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SHORT COMMUNICATION

Spontaneous switch to PIA-independent biofilm formation in an *ica*-positive *Staphylococcus epidermidis* isolate

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Abstract

The ability to form biofilms on abiotic surfaces is considered a major step in *Staphylococcus epidermidis* pathogenesis. In the majority of isolates, biofilm formation is mediated by the production of the polysaccharide intercellular adhesin PIA which is synthesized by enzymes encoded by the *ica* operon. Here, we report on a spontaneous switch to proteinaceous biofilm formation in an *S. epidermidis icaC*::IS*256* insertion mutant. Atomic force microscopy analysis of both PIA-dependent and proteinaceous biofilm revealed remarkable differences in biofilm substructures: the PIA-dependent biofilm was characterized by the presence of fibrous, net-like structures which were absent in proteinaceous biofilm. Transcription of *aap*, encoding the accumulation-associated protein Aap, was enhanced in a variant producing proteinaceous biofilm, while transcription of the Bap-homologous protein gene *bhp* was down-regulated. Regulation of PIA-independent biofilm differed from the wild type. Thus, ethanol induced proteinaceous biofilm formation, whereas NaCl abolished PIA-independent biofilm formation completely. The combined data indicate that biofilm formation in *S. epidermidis* is obviously ensured by more than one mechanism suggesting that this life style represents a crucial factor for this organism.

Keywords: *Staphylococcus epidermidis*; Biofilm; PIA; *aap*; *bhp*; Atomic force microscopy

Introduction

During the past decades, staphylococci, especially *Staphylococcus aureus* and *Staphylococcus epidermidis,* have emerged as a serious health problem in hospital settings. The coagulase-negative *S. epidermidis*, normally a harmless inhabitant of the human skin, is an opportunistic pathogen causing infections in immunocompromised and critically ill patients, leading to acute bacteremia and septicemia. Usually, these infections are associated with the use of medical devices.

Virulence of *S. epidermidis* has been linked to the ability to form thick multilayered biofilms on polymer and metal surfaces (Götz, 2002). Within the biofilm, cells are embedded and protected in a slimy extracellular matrix. The main component of *S. epidermidis* extracellular matrix is polysaccharide intercellular adhesin (PIA) which is synthesized by enzymes encoded by the *ica* operon (Heilmann et al., 1996). PIA is a β-(1,6)-linked glucosaminoglycan with a chain length of at least 130 monomers and different side chain substitutions (Mack et al., 1996). However, non-polysaccharide

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compounds such as extracellular teichoic acids (Sadovskaya et al., 2005) and proteins contribute to the extracellular matrix as well. Recently, two proteins involved in PIA-independent biofilm formation of *S. epidermidis* have been identified: the accumulation-associated protein Aap (Hussain et al., 1997; Rohde et al., 2005) and the biofilm-associated protein Bap (Sadovskaya et al., 2005; Tormo et al., 2005).

In previous work we have described biofilm formation in *S. epidermidis* as a phase-variable process (Ziebuhr et al., 1999). Inactivation of biofilm formation can be caused by insertion of the insertion sequence IS*256* into the *ica* operon. Reversion to a biofilm-positive phenotype after repeated passaging is usually accompanied by precise excision of IS*256* from the target sequence, including the initially duplicated 8-bp target sites. Here, we report that reversion to a biofilm-positive phenotype can also occur without precise excision of IS*256* by a switch to an alternative biofilm, mediated by proteinaceous factors.

Material and methods

Bacterial strains and culture conditions

S. epidermidis CSF41498, a cerebrospinal fluid isolate, was kindly provided by Dr. J. O'Gara. CSF41498 is an *ica*-positive, biofilm-forming strain which is free of naturally occurring IS*256* copies in its genome (Conlon et al., 2002). For inactivation of *icaC* by IS*256*, a 2.3-kb *icaC*::IS*256* insert of pIL1 (Loessner et al., 2002) was amplified using the primers icaC-upper-EcoRI (5′-ATAAACTT-GAATTCGTGTATT-3′) and icaC-lower-KpnI (5′-ATAAAACTCTGGTACCATCATT-3′). The fragment was restricted with KpnI and EcoRI and ligated into the shuttle vector pBT2 (Brückner, 1997). The resulting plasmid was transformed into *S. epidermidis* CSF41498 as described elsewhere (Conlon et al., 2002). Allele replacement was achieved after two rounds of growth in tryptic soy broth (TSB) at 42 °C and subsequent plating on Congo red agar (Ziebuhr et al., 1997). Red colonies were selected and allele replacement was confirmed by Southern blot analysis and sequencing of *icaC*. The resulting *S. epidermidis* CSF41498 *icaC*::IS*256* construct was named *S. epidermidis* CSF41498-1. Passaging and search for biofilm-positive revertants were carried out as described previously (Ziebuhr et al., 1999). *S. epidermidis* PV1 is a biofilm-positive derivative of *S. epidermidis* CSF41498-1 resulting from these experiments. For all experiments, bacteria were grown in TSB at 37 °C. When indicated, 3% ethanol, 4% sodium chloride or 1% glucose were added.

Biofilm formation assay

Biofilm formation assays were performed as described previously (Ziebuhr et al., 1997). Each experiment was carried out at least three times and mean values and standard deviations were calculated. Digestion of biofilm by metaperiodate or proteinase K was carried out as described elsewhere (Wang et al., 2004).

Atomic force microscopy (AFM)

Imaging was done on a Nanoscope IIIa atomic force microscope (Digital Instruments) using the tapping mode as described elsewhere (Balsalobre et al., 2003). Samples were prepared as follows: One to three fresh colonies were scraped off from an agar plate and resuspended in 50 µl ultrapure, 0.22-µm filtered water. Alternatively, biofilm was scraped off from the bottom of a tissue culture flask and resuspended; 25 µl suspension were dropped onto a freshly cleaved ruby red mica, incubated at room temperature for 5 min and excess bacteria were washed off with filtered water. Samples were dried in a desiccator for at least 2 h before imaging in the atomic force microscope. Length measurements were performed using UTHSCSA ImageTool software.

Isolation of RNA and Northern blot

RNA was isolated as described previously (Batzilla et al., 2006). Cells were harvested from shaking cultures in TSB or TSB + 4% NaCl at 37 °C at $OD_{600\,nm}$ 1.5 and $OD_{600\,nm}$ 4.5, respectively. For Northern blot analysis, 10 µg of total RNA were separated on a denaturing gel containing 0.6% agarose. After capillary blotting, the blot was hybridized with ECL-labeled DNA probes specific for *aap* and *bhp*, respectively. For signal detection, the ECL™ Advance detection kit (GE Healthcare, Amersham Biosciences, Freiburg, Germany) was used.

Results

Development of a PIA-independent biofilm by an *icaC*::IS*256* insertion mutant

In previous work we reported phase variation in biofilm formation by insertion and precise excision of IS*256* into and from the *ica* operon (Ziebuhr et al., 1999). When we used an *icaC*::IS*256* insertion mutant (CSF41498-1) of the *ica*-positive, IS*256*-negative strain *S. epidermidis* CSF41498 to study precise excision in more detail, after four to six days of passaging we regularly obtained variants that were capable of producing biofilm while still carrying the *icaC*::IS*256* insertion. The variants displayed a rough phenotype on Congo red agar, comparable to PIA-positive strains. Notably, such variants were detected much more frequently than precise excision of IS*256*. In biofilm formation assays, some of the variants were as