrene microtitre plates was carried out essentially as described by Christensen *et al.* (1985). Briefly, overnight cultures of *S. epidermidis* strains grown in TSB (0.25 % glucose) medium were diluted 1 : 200. The diluted cultures were transferred to wells of polystyrene microtitre plates (200 μ l per well) and incubated at 37 °C for 24 h. At different time points (0, 6 and 12 h), DNase I (2 mg ml⁻¹, Sigma-Aldrich) was added to the wells. After incubation, the wells were washed gently three times with 200 μ l sterile PBS, air-dried and stained with 2 % crystal violet for 5 min. Then, the plate was rinsed under running tap water, air-dried, the crystal violet was redissolved in ethanol and the absorbance was determined at 590 nm.

In the static chamber system. Biofilms were grown in cover-glass cell-culture chambers (Nunc) as described previously (Jager *et al.*, 2005; Qin *et al.*, 2007). Briefly, overnight cultures of *S. epidermidis* strains grown in TSB (0.25 % glucose) medium were diluted to $OD_{600}=0.001$, then inoculated into wells of a chamber (1.5 ml per well) and incubated at 37 °C. After that, the chamber was washed gently four times with 1 ml sterile PBS, then stained by SYTO 9, PI or DDAO for 15 min and observed under the microscope.

In the flow-chamber system. Biofilms were grown in a flow chamber with individual channel dimensions of 1 × 4 × 40 mm. The flow-chamber system was assembled and prepared as described previously (Moller *et al.*, 1998). The flow chambers were inoculated by injecting 350 μ l overnight culture diluted to $OD_{600}=0.001$ into each flow channel with a small syringe. After inoculation, flow channels were

left without flow for 1 h, after which medium flow (0.2 mm s⁻¹) was started using a Watson-Marlow 205S peristaltic pump.

Microscopy and image acquisition. All microscopic observations and image acquisition were performed with a Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena) equipped with detectors and filter sets for monitoring SYTO 9, PI and DDAO fluorescence. Images were obtained using a ×63/1.4 objective or a ×40/1.3i objective. Simulated 3D images and sections were generated using the IMARIS software package (Bitplane).

Cell autolysis assays. Autolysis assays for *S. epidermidis* strains were performed as described by Brunskill & Bayles (1996). Cell samples (50 ml) were collected from exponential-phase cultures growing in TSB medium ($OD_{580}=0.7$) containing 1 M NaCl, and cells were pelleted by centrifugation. The cells were washed twice with 50 ml ice-cold water and resuspended in 50 ml 0.05 M Tris/HCl (pH 7.2) containing 0.05 % (v/v) Triton X-100. The cells were then incubated at 30 °C with shaking, and OD_{580} was measured at 30 min intervals.

RESULTS

Effects of DNase I on biofilm formation of *S. epidermidis*

The effect of DNase I on biofilm formation by *S. epidermidis* in the wells of polystyrene microtitre plates was investigated with six different strains. Strains 1457 and RP62A are reference strains, whereas strains Se886, Se698, Se340 and Se527 are clinical isolates from infected patients. When DNase I (2 mg ml⁻¹) was added to the medium prior to inoculation of the microtitre plates, biofilm formation of all six *S. epidermidis* strains was severely reduced (Fig. 1a). When DNase I was added to established 6 h microtitre-plate-grown biofilms, the majority of the *S. epidermidis* biofilms were affected to some extent by the DNase I treatment, whereas the biofilm formed by strain Se886 appeared not to be affected by the DNase I treatment (Fig. 1a). Established 12 h microtitre-plate-grown biofilms of all the *S. epidermidis* strains appeared resistant to DNase I treatment (Fig. 1a). The presence of RNase (DNase-free, 2 mg ml⁻¹) or heat-inactivated DNase I (2 mg ml⁻¹) did not inhibit biofilm formation (Fig. 1a), indicating that inhibition of biofilm formation by DNase I was not due to a non-specific protein effect. A titration experiment showed that the lower limit for inhibition of biofilm formation by DNase I in the microtitre plate assay was ~0.1 mg DNase I ml⁻¹ (Fig. 1b). The addition of DNase I to growing cultures had no observable effect on the growth rate for any of the *S. epidermidis* strains (data not shown).

Effects of DNase I on initial attachment of *S. epidermidis*

S. epidermidis biofilm formation is a two-step process consisting of initial cell attachment and an accumulative phase (Mack, 1999; Gotz, 2002). We performed a microscopic examination of the effect of DNase I on attachment of *S. epidermidis* in a static chamber setup. After 30 min incubation under static conditions without DNase I,

Fig. 1. (a) Effect of nuclease activity on biofilm formation in polystyrene microtitre plates of various *S. epidermidis* strains. Cultures were grown in TSB medium for 24 h at 37 °C without DNase I, with DNase I (2 mg ml^{-1}) added initially and after 6 and 12 h, with RNase (2 mg ml^{-1}) added initially, or with heat-inactivated DNase I (2 mg ml^{-1}) as a control added initially. (b) Effects of various concentrations of DNase I on biofilm formation in polystyrene microtitre plates of *S. epidermidis* 1457. Cultures were grown in TSB medium for 24 h at 37 °C with DNase I added initially. The biofilm biomass was quantified using a crystal violet assay. The values are means of eight replicates, and the error bars indicate SD.

Fig. 2. Surface attachment of *S. epidermidis* 1457 in static chambers containing medium without DNase I (a) or with DNase I (b). Bars, 20 μm .

numerous *S. epidermidis* 1457 cells had attached to the chamber surface (Fig. 2a). However, in chambers supplemented with DNase I, attachment of *S. epidermidis* 1457 to the surface was significantly reduced (Fig. 2b), indicating that extracellular DNA plays a role in the initial bacterial attachment. Heat-inactivated DNase I was found not to prevent *S. epidermidis* cell attachment (data not shown). Similar results were obtained for *S. epidermidis* strains RP62A, Se886, Se698, Se340 and Se527 (data not shown).

Comparative PCR amplification from chromosomal and extracellular *S. epidermidis* DNA

In order to investigate the nature of the *S. epidermidis* extracellular DNA, we purified extracellular DNA and chromosomal DNA from late-exponential-phase cultures of *S. epidermidis*, and performed comparative PCR. The genes *ycyF*, *trpS*, *srrA*, *sigB* and *isaA*, which are located in different regions of the *S. epidermidis* RP62A chromosome (GenBank accession no. CP000029; Gill *et al.*, 2005), could all be amplified by PCR with both chromosomal and extracellular DNA as template (Fig. 3), indicating that the extracellular DNA is similar to chromosomal DNA.

Role of AtlE in DNA release and biofilm formation of *S. epidermidis*

Heilmann *et al.* (1997) have reported that *atlE*, encoding a major autolysin, is necessary for primary attachment and biofilm development of *S. epidermidis* in the wells of microtitre trays. It was shown that the AtlE protein is located at the cell surface of *S. epidermidis*, but the use of antibodies failed to provide conclusive evidence that the AtlE protein is directly involved in mediating primary attachment. It is therefore possible that AtlE plays an

Fig. 3. PCR of purified *S. epidermidis* chromosomal DNA (C) or extracellular DNA (E) with primers targeting the *ycyF*, *trpS*, *srrA*, *sigB* and *isaA* genes. After PCR, the amplification products were electrophoresed on an agarose gel.

indirect role in *S. epidermidis* biofilm formation by promoting the release of extracellular DNA. In order to investigate the role of *AtlE* in DNA release and biofilm formation, we constructed an *S. epidermidis* 1457 *atlE* mutant (Δ *atlE*). The mutation was confirmed by Southern blotting and direct sequencing (data not shown). The amounts of extracellular DNA in cultures of the Δ *atlE* and parental strains were measured by PI staining in microtitre plates, as described previously by Allesen-Holm *et al.* (2006). Compared with the level in cultures of the 1457 wild-type strain, the amount of extracellular DNA in cultures of the Δ *atlE* strain was dramatically decreased (Fig. 4a). In addition, the Δ *atlE* strain was deficient in biofilm formation in the microtitre plates (Fig. 4a). A complemented strain, Δ *atlE*(pRC21), was capable of releasing extracellular DNA and forming biofilm at levels

similar to that of the wild-type strain (Fig. 4a), whereas the vector control strain Δ *atlE*(pRB473) released extracellular DNA and formed biofilm at levels similar to that of the Δ *atlE* mutant (Fig. 4a). The Δ *atlE* mutant grew at almost the same growth rate as its parental strain (Fig. 4b), indicating that the differences in extracellular DNA release and biofilm formation could not be attributed to differences in growth rate.

Occurrence of extracellular DNA in *S. epidermidis* biofilms

In a previous study we used DDAO to stain extracellular DNA in *P. aeruginosa* biofilms, because of its excellent fluorescent properties and high sensitivity (Allesen-Holm *et al.*, 2006). Here, we used DDAO to stain extracellular DNA, and SYTO 9 to stain bacterial cells in *S. epidermidis* biofilms grown under static or hydrodynamic conditions. The spatial distribution of cells and extracellular DNA in the stained biofilms was visualized by confocal laser scanning microscopy (CLSM).

DDAO staining and subsequent CLSM indicated that extracellular DNA surrounded the wild-type adhering cells in the initial phase of biofilm development in the static chambers (Fig. 5a). However, extracellular DNA was not detected on the surface of the few adhering cells of the Δ *atlE* strain (Fig. 5c). After 24 h in the static chamber, the 1457 wild-type had formed small microcolonies which contained large amounts of extracellular DNA (Fig. 5b), whereas the Δ *atlE* strain had formed scattered small clumps with low amounts of extracellular DNA (Fig. 5d). The Δ *atlE*(pRC21) strain formed microcolonies containing large amounts of extracellular DNA similar to wild-type microcolonies (Fig. 5e, f).

Two-day-old flow-chamber-grown 1457 wild-type biofilms had a mean thickness of $\sim 40\ \mu\text{m}$ and contained large amounts of extracellular DNA (Fig. 6a, b). Two-day-old flow-chamber-grown Δ *atlE* biofilms contained scattered small microcolonies with a mean thickness of $< 10\ \mu\text{m}$, and only low amounts of extracellular DNA (Fig. 6c, d). The Δ *atlE*(pRC21) strain formed biofilms with extracellular DNA similar to that of wild-type biofilms (Fig. 6e, f).

Occurrence of dead cells in *S. epidermidis* biofilms

In addition to extracellular DNA, DDAO staining will also stain dead cells. It is therefore likely that part of the observed DDAO staining is due to the presence of dead cells in the biofilms. In order to investigate whether dead cells were present in the *S. epidermidis* biofilms we employed PI staining. Although PI can be used to map the location of extracellular DNA in biofilms if the sensitivity of the CLSM microscope is set to a very high level (Allesen-Holm *et al.*, 2006), PI will visualize dead cells (which are permeable and have a very high DNA concentration) when normal settings of the microscope are

Fig. 4. (a) Relative amounts of extracellular DNA per OD₆₀₀ unit (black bars), and relative amounts of biofilm (grey bars), in microtitre plate cultures of *S. epidermidis* 1457 wild-type, *atlE*, *atlE*(pRC21) and *atlE*(pRB473). The cultures were grown for 24 h in minimal medium supplemented with 0.05 mM PI, whereupon PI absorbance (A_{480}) and cell density (OD₆₀₀) were measured. The biofilm biomass was quantified using a crystal violet assay. The values are means of eight replicates, and the error bars indicate SD. (b) Growth curves of the *S. epidermidis* 1457 wild-type and *atlE* mutant cultivated in TSB batch cultures.

Fig. 5. Biofilms of *S. epidermidis* 1457 wild-type (a, b), $\Delta atlE$ (c, d) and $\Delta atlE(pRC21)$ (e, f) were grown in static chambers with TSB medium for 1 h (a, c, e) or 24 h (b, d, f), and then stained with SYTO 9 (green) and DDAO (red), upon which microscopic investigation was performed by the use of CLSM. The green and red signals are shown in the same micrographs, whereby cells surrounded by extracellular DNA appear yellow. Bars, 20 μm .

used (Haagensen *et al.*, 2007). With appropriate settings of the CLSM microscope, the Live_Dead reagents (SYTO 9 and PI) will therefore stain live cells green and dead cells red.

Live_Dead staining indicated that virtually all of the attached cells [1457 wild-type, $\Delta atlE$ strain and $\Delta atlE(pRC21)$ strain] in newly inoculated flow chambers were alive (data not shown). Live_Dead staining in two-day-old flow-chamber-grown *S. epidermidis* biofilms suggested that the microcolonies formed by the wild-type contained dead cells in the centre (Fig. 7a), whereas the small cell aggregates formed by the $\Delta atlE$ strain contained almost no dead cells (Fig. 7b); the microcolonies formed by the $\Delta atlE(pRC21)$ strain contained dead cells in the centre similar to the wild-type microcolonies (Fig. 7c).

Autolysis of *S. epidermidis* 1457 wild-type and $\Delta atlE$ mutant cells

Because our experiments indicated that the presence of extracellular DNA in *S. epidermidis* cultures and biofilms is mainly due to AtlE-mediated cell lysis, we compared autolysis of the *S. epidermidis* 1457 wild-type and $\Delta atlE$ strains. As demonstrated in Fig. 8, the $\Delta atlE$ cells exhibited a much lower rate of autolysis than those of the parental strain, confirming that AtlE has autolytic activity in our *S. epidermidis* strain.

DISCUSSION

In the present study we provide evidence that extracellular DNA is a major component contributing to initial

Fig. 6. Biofilms of *S. epidermidis* 1457 wild-type (a, b), $\Delta atlE$ (c, d) and $\Delta atlE(pRC21)$ (e, f) were grown for 2 days in flow chambers irrigated with minimal medium, and were then stained with SYTO 9 and DDAO, upon which microscopic investigation was performed by CLSM. Cells (green) and extracellular DNA (red) are shown in separate micrographs acquired at the same locations. The central pictures show top-down views, and the flanking pictures show side views. Bars, 50 μ m.

attachment of *S. epidermidis* to both plastic and glass surfaces. DNase I treatment prevented attachment and biofilm formation of *S. epidermidis*, whereas RNase treatment and the presence of heat-inactivated DNase I had no effect. Extracellular DNA was found to be abundant in *S. epidermidis* biofilms under both static and hydrodynamic conditions. Because extracellular DNA can function as a cell-to-cell connector (Whitchurch *et al.* 2002; Nemoto *et al.*, 2003; Petersen *et al.*, 2004, 2005), it is likely that extracellular DNA also has a role in the accumulative phase of *S. epidermidis* biofilm formation. In support of this hypothesis, about half of the biofilm biomass was dispersed when DNase I was added to six-hour-old *S. epidermidis* biofilms (except for one out of the six strains tested). However, DNase I treatment could not disperse more mature biofilms of *S. epidermidis*, indicating that extracellular DNA may be most critical as a matrix component

in the initial phase of *S. epidermidis* biofilm formation. The possibility that proteases or macromolecules which may interfere with DNase I activity are present in mature *S. epidermidis* biofilms has not been investigated. Preliminary experiments indicated that addition of exogenous DNA to microtitre-plate cultures did not stimulate biofilm formation (data not shown), indicating that the DNA has to be released directly from the cells in order to stimulate biofilm formation. However, further investigations are needed before firm conclusions can be drawn on this issue.

Comparative PCR analysis suggested that the extracellular DNA present in *S. epidermidis* cultures is similar to genomic DNA, which is in accordance with the hypothesis that it originates from lysis of a small subpopulation of the *S. epidermidis* bacteria. DNA release from *S. epidermidis* appears to be mainly mediated by the autolysin protein

Fig. 7. Biofilms of *S. epidermidis* 1457 wild-type (a), $\Delta atlE$ (b) and $\Delta atlE(pRC21)$ (c) were grown for 2 days in flow chambers irrigated with minimal medium, and were then stained with SYTO 9 and PI, upon which microscopic investigation was performed by CLSM. The central pictures show horizontal optical sections, and the flanking pictures show side views. Live cells appear green and dead cells appear yellow. Bars, 50 μ m.

AtlE, since inactivation of *atlE* reduced DNA release by more than 90%. Because AtlE has autolytic activity, it is likely that AtlE activity results in lysis of a small fraction of the bacteria, resulting in the production of extracellular DNA which promotes surface attachment of the remaining population. AtlE-mediated cell lysis may also occur in established biofilms, as extracellular DNA was found in biofilms of the *S. epidermidis* wild-type but not in microcolonies of the *atlE* mutant. Similar mechanisms for extracellular DNA production have been reported for *Streptococcus pneumoniae*, *Acinetobacter calcoaceticus* and *P. aeruginosa* (Steinmoen *et al.*, 2002; Palmen & Hellingwerf, 1995; Allesen-Holm *et al.*, 2006).

The dead cells observed in the centre of wild-type microcolonies in flow-chamber-grown *S. epidermidis* biofilms may be connected to AtlE-mediated cell lysis if the kinetics of the process are such that the intermediary dead cells are present for some time before they lyse. This would be in accordance with the fact that virtually no dead cells were observed in biofilms formed by the *atlE* mutant. However, the fact that no dead cells were detected in the initial phase of biofilm development of the wild-type indicates that AtlE-mediated lysis is a fast process, where intermediary dead cells are present only for a very short time. It is possible therefore that AtlE-mediated DNA release occurs in *S. epidermidis* biofilms without the presence of detectable amounts of dead cells. Although the dead cells in the

centre of the microcolonies in *S. epidermidis* wild-type biofilms are anticipated to eventually lyse and contribute to the pool of extracellular DNA in the biofilm, the process may not be connected to AtlE activity. The finding that the wild-type biofilms contained more dead cells than the *AtlE* biofilms could be related to the differences in microcolony size, which may in turn result in different nutritional conditions and different concentrations of waste products.

There may be factors other than AtlE involved in DNA release from *S. epidermidis*. Recently, another autolysin protein was identified in *S. epidermidis*, Aae, which has bacteriolytic activity and adhesive properties, although its role in DNA release remains to be investigated (Heilmann *et al.*, 2003). More recently, Resch *et al.* (2005) have reported the presence of phage activities in both planktonic and biofilm cells of *Staphylococcus aureus*, and phages were present over a much longer period in biofilm cultures than in planktonic supernatants. It is reasonable to believe that phage release resulting in cell lysis may occur during biofilm formation of *S. epidermidis* as well. We have found a locus in the genome of *S. epidermidis* which is homologous to the *comE* locus (*comEA*, *comEB* and *comEC*) reported to be associated with genetic transformation and DNA release in *Bacillus subtilis* (Inamine & Dubnau, 1995). However, further investigation is needed to elucidate the role of this locus in *S. epidermidis*.

Biofilm formation and extracellular DNA release have been reported to be regulated by quorum sensing in *P. aeruginosa* (Davies *et al.*, 1998; Allesen-Holm *et al.*, 2006). In *S. epidermidis* it has been reported that the expression of AtlE and biofilm formation are increased in an *agr* quorum-sensing mutant (Vuong *et al.*, 2003), implying that AtlE-mediated DNA release may be regulated by the *agr* quorum-sensing system. Although quorum sensing may be a common denominator in the regulation of DNA release in biofilms of *P. aeruginosa* and *S. epidermidis*, the available evidence suggests that it has opposite effects in the two organisms.

Extracellular DNA has now been shown to play a role in biofilm formation for representatives of the genera *Pseudomonas* (Whitchurch *et al.*, 2002; Nemoto *et al.*, 2003), *Streptococcus* (Petersen *et al.*, 2004, 2005) and *Staphylococcus* (this study). Because virtually all bacterial populations generate extracellular DNA (e.g. Lorenz *et al.*, 1991), and because most bacterial species bind to DNA (e.g.

Fig. 8. Autolysis of *S. epidermidis* 1457 wild-type (◆) and $\Delta atlE$ (■) strains in medium with Triton X-100.

Dubnau, 1999), extracellular DNA may be a common matrix component in bacterial biofilms.

ACKNOWLEDGEMENTS

We thank Professor Friedrich Götz, University of Tübingen, for providing plasmids pRC21 and pRB473, and Janus Haagenen for help with confocal microscopy. This work was supported by the State Key Program of Basic Research of China (973) (2002CB512803), the Hi-Tech Program of China (863) (2004AA223080), the Scientific Technology Development Foundation of Shanghai (055407069), the China International Science and Technology Cooperation Projects funded by the Ministry of Science and Technology of China (2003DFB00023, 2006DFA32760), and the Danish Research Councils. Z. Q. was supported by the Danida fellowship during his visit at BioCentrum-DTU.

REFERENCES

- Allesen-Holm, M., Barken, K. B., Yang, L., Klausen, M., Webb, J. S., Kjelleberg, S., Molin, S., Givskov, M. & Tolker-Nielsen, T. (2006). A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Mol Microbiol* **59**, 1114–1128.
- Bruckner, R. (1997). Gene replacement in *Staphylococcus carnosus* and *Staphylococcus xylosus*. *FEMS Microbiol Lett* **151**, 1–8.
- Brunskill, E. W. & Bayles, K. W. (1996). Identification and molecular characterization of a putative regulatory locus that affects autolysis in *Staphylococcus aureus*. *J Bacteriol* **178**, 611–618.
- Christensen, G. D., Simpson, W. A., Bisno, A. L. & Beachey, E. H. (1982). Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect Immun* **37**, 318–326.
- Christensen, G. D., Simpson, W. A., Younger, J. J., Baddour, L. M., Barrett, F. F., Melton, D. M. & Beachey, E. H. (1985). Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol* **22**, 996–1006.
- Clark, J. D. & Maaløe, O. (1967). DNA replication and the cell cycle in *Escherichia coli* cells. *J Mol Biol* **23**, 99–112.
- Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R. & Lappin-Scott, H. M. (1995). Microbial biofilms. *Annu Rev Microbiol* **49**, 711–745.
- Davies, D. G., Parsek, M. R., Pearson, J. P., Iglewski, B. H., Costerton, J. W. & Greenberg, E. P. (1998). The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* **280**, 295–298.
- Dubnau, D. (1999). DNA uptake in bacteria. *Annu Rev Microbiol* **53**, 217–244.
- Flemming, H. C., Wingender, J., Griegbe, T. & Mayer, C. (2000). Physicochemical properties of biofilms In *Biofilms: Recent Advances in their Study and Control*, pp. 19–34. Edited by L. V. Evans. Amsterdam: Harwood Academic Publishers.
- Gill, S. R., Fouts, D. E., Archer, G. L., Mongodin, E. F., Deboy, R. T., Ravel, J., Paulsen, I. T., Kolonay, J. F., Brinkac, L. & other authors (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *J Bacteriol* **187**, 2426–2438.
- Gotz, F. (2002). Staphylococcus and biofilms. *Mol Microbiol* **43**, 1367–1378.
- Gotz, F., Heilmann, C. & Cramton, S. E. (2000). Molecular basis of catheter associated infections by staphylococci. *Adv Exp Med Biol* **485**, 103–111.
- Haagenen, J. A., Klausen, M., Ernst, R. K., Miller, S. I., Folkesson, A., Tolker-Nielsen, T. & Molin, S. (2007). Differentiation and distribution of colistin- and sodium dodecyl sulfate-tolerant cells in *Pseudomonas aeruginosa* biofilms. *J Bacteriol* **189**, 28–37.
- Heilmann, C., Gerke, C., Perdreau-Remington, F. & Gotz, F. (1996a). Characterization of Tn917 insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. *Infect Immun* **64**, 277–282.
- Heilmann, C., Schweitzer, O., Gerke, C., Vanittanakom, N., Mack, D. & Gotz, F. (1996b). Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Mol Microbiol* **20**, 1083–1091.
- Heilmann, C., Hussain, M., Peters, G. & Gotz, F. (1997). Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. *Mol Microbiol* **24**, 1013–1024.
- Heilmann, C., Thumm, G., Chhatwal, G. S., Hartleib, J., Uekotter, A. & Peters, G. (2003). Identification and characterization of a novel autolysin (Aae) with adhesive properties from *Staphylococcus epidermidis*. *Microbiology* **149**, 2769–2778.
- Inamine, G. S. & Dubnau, D. (1995). ComEA, a *Bacillus subtilis* integral membrane protein required for genetic transformation, is needed for both DNA binding and transport. *J Bacteriol* **177**, 3045–3051.
- Jager, S., Mack, D., Rohde, H., Horstkotte, M. A. & Knobloch, J. K. (2005). Disintegration of *Staphylococcus epidermidis* biofilms under glucose-limiting conditions depends on the activity of the alternative sigma factor σ^B . *Appl Environ Microbiol* **71**, 5577–5581.
- Lorenz, M. G., Gerjets, D. & Wackernagel, W. (1991). Release of transforming plasmid and chromosomal DNA from two cultured soil bacteria. *Arch Microbiol* **156**, 319–326.
- Mack, D. (1999). Molecular mechanisms of *Staphylococcus epidermidis* biofilm formation. *J Hosp Infect* **43** (Suppl.), S113–S125.
- Mack, D., Siemssen, N. & Laufs, R. (1992). Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for functional relation to intercellular adhesion. *Infect Immun* **60**, 2048–2057.
- Mah, T. F. & O'Toole, G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9**, 34–39.
- Moller, S., Sternberg, C., Andersen, J. B., Christensen, B. B., Ramos, J. L., Givskov, M. & Molin, S. (1998). In situ gene expression in mixed-culture biofilms: evidence of metabolic interactions between community members. *Appl Environ Microbiol* **64**, 721–732.
- Nemoto, K., Hirota, K., Murakami, K., Taniguti, K., Murata, H., Viducic, D. & Miyake, Y. (2003). Effect of Varidase (streptodornase) on biofilm formed by *Pseudomonas aeruginosa*. *Chemotherapy* **49**, 121–125.
- Palmen, R. & Hellingwerf, K. J. (1995). *Acinetobacter calcoaceticus* liberates chromosomal DNA during induction of competence by cell lysis. *Curr Microbiol* **30**, 7–10.
- Petersen, F. C., Pecharki, D. & Scheie, A. A. (2004). Biofilm mode of growth of *Streptococcus intermedius* favored by a competence-stimulating signaling peptide. *J Bacteriol* **186**, 6327–6331.
- Petersen, F. C., Tao, L. & Scheie, A. A. (2005). DNA binding-uptake system: a link between cell-to-cell communication and biofilm formation. *J Bacteriol* **187**, 4392–4400.
- Qin, Z., Yang, X., Yang, L., Jiang, J., Ou, Y., Molin, S. & Qu, D. (2007). Formation and properties of in vitro biofilms of *ica*-negative *Staphylococcus epidermidis* clinical isolates. *J Med Microbiol* **56**, 83–93.

- Resch, A., Fehrenbacher, B., Eisele, K., Schaller, M. & Gotz, F. (2005).** Phage release from biofilm and planktonic *Staphylococcus aureus* cells. *FEMS Microbiol Lett* **252**, 89–96.
- Rupp, M. E. & Archer, G. L. (1994).** Coagulase-negative staphylococci: pathogens associated with medical progress. *Clin Infect Dis* **19**, 231–243.
- Rupp, M. E., Fey, P. D., Heilmann, C. & Gotz, F. (2001).** Characterization of the importance of *Staphylococcus epidermidis* autolysin and polysaccharide intercellular adhesin in the pathogenesis of intravascular catheter-associated infection in a rat model. *J Infect Dis* **183**, 1038–1042.
- Steinmoen, H., Knutsen, E. & Havarstein, L. S. (2002).** Induction of natural competence in *Streptococcus pneumoniae* triggers lysis and DNA release from a subfraction of the cell population. *Proc Natl Acad Sci U S A* **99**, 7681–7686.
- Stewart, P. S. & Costerton, J. W. (2001).** Antibiotic resistance of bacteria in biofilms. *Lancet* **358**, 135–138.
- Sutherland, I. W. (2001).** The biofilm matrix – an immobilized but dynamic microbial environment. *Trends Microbiol* **9**, 222–227.
- Vuong, C., Gotz, F. & Otto, M. (2000).** Construction and characterization of an *agr* deletion mutant of *Staphylococcus epidermidis*. *Infect Immun* **68**, 1048–1053.
- Vuong, C., Gerke, C., Somerville, G. A., Fischer, E. R. & Otto, M. (2003).** Quorum-sensing control of biofilm factors in *Staphylococcus epidermidis*. *J Infect Dis* **188**, 706–718.
- Whitchurch, C. B., Tolker-Nielsen, T., Ragas, P. C. & Mattick, J. S. (2002).** Extracellular DNA required for bacterial biofilm formation. *Science* **295**, 1487.

Edited by: P. E. Kolenbrander