Evaluation of the type I signal peptidase as antibacterial target for biofilm-associated infections of Staphylococcus epidermidis

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The development of antibacterial resistance is inevitable and is a major concern in hospitals and communities. Moreover, biofilm-grown bacteria are less sensitive to antimicrobial treatment. In this respect, the Gram-positive Staphylococcus epidermidis is an important source of nosocomial biofilm-associated infections. In the search for new antibacterial therapies, the type I signal peptidase (SPase I) serves as a potential target for development of antibacterials with a novel mode of action. This enzyme cleaves off the signal peptide from secreted proteins, making it essential for protein secretion, and hence for bacterial cell viability. S. epidermidis encodes three putative SPases I (denoted Sip1, Sip2 and Sip3), of which Sip1 lacks the catalytic lysine. In this report, we investigated the active S. epidermidis SPases I in more detail. Sip2 and Sip3 were found to complement a temperature-sensitive Escherichia coli lepB mutant, demonstrating their in vivo functional activity. In vitro functional activity of purified Sip2 and Sip3 proteins and inhibition of their activity by the SPase I inhibitor arylomycin A2 were further illustrated using a fluorescence resonance energy transfer (FRET)-based assay. Furthermore, we demonstrated that SPase I not only is an attractive target for development of novel antibacterials against free-living bacteria, but also is a feasible target for biofilm-associated infections.

INTRODUCTION

Bacterial infections cause over 13 million deaths each year, making them the second-leading cause of death worldwide. In this respect, an important threat is the increasing problem of antibacterial resistance (Cohen, 2000; Yoneyama & Katsumata, 2006). Moreover, the biofilm mode of growth renders bacteria less susceptible to antibacterial treatment and, as a consequence, biofilm-based infections can rarely be resolved (Hall-Stoodley et al., 2004).

Biofilms protect bacteria from hostile conditions and play a central role in the pathogenesis of serious infections, such as medical device-related infections (Schulin & Voss, 2001). Due to the widespread use of implanted medical devices, the biofilm-producing Gram-positive coagulase-negative Staphylococcus epidermidis has emerged as a common cause of life-threatening foreign body-associated blood infections in immunocompromised patients (O’Gara & Humphreys, 2001).

Due to this growing number of hard-to-treat bacterial infections, the future availability of new antibacterials with a novel mode of action is highly desirable and antibacterials that target biofilm-embedded bacteria are desperately needed. An attractive novel antibacterial target is the type I signal peptidase (SPase I), which plays a vital role in protein secretion, an essential process for cell viability. SPases I are integral membrane-bound serine endopeptidases and are indispensable for transport and maturation of secretory proteins, as they cleave off the N-terminal signal peptide during or shortly after translocation across the cytoplasmic membrane (Dalbey et al., 1997). Bacterial SPases I belong to a special group of serine proteases, which use a catalytic Ser-Lys dyad mechanism (Black, 1993; Tschantz et al., 1993). The Escherichia coli SPase I is the best characterized, and several crystal structures (Paetzel et al., 1998, 2002, 2004) and solution NMR data have been published (Musial-Siwek et al., 2008). SPase I
cannot be inhibited by standard protease inhibitors (Black & Bruton, 1998), and only few compounds are so far known to inhibit bacterial SPase I (Allsop et al., 1996; Black & Bruton, 1998; Bruton et al., 2003; Kulanthaivel et al., 2004; Paetzel et al., 2004). However, until now, it has remained unclear whether the bacterial SPase I is also a favourable target in biofilm-based infections.

In this work, the functional activity of \textit{S. epidermidis} Sip2 and Sip3 was evidenced \textit{in vivo} and \textit{in vitro}. Inhibition of their \textit{in vitro} activity by the known lipopeptide SPase I inhibitor arylomycin A\textsubscript{2} (Paetzel et al., 2004) was tested using a fluorescence resonance energy transfer (FRET)-based assay. A second part of this study deals with the question of whether SPase I is also a potential target in biofilm-based infections. Therefore, the effect of arylomycin A\textsubscript{2} on preformed \textit{S. epidermidis} biofilms was investigated.

**METHODS**

**Bacterial strains, media and growth conditions.** \textit{E. coli} TG1 (Sambrook et al., 1989) served as host for cloning purposes and \textit{E. coli} BL21(DE3)pLysS (Studier & Moffatt, 1986) was used for the overproduction of target proteins. These strains were routinely grown at 37 \textdegree C (300 r.p.m.) in Luria–Bertani (LB) broth (Miller, 1972) supplemented, when applicable, with ampicillin (50 \textmu g ml\textsuperscript{-1}) and/or chloramphenicol (25 \textmu g ml\textsuperscript{-1}).

Leader peptidase (LepB) complementation assay using \textit{E. coli} IT89 (Inada et al., 1989) was performed as described by Cregg et al. (1996). The OD\textsubscript{595} was recorded at 30 min intervals for 7.5 h in 24-well plates (1 ml total volume).

For biofilm formation experiments, cultures were grown in brain heart infusion (BHI) (BD Diagnostic Systems BBL) in a shaking incubator at 37 \textdegree C. Strains used were \textit{S. epidermidis} 1457, a biofilm-positive clinical isolate (Mack et al., 1992), and \textit{S. epidermidis} RP62A (ATCC 35984), a slime-producing \textbeta-lactamase-secreting strain isolated from a patient with intravascular catheter-associated sepsis (Christensen et al., 1982).

**RNA techniques**

RT-PCR. For transcriptional analysis, RT-PCR was carried out using primer pairs Sip1Hisf and Sip1r, Sip2Hisf and Sip2r, Sip3Hisf and Sip3r, and Sip1Hisf and Sip2r (Supplementary Table S1), as previously described (Lammertyn et al., 2004), on 100 ng total RNA using the Access RT-PCR system (Promega). Total RNA was isolated from an \textit{S. epidermidis} 1457 culture grown to late-exponential growth phase, as described by Vandecasteele et al. (2002).

**DNA techniques**

Standard DNA manipulations. DNA manipulations were carried out using standard techniques (Sambrook et al., 1989). Oligonucleotides and plasmids used in this study are listed in Supplementary Tables S1 and S2, respectively.

**Cloning of sip genes in \textit{E. coli}.** The \textit{S. epidermidis} sip2 and sip3 genes were amplified by PCR using \textit{S. epidermidis} strain 1457 chromosomal DNA as template. After cloning the PCR-amplified DNA fragments in pgEM-T Easy (Promega), DNA sequences were verified. For \textit{E. coli} LepB complementation experiments, pEX50Sip2 and pEX50Sip3 were constructed by cloning the respective PCR-amplified sip genes after the tac promoter in the low-copy-number plasmid pEX50. In order to overproduce \textit{S. epidermidis} SPases, the expression vectors pET3aSip2 and pET3aSip3 were constructed by cloning the respective PCR-amplified sip genes behind the T7 promoter in pET3a (Novagen).

**Protein expression and analysis**

Overproduction, solubilization and purification of Sip proteins in \textit{E. coli}. For expression of Sip2 and Sip3, \textit{E. coli} BL21(DE3)pLysS was transformed with pET3aSip2 and pET3aSip3, respectively, grown at 37 \textdegree C and induced with 1 mM IPTG as described by Studier et al. (1990). From a 600 ml IPTG-induced culture, cells were harvested 4 h post-induction. Sip2 and Sip3 enzymes were purified by extraction of membrane proteins as described previously (Geukenks et al., 2002) and loaded on a Ni\textsuperscript{2+}–NTA Sepharose (IBA) column as outlined in the QIAexpressionist (Qiagen). Purity was analysed by SDS-PAGE using 12.5 % (w/v) resolving gels (Laemmli, 1970) and Coomassie brilliant blue (CBB) staining.

**In vitro functional activity of purified Sip proteins and inhibition by the lipopeptide SPase I inhibitor arylomycin A\textsubscript{2}**. The design and validation of the fluorescent quenched synthetic peptide (Dabcy1)AGHDAHASET(EDANS) and the FRET-based assay are described elsewhere (Bockstael et al., 2009). Briefly, in this fluorescence assay, following 5 min pre-incubation of the SPase with or without arylomycin A\textsubscript{2} in assay buffer [50 mM Tris/HCl, pH 8.0, 0.5 % (v/v) Triton X-100] at 37 \textdegree C, the reaction was initiated by the addition of peptide substrate. Control reactions, respectively without SPase, without arylomycin A\textsubscript{2}, and with DMSO instead of inhibitor, were included. The fluorescence emission signal was measured at 510 nm as a function of time following excitation at 340 nm.

**Susceptibility testing under planktonic growth conditions.** The MICs of arylomycin A\textsubscript{2} (Basilea Pharmaceutica) and rifampicin (Aventis Pharma) for the \textit{S. epidermidis} strains were determined using the broth microdilution assay in LB broth following the standard procedure (NCCLS, 2003). The MIC was defined as the lowest concentration of antibacterial that resulted in the complete inhibition of visible growth after 16 h incubation at 37 \textdegree C.

**Biofilm formation.** Biofilm experiments were performed in Cellstar polystyrene 24-well cell culture plates (Greiner Bio-One). Wells were inoculated with 1 ml of a 1:50-diluted overnight culture (~5 \times 10\textsuperscript{7} c.f.u. ml\textsuperscript{-1}) and control wells were filled with BHI medium. Plates were incubated at 37 \textdegree C without shaking. Stock solutions of arylomycin A\textsubscript{2} and rifampicin (10 \mu g ml\textsuperscript{-1} in DMSO) were made and diluted in BHI broth to concentrations corresponding to 30 \times MIC and 300 \times MIC (less than 5 % DMSO final concentration). Following 8 and 24 h of biofilm formation, the supernant (containing non-adhered cells) was removed from each well and plates were gently rinsed twice with 1 ml PBS (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4). Subsequently, 1 ml fresh medium containing arylomycin A\textsubscript{2} or rifampicin was added to each well and plates were further incubated for 16 h at 37 \textdegree C. Positive controls were untreated biofilms grown in 1 ml fresh medium, containing the same amount of DMSO as the test samples. Negative controls were BHI alone without added bacteria. After 16 h incubation (leading to 24 and 40 h biofilms), the supernatant was removed and the wells were gently rinsed twice with 1 ml PBS.

**Biofilm quantification**

**Crystal violet (CV) assay.** \textit{S. epidermidis} biofilm biomass formation was quantified by using the microtitre plate assay first described by Christensen et al. (1985) and modified as follows. Briefly, 1 ml 0.1 % (w/v) CV was added to the wells after rinsing. After 10 min, the excess CV was removed and wells were rinsed twice with distilled water.
Finally, bound CV was released by adding 1 ml 33% (v/v) acetic acid (ChemLab). Samples were diluted 1:10 in 33% (v/v) acetic acid and absorbance was measured at 590 nm.

**Resazurin assay.** Using a filter-sterilized 0.01% aqueous solution of resazurin (Sigma), the metabolic activity of treated and untreated biofilms was quantified with a slight modification of the method of Pettit et al. (2005). Briefly, after rinsing, 1 ml PBS was added to each well followed by addition of 50 ml resazurin solution. Plates were gently shaken and incubated for 1 h at 37 °C. After gently shaking again, absorbance at 570 and 600 nm was measured. Negative controls included media plus drug concentrations equal to experimental wells. The percentage reduction of resazurin was calculated using the formula described by Pettit et al. (2005).

**Biofilm c.f.u. counting.** Viable counts were obtained from the same wells that were used in the resazurin assay. After obtaining resazurin absorbance readings, wells were rinsed with PBS and scaped thoroughly for dilution plating. Well contents were removed and 100 μl of 10-fold dilutions in PBS was plated on BHI agar. Colonies were counted manually after 24 h incubation at 37 °C.

**Biofilm imaging by confocal laser scanning microscopy (CLSM).** Aliquots (3 ml) of 1:50 diluted overnight culture were used to grow S. epidermidis 1457 biofilms on 35 mm glass-bottomed culture dishes coated with poly-D-lysine (MatTek). Biofilm formation was achieved as described above. At the end of the experiment, medium was removed and dishes were rinsed once with 2 ml PBS. Wheatgerm agglutinin (WGA), which binds to polysaccharide intracellular adhesin (PIA), conjugated with fluorescent Alexa Fluor 633 (Molecular Probes) (Strathmann et al., 2002) was used to study matrix production in biofilms (staining red). A 160 μl volume of a 500 μg ml⁻¹ working solution of WGA in PBS was carefully applied directly on top of the biofilm. After incubation for 30 min in the dark at room temperature, excess staining solution was removed by washing twice with PBS. Staining of bacterial cells within the biofilm was performed by use of the fluorescent DNA-binding stain SYTO 9 (Molecular Probes) for living bacteria (staining green) and SYTOX orange (Molecular Probes) for dead bacteria (staining yellow). Subsequent to WGA staining, the biofilms were treated with 160 μl freshly prepared staining solution containing 5 μM SYTO 9 and 5 μM SYTOX orange in PBS. Biofilms were incubated for 30 min in the dark at room temperature and excess staining solution was removed by washing once with PBS. Arylomycin A₂-treated and untreated biofilms were examined on a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems). Images were obtained using an HCX PL APO × 63 [numerical aperture (NA) 1.20] water immersion lens and an HC PL APO CS × 20 (NA 0.70) lens. Images were recorded at 476 nm excitation and 479–538 nm emission wavelengths for SYTO 9 (green channel), 561 nm excitation and 566–615 nm emission wavelengths for SYTOX orange (yellow channel), and at 633 nm excitation and 652–752 nm emission wavelengths for WGA–Alexa Fluor 633 (red channel). Digital images were recorded using the standard Leica software (Leica LAS AF version 2.0).

**β-Lactamase secretion assay.** Secreted β-lactamase activity was measured using a modified version of the nitrocefin assay (Novick, 1991). Briefly, after 24 and 40 h of biofilm formation, culture medium was removed from each well and pelleted by centrifugation. A 100 μl volume of supernatant was loaded onto a flat-bottomed transparent polystyrene microtitre plate (Nunclon), and 100 μl of 0.1 mM nitrocefin (Oxoid) in 50 mM phosphate buffer, pH 7.0, with 1 mM EDTA was added to start the reaction. Hydrolysis of nitrocefin was immediately monitored by recording A486 at 10 min intervals for 1 h at 37 °C. The secreted β-lactamase activity was calculated based upon ΔA486 and converted into μmol nitrocefin hydrolysed min⁻¹ ml⁻¹ using a molar extinction coefficient for hydrolysed nitrocefin of 20 500 M⁻¹ cm⁻¹ at 486 nm.

**Measurement of fluorescence and absorbance signals.** Fluorescence and absorbance measurements were performed on an Infinite M200 automated microplate reader (Tecan).

**Statistical analysis.** In vitro biofilm quantification assays were analysed by Student’s t test (Excel, Microsoft). Two-sided P≤0.05 was considered to be significant.

## RESULTS AND DISCUSSION

### Type I signal peptidases in S. epidermidis

It has already been reported that S. epidermidis (ATCC 12228) contains three sip-like genes (Zhang et al., 2003), which have been designated sip1, sip2 and sip3 (GenBank accession number AE015929, loci SE_0660, SE_0662 and SE_2397, respectively). RT-PCR experiments showed that sip1 and sip2 belong to the same operon (data not shown), in analogy with spoA and spoB, which are also co-transcribed on the chromosome of *Staphylococcus aureus* (Cregg et al., 1996).

It is noteworthy that the Lys residue of Box D, known to be essential for catalytic activity in SPases I, is not present in Sip1. It could therefore be concluded that sip1 most probably encodes an inactive enzyme, because Sip1 cannot catalyse the hydrolysis of peptide bonds to a physiologically significant extent, reminiscent of SpsA of *S. aureus*, which has recently proven to be inactive (Kavanaugh et al., 2007). Therefore, we focused in this work only on the active S. epidermidis Sip2 and Sip3 enzymes. *S. epidermidis* Sip2 shows high homology with *S. aureus* SpoSb (90% similarity). However, one of the key differences between *S. aureus* and *S. epidermidis* with respect to SPases is the presence of a third SPase (Sip3) in *S. epidermidis*. In bacteria having multiple SPases, the individual enzymes may only be required for the processing of a particular group of proteins. Construction of mutants could give further insights into whether one sip gene by itself is sufficient for cell viability in *S. epidermidis* or whether both Sip2 and Sip3 have to be inactivated simultaneously for the bacteria to become non-viable. However, this is beyond the scope of this paper. It has already been proven that SPase activity in general is essential for bacterial viability (van Roosmalen et al., 2004), as also shown below. Furthermore, when looking for an antibiotic agent, compounds targeting both SPases can be found, as exemplified by the inhibition of both enzymes by arylomycin A₂.

### Expression and functional analysis of S. epidermidis sip2 and sip3 genes in *E. coli*

**In vivo** functional activity of *S. epidermidis* Sip2 and Sip3 was assessed by complementation of the temperature-sensitive (Ts) LepB mutant of *E. coli* IT89 (Inada et al., 1989). *E. coli* IT89 has an amber mutation in the *lepB* gene at codon 39 rendering pre-protein processing temperature-
sensitive. The strain shows normal growth at 27 °C, but stops growing at the non-permissive temperature of 42 °C. Growth can be restored by complementation of the mutant lepB with plasmid-borne active SPase I (Inada et al., 1989). For this purpose, E. coli IT89 was transformed with pEX50Sip2, pEX50Sip3 or with the control plasmid pEX50. Growth of E. coli IT89(pEX50Sip2), E. coli IT89(pEX50Sip3) and E. coli IT89(pEX50) at the non-permissive temperature of 42 °C was monitored by measuring OD_{595} as a function of time for 7.5 h in the absence of IPTG.

Fig. 1(a) shows that both Sip2 and Sip3 of S. epidermidis support growth at the non-permissive temperature, demonstrating that S. epidermidis Sip2 and Sip3 correctly insert into the E. coli inner membrane and that they can process all E. coli proteins necessary for cell viability. However, the growth rate of E. coli IT89(pEX50Sip2) was retarded compared with that of E. coli IT89(pEX50Sip3). Induction with 1 mM IPTG did not change this (data not shown). The expression levels of S. epidermidis Sip2 and Sip3 in E. coli IT89 were analysed by immunoblotting using a Ni\(^{2+}\)-NTA conjugate (HisDetector Western blot kit, AP Colorimetric, KPL) and the chromogenic substrate solution NBT/BCIP (Roche). A protein of approximately 23 kDa was detected in the E. coli IT89 strains harbouring pEX50Sip2 and pEX50Sip3 that was not present in the control (Fig. 1b).

The presence of a faint band for Sip2 on the immunoblot together with the retarded cell growth rate could indicate that there is less expression of Sip2 than Sip3 in the lepB mutant. The lower level of Sip2 expression and hence lower level of Sip2 complementation of the lepB mutation in comparison with Sip3 results in the observed growth retardation. Another explanation for the retarded growth of the Sip2-complemented strain could be that Sip2 was not able to fully complement E. coli LepB, most likely because Sip2 is not capable of (efficiently) processing all proteins necessary for viability in E. coli.

Since E. coli IT89 is known to easily revert to the wild-type through a back-mutation in the TAG amber termination codon in the lepB gene, we verified that the sequence of the mutant lepB gene was not changed during the course of the experiment. In all cases, sequencing showed the TAG amber termination codon to be still present. Therefore, we can conclude that growth of E. coli IT89(pEX50Sip2) and E. coli IT89(pEX50Sip3) was due to functional production of catalytically active S. epidermidis Sip2 and Sip3.

### Overproduction and purification of the S. epidermidis Sip proteins

The two active SPases I of S. epidermidis, Sip2 and Sip3, were purified and tested for their in vitro functional activity. The His-tagged Sip2 and Sip3 were overproduced in E. coli BL21(DE3)pLysS using the pET system. The Sip proteins were His-tagged at the N terminus to facilitate purification. After IPTG induction, His–Sip2 and His–Sip3 enzymes were observed as clear protein bands on SDS-PAGE with CBB staining, both with molecular masses of ~23 kDa (data not shown). Immunoblotting confirmed the production of the two His-tagged proteins (data not shown). For the purification of the His-tagged Sip2 and Sip3 proteins, membrane fractions were isolated from E. coli BL21(DE3)pLysS cells producing the respective Sip protein. From the clarified membrane protein extracts, the His-tagged Sip proteins were purified by native affinity chromatography (data not shown).

### In vitro functional activity of purified Sip proteins and inhibition by the lipohexapeptide SPase I inhibitor arylomycin A₂

The validated peptide (Dabcyl)AGHDAHASET(EDANS), based on the C-terminal region of the S. epidermidis pre-SceD protein and containing the native SPase I cleavage site (Bockstael et al., 2009), was used to assess the in vitro functional activity of purified Sip2 and Sip3. Growth curve of E. coli IT89 transformants containing the plasmids pEX50 (control), pEX50Sip2 and pEX50Sip3. Cultures were grown at 42 °C and OD_{595} was monitored as a function of time. Immunoblot analysis of the expression of His-tagged S. epidermidis Sip2 and Sip3 in E. coli IT89 using a Ni\(^{2+}\)-NTA conjugate and the chromogenic substrate solution NBT/BCIP. Proteins present in total cell lysates of E. coli IT89 cultures grown at 42 °C for 7.5 h were separated on a 12.5% (w/v) SDS-polyacrylamide gel. Lanes: 1, E. coli IT89(pEX50Sip2); 2, E. coli IT89(pEX50Sip3); 3, E. coli IT89(pEX50) (control).
SPases are the only target of arylomycin A2. Inhibition by substrate binding site. Therefore, one could infer that from a mutation in a conserved region of the SPase due to the natural resistance of these bacteria, resulting in Sip2 and Sip3 and by E. coli LepB, as detected by both HPLC and fluorescence spectrometry. The two Sip enzymes are also able to process a purified preprotein, confirming their enzymatic activity.

The lipohexapeptide arylomycin A2 is one of the few compounds so far known to inhibit bacterial SPase I, as shown by Paetzel et al. (2004), since the reported X-ray crystal structure of arylomycin A2 in complex with the E. coli SPase I Δ2-75 establishes the mechanism of action of this antibiotic. In addition, it was recently reported by Romesberg (2009) that the insufficient potency of arylomycin A2 in vivo against some bacterial strains could be due to the natural resistance of these bacteria, resulting from a mutation in a conserved region of the SPase substrate binding site. Therefore, one could infer that SPases are the only target of arylomycin A2. Inhibition by arylomycin A2 of processing of the fluorogenic peptide by Sip2 and Sip3 (Fig. 2) indicates that these enzymes indeed employ a Ser/Lys catalytic dyad mechanism. For this reason, we decided to use arylomycin A2 in the experiments described below to investigate whether SPase I is a possible target for bacteria in biofilms.

Biofilm formation and quantification in the presence of an SPase I inhibitor

In an attempt to answer the question whether SPase I is a feasible novel antibacterial target to treat biofilm-associated infections, we investigated the effect of the known SPase I inhibitor arylomycin A2 on 8 and 24 h-old S. epidermidis biofilms and compared this with the effect of rifampicin. Rifampicin, a transcription inhibitor, ranks as one of the most effective of the few antibacterial agents that are active against biofilm-related infections. This is probably due to its advantageous physico-chemical and structural properties (e.g. hydrophobicity and size), which allow rifampicin to penetrate the protective exopolysaccharide matrix. In addition, its intracellular target, the RNA polymerase, seems to be a favourable target and of particular importance for survival in biofilms (Villain-Guillot et al., 2007). Quantification of the antibacterial activity of both compounds against biofilms was done by using simple methods for in vitro biofilm quantification. Although not much is known about the diffusion of arylomycin A2 through an S. epidermidis biofilm, we do know that it exhibits very potent antibacterial activity against free-living S. epidermidis cells (Roberts et al., 2007). The MIC values against planktonic S. epidermidis 1457 and RP62A are 0.235 and 0.058 μg ml⁻¹ for arylomycin A2 and 0.0017 and 0.0001 μg ml⁻¹ for rifampicin, respectively. Since the antibiotic concentration required to kill bacteria in a biofilm is 100–1000 times higher than that needed to kill their planktonic counterparts (Donlan & Costerton, 2002) and in view of the limited availability of the antibiotics, concentrations of 30× and 300× the MIC values of arylomycin A2 and rifampicin were used to determine their effect on biofilm formation.

CV assay

CV-staining is the standard method to quantify total biofilm biomass (Christensen et al., 1985). CV binds to negatively charged surface molecules and polysaccharides in the extracellular matrix (Li et al., 2003). Because both living and dead cells, as well as matrix, are stained with CV, this method provides no information about viability (Pitts et al., 2003). Fig. 3 shows the relative biofilm formation compared with untreated control samples obtained after CV-staining of untreated and treated biofilms. Experiments were performed on both S. epidermidis strains, namely S. epidermidis 1457 (Fig. 3a) and RP62A (Fig. 3b), with strain 1457 displaying higher biofilm-forming capacity under the conditions used. Eight and 24 h-old biofilms were treated with a control solution, arylomycin A2 or rifampicin. At 16 h after incubation, resulting in 24 and 40 h-old biofilms, the addition of both arylomycin A2 and rifampicin resulted in a decrease in absorbance after CV-staining for S. epidermidis 1457 and RP62A. Therefore, it can be concluded for both strains that treatment with

**Fig. 2.** Continuous fluorometric assay showing the inhibition of the in vitro activity of Sip2 (left) and Sip3 (right) by the lipohexapeptide SPase I inhibitor arylomycin A2 (arylo). FL, fluorescence intensity.
Arylomycin A₂ or rifampicin resulted in a similar dose-dependent biofilm reduction compared with untreated biofilms.

**Resazurin assay and biofilm viable cell counts**

To discriminate between living and dead or metabolically inactive cells, a quantification method based on the chemical reduction of resazurin by metabolically active cells was used. This method has already been applied in susceptibility testing of *S. epidermidis* (Pettit *et al.*, 2005). Resazurin, the main component of Alamar Blue, is a blue redox indicator that can be reduced by viable bacteria in the biofilm to pink resorufin, which is fluorescent (O’Brien *et al.*, 2000). Continued growth maintains a reduced environment (pink), while inhibition of growth maintains an oxidized environment (blue). The extent of conversion from blue to pink is a reflection of cell viability (Pettit *et al.*, 2005) and gives an estimate of the effect of antibacterial agents. Another common method of assessing bacterial biofilm susceptibility is quantification of treated versus untreated biofilms by conventional plating for the determination of viable cell counts (c.f.u. ml⁻¹) after disruption of the biofilm (Donlan & Costerton, 2002). Since resazurin is non-toxic, cells in the wells could be plated directly after absorbance readings for determination of viable cell counts (Pettit *et al.*, 2005).

Fig. 4 shows the percentage of resazurin reduction and viable cell counts obtained for untreated and treated *S. epidermidis* biofilms. Eight and 24 h-old biofilms were
treated with a control solution, arylomycin A$_2$ or rifampicin. The two susceptibility measurement methods showed good correlation. At 16 h after incubation in the presence of the antibiotic, both arylomycin A$_2$ and rifampicin gave a dose-dependent reduction in cell viability compared with untreated biofilms. However, metabolic activity was not completely suppressed. An important consideration is the high drug concentrations required to eradicate biofilms, so it is conceivable that higher concentrations would completely eradicate the biofilms. With an 8 h-old S. epidermidis 1457 biofilm (Fig. 4a), arylomycin A$_2$ gave a marked reduction in metabolic activity.
activity at both concentrations (fourfold and sixfold reduction for 30 x and 300 x MIC, respectively), which was greater than that due to rifampicin. This was also reflected in a three-log reduction in c.f.u. ml$^{-1}$ at 300 x MIC. The reduction of viability in a 1 day-old S. epidermidis 1457 biofilm was smaller, but still significant at 300 x MIC. Here, the antibacterial activity of rifampicin proved slightly greater. Arylomycin A$_2$ and rifampicin also showed strong suppression of metabolic activity at 30 x and 300 x MIC for S. epidermidis RP62A (Fig. 4b), with their antibacterial activities being similar. However, a severe reduction in c.f.u. ml$^{-1}$ was only seen for rifampicin at 300 x MIC, although resazurin reduction for both compounds at that concentration remained similar.

### Biofilm imaging by CLSM

CLSM was first used to confirm biofilm formation following 8 and 24 h incubation prior to treatment with arylomycin A$_2$ and rifampicin. In both cases the presence of an S. epidermidis biofilm was confirmed by a dense, uniform layer of staphylococcal cells embedded in an extracellular matrix, characteristic for biofilm formation (Sutherland, 2001) (data not shown).

Furthermore, CLSM with live/dead staining was helpful to further elucidate any effects after treatment of 8 and 24 h-old S. epidermidis 1457 biofilms with different concentrations of arylomycin A$_2$. CLSM investigations showed marked differences in a concentration-dependent manner.

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**Fig. 5.** CLSM of a 24 h-old untreated S. epidermidis 1457 biofilm formed on 35 mm glass-bottomed culture dishes coated with poly-D-lysine and a biofilm after 16 h of contact with arylomycin A$_2$ at 30 x MIC (24 h total time). Biofilms grown for 8 h were treated for 16 h with a control solution (a, c) or with arylomycin A$_2$ (b, d). The biofilms were stained with SYTO 9, SYTOX orange and Alexa fluor 633 and investigated using CLSM. (a) $xy$ Plane of untreated biofilm; (b) $xy$ plane of biofilm treated for 16 h with arylomycin A$_2$ (30 x MIC); (c) orthogonal image ($xz$ plane) of untreated biofilm; (d) orthogonal image ($xz$ plane) of biofilm treated for 16 h with arylomycin A$_2$ (30 x MIC). Biofilms were viewed at $\times 20$ (a, b) and $\times 63$ (c, d) magnification.
in the appearance of the biofilms following treatment with arylomycin A2. Fig. 5 highlights 16 h treatment of an 8 h biofilm with arylomycin A2 at 30\( \times \)MIC (Fig. 5b, d), leading to a reduction in the thickness and the density of the biofilm with the formation of holes, and a reduction of the number of viable cells compared with untreated biofilm (Fig. 5a, c). A comparison of Fig. 5(c) with Fig. 5(d) reveals a reduction in biofilm thickness after treatment with arylomycin A2. However, most bacteria were not killed at this concentration and the presence of dead cells (staining yellow) in the treatment group could not be clearly detected. Similar results were obtained for the other tested conditions (data not shown).

Overall, from the results of the biofilm experiments, we can deduce that SPase I must be functionally active and have a role in sessile cells. The effect of the SPase I inhibitor arylomycin A2 resembles the antibacterial effect of rifampicin towards S. epidermidis biofilms, and arylomycin A2 even proves to be slightly more active in young staphylococcal biofilms. However, the effect of rifampicin was slightly greater towards older and thicker biofilms. This might be a consequence of differences in physico-chemical properties between the two antibiotics evaluated here. The relevance of the use of young 8 h biofilms for the investigation of the treatment of biofilms with antibiotics might be questionable, but the results obtained were similar to the results for 24 h biofilms. In addition, the fact that there are still staphylococcal cells present after 16 h exposure to 30\( \times \) and 300\( \times \)MIC of antibiotics also confirms the biofilm nature of the cells, since planktonic cells would have been killed at the high concentrations of antibiotics administered.

Furthermore, immunoblot analysis using anti-Sip2 polyclonal antibodies revealed the presence of Sip2 in one-day-old staphylococcal biofilms (Fig. 6). In addition, the activity of \( \beta \)-lactamase, an SPase I-dependent secreted protein, was detected in the culture medium of S. epidermidis RP62A biofilms by use of a nitrocefin assay, proving the in vivo functional activity of SPases I in S. epidermidis biofilms (Fig. 7). For the non-\( \beta \)-lactamase-secreting S. epidermidis 1457 strain, negligible amounts of \( \beta \)-lactamase were detected.

Altogether, the obtained results are very promising, as they demonstrate that SPase I is a valuable target for the development of antibacterials against biofilm-based infections.

**Conclusions**

Since SPase I is a promising target for the development of antibacterials with a novel mode of action, we investigated the SPases I of S. epidermidis, an important cause of biofilm-associated foreign body infections, as possible targets for the treatment of biofilm-related infections.

Since the use of the SPase I inhibitor arylomycin A2 reduces biofilm formation markedly, it is evident that SPase I is still functional in biofilms, making SPase I a feasible antibacterial target for biofilm-based infections. Furthermore, SpsB from S. aureus has recently been shown to be responsible for the removal of the N-terminal leader of
AgrD in vitro, suggesting a role for SPase I in quorum sensing and biofilm formation (Kavanaugh et al., 2007).

Hence, we conclude that SPase I is an attractive target for the development of new antibacterials not only towards planktonic cells but also, most importantly, towards biofilm-associated cells. Further research in this field should be encouraged, as it could lead to the design or discovery of more active SPase I inhibitors with favourable physico-chemical properties that promote diffusion through staphylococcal biofilms, with a view to the treatment of biofilm-based infections.

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