Molecular Characterization of a Novel *Staphylococcus Aureus* Surface Protein (SasC) Involved in Cell Aggregation and Biofilm Accumulation

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Abstract

**Background:** Staphylococci belong to the most important pathogens causing implant-associated infections. Colonization of the implanted medical devices by the formation of a three-dimensional structure made of bacteria and host material called biofilm is considered the most critical factor in these infections. To form a biofilm, bacteria first attach to the surface of the medical device, and then proliferate and accumulate into multilayered cell clusters. Biofilm accumulation may be mediated by polysaccharide and protein factors.

**Methodology/Principal Findings:** The information on *Staphylococcus aureus* protein factors involved in biofilm accumulation is limited, therefore, we searched the *S. aureus* Col genome for LPXTG-motif containing potential surface proteins and chose the so far uncharacterized *S. aureus* surface protein C (SasC) for further investigation. The deduced SasC sequence consists of 2186 amino acids with a molecular mass of 238 kDa and has features typical of Gram-positive surface proteins, such as an N-terminal signal peptide, a C-terminal LPXTG cell wall anchorage motif, and a repeat region consisting of 17 repeats similar to the domain of unknown function 1542 (DUF1542). We heterologously expressed sasC in *Staphylococcus carnosus*, which led to the formation of huge cell aggregates indicative of intercellular adhesion and biofilm accumulation. To localize the domain conferring cell aggregation, we expressed two subclones of sasC encoding either the N-terminal domain including a motif that is found in various architectures (FIVAR) or 8 of the DUF1542 repeats. SasC or its N-terminal domain, but not the DUF1542 repeat region conferred production of huge cell aggregates, higher attachment to polystyrene, and enhanced biofilm formation to *S. carnosus* and *S. aureus*. SasC does not mediate binding to fibrinogen, thrombospondin-1, von Willebrand factor, or platelets as determined by flow cytometry.

**Conclusions/Significance:** Thus, SasC represents a novel *S. aureus* protein factor involved in cell aggregation and biofilm formation, which may play an important role in colonization during infection with this important pathogen.

Introduction

In the past two decades, *Staphylococcus aureus* has emerged one of the most important pathogens causing infections with indwelling medical devices, such as prosthetic heart valves, intravascular catheters, and cerebrospinal fluid shunts, which creates an increasing health care problem [1]. For example, prosthetic joint infections occur at a frequency of 1.5–2.5% in primary total hip or total knee arthroplasty with a mortality rate of up to 2.5% [2]. By far the most frequently isolated species from these infections are *Staphylococcus* species, i.e. *S. aureus* (22–39%) and coagulase-negative *staphylococci* (15–37.5%) [2].

The pathogenesis of device-associated infections with *staphylococci* is mainly characterized by the pathogens ability to colonize the surfaces of the implanted medical device by the formation of a three-dimensional structure of microorganisms embedded in a thick extracellular matrix composed of polysaccharides, proteins, extracellular DNA, and host factors, known as biofilm [3,4,5]. Microorganisms within a biofilm are protected against antimicrobial chemotherapy as well as against the immune system of the host.

Biofilm formation occurs in a two-step process. The first step involves the adherence of the bacteria to artificial surfaces that can occur either directly or via host factors acting as bridging molecules, such as the extracellular matrix and plasma proteins fibrinogen (Fg) and fibronectin (Fn) or platelets [6]. In the second step, the bacteria proliferate and accumulate into a biofilm requiring intercellular adhesion. Direct *S. aureus* adherence to the unmodified artificial surface may be mediated by the major autolysin Atl [7], which is highly homologous to the *S. epidermidis* autolysin/adenosin AtlE shown to be involved in the attachment to...
polymer surfaces [8]. *S. aureus* host-factor binding proteins that typically belong to the family of microbial surface components recognizing adhesive matrix molecules (MSCRAMM) are involved in binding to host-factor-coated foreign material, among them Fn-binding proteins (FnBPA, FnBPB, Ehh), Fg-binding proteins (ClfA, ClfB), collagen-binding protein (Cna), baterialin-binding protein (Bap), and von Willebrand factor (vWF)-binding protein A (Spa) [9,10,11,12,13,14,15,16]. Staphylococcal biofilm accumulation is mediated by polysaccharide as well as protein factors. The intercellular polysaccharide adhesin (PIA), a β-1,6-N-acetylglicosaminoglycan [3], is produced by the gene products encoded by the *icaADBC* operon that was first identified in *S. epidermidis* [17] and is also present in *S. aureus* [18]. Surface proteins conferring biofilm accumulation include the accumulation-associated protein (Aap) from *S. epidermidis* [19,20] and the homologous *S. aureus* surface protein G (SasG) [21]. In *S. aureus*, another protein, the biofilm-associated protein (Bap), is involved in biofilm accumulation [22]. However, so far the *bap* gene has not been found in any *S. aureus* isolate of human origin, but has only been identified within bovine mastitis isolates [22].

A recent study demonstrated that all 18 *S. aureus* isolates from prosthetic joint infections carry the *icaADBC* operon, produce PIA, and are biofilm-positive [23]. Surprisingly, the biofilms of all 18 *S. aureus* isolates could be almost completely eradicated by the treatment with dispersin B (DspB), an enzyme with specific β-1,6-hexosaminidase activity as well as by the treatment with trypsin suggesting that both, proteinaceous adhesins and PIA contribute to biofilm formation in these *S. aureus* isolates. This was in contrast to *S. epidermidis* isolates from prosthetic joint infections. Only 62% of the 52 *S. epidermidis* isolates carry the *icaADBC* operon and *S. epidermidis* biofilms produced by *icaADBC*-positive strains were disintegrated by DspB, but not by proteases. Furthermore, biofilms produced by *icaADBC*-negative strains were disintegrated by proteases, but not by DspB [23]. Thus, different mechanisms seem to be involved in biofilm formation in clinical *S. aureus* and *S. epidermidis*-associated prosthetic joint infection isolates. More specifically, in *S. aureus*-associated prosthetic joint infections, polysaccharide and protein factors seem to act synergistically in biofilm formation. Only 33% of the analyzed *S. aureus* strains carry the *sasG* gene and none of them carry the *bap* gene, indicating the existence of further, not yet identified surface proteins involved in biofilm accumulation of *S. aureus*.

Upon a search for LPXTG-motif containing surface-anchored proteins encoded by the *S. aureus* Col genome (http://www.tigr.org), we chose to study the so far uncharacterized SasC. We heterologously expressed *sasC* from *S. aureus* Col and from the clinical *S. aureus* isolate 4074 in *Staphylococcus carnosus* under the control of a xylose-inducible promoter. *S. carnosus* expressing *sasC* formed huge cell aggregates indicative of intercellular adhesion, which were disintegrated by protease treatment. Upon plasmid-encoded expression of *sasC*, *S. carnosus* as well as *S. aureus* not only formed huge cell aggregates, but also formed a much more pronounced biofilm in microtiter plates as well as in glass tubes than the respective wild-type strains. The domain conferring cell aggregation and biofilm formation was localized to the N-terminal domain of SasC. In conclusion, we identified SasC as a novel *S. aureus* factor involved in intercellular adhesion and biofilm accumulation.

### Results

**Identification and cloning of the *sasC* gene of *S. aureus***

As a candidate gene conferring biofilm formation in *S. aureus*, we amplified a DNA fragment containing the *sasC* gene including the ribosome binding site by polymerase chain reaction (PCR) from *S. aureus* 4074 and *S. aureus* Col genomic DNA using the primers CHsasCfor and CHsasCrev yielding 6577 bp DNA fragments. The DNA fragments were cloned into the BamHI and SalI sites of the vector pCX19 in *S. carnosus*, creating plasmids pSasC4074 or pSasCCol.

**Nucleotide sequence of *sasC* and amino acid sequence analysis of the deduced protein**

The nucleotide sequence of the cloned *sasC* gene from *S. aureus* 4074 was determined on both strands. *sasC* consists of 6558 nucleotides and encodes a deduced protein of 2186 amino acids (aa) with a predicted molecular mass of 237.9 kDa. The ATG start codon is preceded by a putative ribosome binding site at a distance of 8 bp. Putative −10 (TATATT, nucleotides −61 to −56) and −35 (TAAACA, nucleotides −80 to −75) promotor sequences were deduced from homologous DNA sequences from strain *S. aureus* Col. A putative ρ-independent terminator consisting of two stem-loops is located downstream of the TAA stop codon. The deduced SasC sequence contains a putative signal peptide in the first 37 aa that contains an YSIRK motif, which seems to play a role in signal peptide processing [24]. The predicted *sasC* gene product is composed of 23.1% hydrophilic, 12.1% basic, and 13.2% acidic aa. The theoretical pl value of SasC is 5.98. The deduced aa sequence of *SasC* of strain 4074 shares 97% identical aa with homologous proteins from strains *S. aureus* MW2 [25] and MSSA476 [26], 96% identical aa with strains USA300 [27], COL [28], Newman [29], NCTC8325 (accession number: Q2FXH4 [30]), and N315 [31] and 89% identical aa with strain MRSAS252 [26]. Besides, SasC shares 31% identical aa with Mrp and Fmb proteins from strain Col that have been implicated in methicillin-resistance [32,33].

Sequence comparison of the deduced SasC sequence with known protein sequences in databases revealed a domain structure of SasC (Fig. 1A). The central portion of SasC contains a domain, which is similar to the motif found in various architectures (FIVAR; 54 aa, starting at N-590 and ending at D-643). The FIVAR domain is followed by 17 direct repeated sequences of 72 aa each separated by a stretch of 5 aa, which are homologous to a domain of unknown function (DUF1542) also found in other cell surface proteins. The first DUF1542 repeat starts at Q-671 and the last ends at I-1974. The DUF1542 repeats share between 18 and 47% identical aa (Fig. 1B). The FIVAR motif and the DUF1542 domain are also present within Mrp and Fmb (see above) as well as within the cell surface protein Ehh from *S. aureus* [10] and the homologous Eemb from *S. epidermidis* [34].

**SasC mediates strong cell aggregation in *S. carnosus***

After overnight growth in tryptic soy (TS) broth supplemented with 1% xylose, *S. carnosus* expressing *sasC* formed huge cell clusters that were visible macroscopically (Fig. 2A) and microscopically (Fig. 2C). In contrast, the strains did not form cell clusters without induction by xylose (Fig. 2A). The cell clusters were dissolved upon treatment with trypsin (Fig. 2B and C) or proteinase K (Fig. 2B). Disruption of cell clusters by trypsin was concentration-dependent (Fig. 2C).

**Characterization of *S. carnosus* and *S. aureus* expressing *sasC* or *sasC*-subclones**

In order to dissect the functional domains within SasC, we constructed subclones of *sasC* expressing either the N-terminal domain including the FIVAR motif (subclone 1) or 8 of the 17 DUF1542 repeats (subclone 2) in *S. carnosus* yielding *S. carnosus*
**Figure 1. Schematic model of SasC and amino acid alignment of the DUF1542 repeats.**

A: Model of SasC. The positions of the N-terminal signal peptide including the YSIRK motif, the C-terminal LPXTG motif, the FIVAR motif (aa N-590 to D-643), and the 17 DUF1542 repeats (aa Q-671 to I-1974) are indicated. The DUF1542 repeats consist of 72 aa each separated by 5 aa. The SasC domains expressed by subclone 1 (aa T-654 is fused to N-1987) and subclone 2 (aa T-40 is fused to E-1359) are indicated. The SasC domain expressed as His-tagged fusion protein is shown: His-DUF1542 (aa Q-902 to Q-1515). + indicates cell aggregation mediated by the respective clone; − indicates no cell aggregation.

B: Alignment of the deduced amino acid sequences of the DUF1542 repeats. The consensus sequence indicates the DUF1542 domain (Pfam accession number: PF07564). Bold letters indicate amino acids that match the consensus sequence. Asterisks indicate identical amino acids, colons indicate very similar amino acids. Gaps (dashes) were filled in to maximize homologies.

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(pSasCsub1) or *S. carnosus* (pSasCsub2), respectively (see Fig. 1). An inverse PCR with the plasmid pSasC4074 as a template was carried out for the construction of subclone 1 and subclone 2 by using the primers CHsasCSub1rev/CHsasCSub1for and CHsasCSub2rev/CHsasCSub2for, respectively.

The resulting PCR fragments were ligated and subsequently, *S. carnosus* was transformed with the ligation mixtures. Correct clones were verified by PCR and DNA sequencing. However, DNA sequence analysis of subclone 1 revealed a 33-bp deletion in the C-terminal region so that aa T-654 is fused to N-1987 rather than to D-1976 (Fig. 1A). To functionally characterize *sasC* also in the *S. aureus* background, we transformed *S. aureus* 4074 and *S. aureus* SH1000, which is a 8325-4 derivative reconstituted for its *rsbU* mutation [35], with the plasmids pSasC4074, pSasCsub1, or pSasCsub2 leading to overexpression of *sasC* and its subfragments in these strains.

To verify the production of the whole SasC or the truncated SasC proteins, cell lysates of the strains were prepared and analyzed by SDS-PAGE (Fig. 3A). *S. carnosus* and the *S. aureus* strains expressing *sasC* revealed an additional protein band corresponding to the size of SasC of 238 kDa. The strains expressing subfragments 1 or 2 revealed additional protein bands at 93 or 96 kDa, respectively. The strains expressing *sasC* or the subfragments 1 or 2 were further characterized.
In contrast to the wild-type strains, \textit{S. carnosus} (pSasC4074), \textit{S. aureus} 4074 (pSasC4074) and \textit{S. aureus} SH1000 (pSasC4074) as well as \textit{S. carnosus} (pSasCsub1) and \textit{S. aureus} SH1000 (pSasCsub1) formed huge cell aggregates that were visible macroscopically in cultures grown overnight on TS agar supplemented with 1% xylose and resuspended in PBS on glass slides (Fig. 4). The cell aggregates formed by \textit{S. aureus} 4074 (pSasCsub1) were somewhat smaller in size (comparable to those mediated by the \textit{icaADBC} operon encoding the production of the polysaccharide intercellular adhesin, PIA [17] Fig. 4–13) corresponding to a lower expression level of the fusion protein (see Fig. 3). In contrast, the parent strains or strains expressing the subfragment 2 did not form visible cell aggregates (Fig. 4). Thus, \textit{sasC} and its subfragment 1 confer cell aggregation.

Biofilm formation on polystyrene and glass

Bacterial biofilm formation results from initial attachment of bacterial cells to a surface and subsequent accumulation into multilayered cell clusters, which requires intercellular adhesion visible as cell aggregation. To determine, whether \textit{sasC} not only mediates cell aggregation, but also initial attachment, we analyzed the capacity of the respective strains for attachment to a polystyrene or a glass surface. Initial attachment of \textit{S. carnosus} (pCX19) to polystyrene was very low indicated by a low number of planktonic cells (Fig. 5A). In \textit{S. aureus}, the expression of \textit{sasC} or the subfragment 1 also led to a markedly increased biofilm formation as shown for strain SH1000 [\textit{A}490 value of 1.5 or 1.4 for \textit{S. aureus} SH1000 (pSasC4074) or \textit{S. aureus} SH1000 (pSasCsub1), respectively, versus \textit{A}490 value of 0.7 for \textit{S. aureus} SH1000] (Fig. 5A). As observed with \textit{S. carnosus}, the biofilm formation of \textit{S. aureus} SH1000 (pSasCsub2) is comparable with that of its wild type (\textit{A}490 value: 0.3). The results for \textit{S. aureus} 4074 are similar (not shown). The stronger capacity for biofilm formation mediated by \textit{sasC} or the subfragment 1 also could be observed on a glass surface with \textit{S. carnosus} as well as \textit{S. aureus} (pCN27) producing PIA [17] (Fig. 5B). Thus, \textit{sasC} and its subfragment 1 mediate biofilm formation on polystyrene and on glass.

Initial attachment to polystyrene and to glass

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(i) Cell aggregation

In contrast to the wild-type strains, \textit{S. carnosus} (pSasC4074), \textit{S. aureus} 4074 (pSasC4074) and \textit{S. aureus} SH1000 (pSasC4074) as well as \textit{S. carnosus} (pSasCsub1) and \textit{S. aureus} SH1000 (pSasCsub1) formed huge cell aggregates that were visible macroscopically in cultures grown overnight on TS agar supplemented with 1% xylose and resuspended in PBS on glass slides (Fig. 4). The cell aggregates formed by \textit{S. aureus} 4074 (pSasCsub1) were somewhat smaller in size (comparable to those mediated by the \textit{icaADBC} operon encoding the production of the polysaccharide intercellular adhesin, PIA [17] Fig. 4–13) corresponding to a lower expression level of the fusion protein (see Fig. 3). In contrast, the parent strains or strains expressing the subfragment 2 did not form visible cell aggregates (Fig. 4). Thus, \textit{sasC} and its subfragment 1 confer cell aggregation.

(ii) Biofilm formation on polystyrene and glass

Biofilm formation on polystyrene was determined in the quantitative biofilm assay. Whilst \textit{S. carnosus} (pCX19) revealed a biofilm-negative phenotype (\textit{A}490 value: 0.11), \textit{S. carnosus} (pSasC4074) and \textit{S. carnosus} (pSasCsub1) showed an enhanced biofilm formation corresponding to an \textit{A}490 value of 0.43 and 0.4 (Fig. 5A). In contrast, biofilm formation of strain \textit{S. carnosus} (pSasCsub2) is comparable to that of the wild type (\textit{A}490 value: 0.05).
attached bacteria (Fig. 6A). *S. carnosus* strains expressing *sasC* or its subfragments showed higher initial attachment with *S. carnosus* (pSasCsub1) yielding the highest numbers of attached bacteria (Fig. 6A). Essentially the same was observed with *S. aureus* SH1000 strains albeit at a higher level of attachment (Fig. 6A) and with *S. aureus* SH1000 (pSasCsub2) showing numbers of attached bacteria that were comparable with the wild type.

Initial attachment of the strains to a glass surface generally was higher (Fig. 6B) and similar with *S. carnosus* and *S. aureus* SH1000 wild-type strains and the same strains expressing *sasC* or the subfragment 2. In contrast, both strains expressing subfragment 1 showed a slightly lower number of attached bacterial cells (Fig. 6B). Thus, expression of *sasC* slightly increased attachment to a polystyrene surface, but did not increase attachment to glass.

(iv) Binding to Fg, vWF, thrombospondin-1 (TSP-1), and platelets

The ability of *S. aureus* to bind to extracellular matrix and plasma proteins and to host cells determines its capacity for tissue colonization. The potential of *sasC* to mediate binding to the extracellular matrix and plasma proteins Fg, TSP-1, and vWF as well as to platelets was analyzed by flow cytometry. While *S. aureus* 4074 bound to Fg, TSP-1, and vWF as well as to activated platelets in a dose-dependent fashion, *S. carnosus* and *S. carnosus* (pSasCsub2) did not (Fig. 7). Thus, *sasC* does not mediate binding to these extracellular matrix proteins or to activated platelets.

**Biofilm formation of a sasC transposon (Tn)917 insertion mutant (SMH2035)**

To further support the role for SasC in biofilm formation, we analyzed the capacity of a *sasC*Tn917 insertion mutant (SMH2035) for biofilm formation in comparison to its wild-type strain *S. aureus* SH1000. Under all conditions tested, the *sasC* mutant strain showed reduced biofilm formation in microtiter plates, i.e. the addition of 1% xylene [4490 value of 0.4 for SMH2035 versus 4490 value of 0.7 for SH1000], 0.25% glucose [4490 value of 1.6 for SMH2035 versus 4490 value of 1.8 for SH1000] or no additional carbohydrate source in the biofilm assay [4490 value of 0.5 for SMH2035 versus 4490 value of 1.0 for SH1000] (Fig. 8). The biofilm-forming capacity of both strains was most pronounced upon addition of 0.25% glucose, which is known to induce the production of PIA in *S. epidermidis* [36] and probably leads to enhanced PIA production also in *S. aureus*, which might partially obscure the function of SasC. The presence of 1% xylene seems to slightly reduce biofilm formation. So far, the reason for this is unknown.

Expression and purification of the 6 x Histidine (His)-DUF1542 fusion protein in *Escherichia coli*

For expression of the *sasC* portion encoding 8 of the 17 DUF1542-repeats in *E. coli*, the PCR-amplified fragments were cloned into the expression vector pQE30Xa. One representative clone expressing the DUF1542 repeats (6 x His-DUF1542) contained the plasmid pHis-DUF1542. Subsequently, the 6 x His-DUF1542 fusion protein was purified from *E. coli* (pHis-DUF1542) via its His-tag using Ni-NTA affinity chromatography under native conditions. SDS-PAGE of the affinity-purified fusion proteins revealed an approximately 70-kDa protein for the 6 x His-DUF1542 (lane 3), and purified 6 x His-DUF1542 (1.5 μg) (lane 4). The size of one marker protein is shown on the left (lane 1; prestained protein ladder, Fermentas).

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To detect the surface location of SasC, the antiserum against the 6 x His-DUF1542 fusion protein that was raised in rabbits was used in immunofluorescence microscopy. The anti-His-DUF1542 antiserum strongly reacted with cells of *S. carnosus* (pSasC4074) (Fig. 9) and *S. aureus* 4074 (pSasC4074) (not shown) indicating the cell surface location of SasC. With *S. carnosus* (pSasC4074), no immunofluorescence was detected with the preimmune serum (not shown) and with *S. carnosus* (pCX19), no immunofluorescence was detected with the anti-His-DUF1542 antiserum (Fig. 9). However, there was some immunofluorescence detectable with the preimmune serum and strain *S. aureus* 4074 (not shown), which may be due to the IgG-binding and surface-associated proteins A and Eap [37,38].

**Characterization of sasC expression in *S. aureus* strains**

To analyze the production of SasC in *S. aureus*, we performed Western blot analysis using the anti-His-DUF1542 antiserum. In cultures of *S. aureus* 4074, Col, and SH1000, a faint band corresponding to SasC was detected in lysostaphin lysates of cultures after 10, 16, 24, and 48 h of growth (not shown). A strong production of SasC was observed in control strains *S. aureus* 4074.
Be present in 97% (66/68) of the clinical isolates, we performed PCR analysis using primers encoding a portion of the N-terminal SasC domain was found to be involved in biofilm formation. To date, several SasC isolates have been implicated in biofilm formation, among them SMH2035 (pCX19); 2, S. aureus (pSasCSub1); 4, S. carnosus (pSasCSub2); 5, S. aureus SH1000; 6, S. aureus SH1000 (pSasC4074); 7, S. aureus SH1000 (pSasCSub1); 8, S. aureus SH1000 (pSasCSub2). B: Biofilm formation on glass. Lanes: 1, S. carnosus (pSasCSub1); 2, S. carnosus (pSasCSub2); 3, S. carnosus (pSasC4074); 4, S. carnosus (pSasCSub2); 5, S. aureus SH1000; 6, S. aureus SH1000 (pSasC4074); 7, S. aureus SH1000 (pSasCSub1); 8, S. aureus SH1000 (pSasCSub2); 9, S. carnosus (pCN27).

Prevalence of sasC

To determine the prevalence of the sasC gene among clinical S. aureus isolates, we performed PCR analysis using primers CHsasC1for and CHsasC1rev. An approximate 500 bp fragment encoding a portion of the N-terminal SasC domain was found to be present in 97% (66/68) of the clinical S. aureus strains that were tested (not shown). This indicates a very high prevalence of the sasC gene among clinical S. aureus isolates.

Discussion

The most frequently isolated bacteria from implant-associated infections are S. aureus and coagulase-negative staphylococci causing significant morbidity and mortality. The pathogenicity of these infections is characterized by the pathogens pronounced ability to form biofilms. To date, several S. aureus genes have been implicated in biofilm formation, among them atl [7], dltA [39], and the seaIDBC gene cluster [18]. S. aureus surface proteins reported to be involved in biofilm formation include SasG [21], the Fn- and Fg-binding proteins FnBPA and FnBPB [40], and the biofilm-associated protein Bap [22]. SasG is homologous to the accumulation-associated protein Aap, which mediates biofilm accumulation in S. epidermidis [19,20], and the plasmin-sensitive surface protein Pls [41], which so far has not been implicated in biofilm formation. Furthermore, extracellular genomic DNA (eDNA) has been established as another important component of S. aureus biofilms [5]. eDNA is released from the bacteria by cell lysis and may implicate an additional role for the major S. aureus autolysin Atl in biofilm development besides its function in initial attachment [5].

However, a recent study indicated the existence of further, yet unidentified surface proteins contributing to S. aureus biofilm formation [25]. As a potential candidate, we identified SasC in the S. aureus genome and expressed its gene heterologously in S. carnosus as well as in the S. aureus strains SH1000 and 4074 under a xylose-inducible promoter. All strains expressing sac showed a high-level production of a protein with a molecular size of approximately 240 kDa corresponding to SasC. Expression of sac led to strong cell cluster formation, intercellular adhesion, and biofilm formation. Furthermore, a sac Tn917 insertion mutant (SMH2035) showed a reduced capability for biofilm formation in comparison to its wild type.

SasG and Aap promote biofilm formation via their B-repeats. Each B-repeat also known as G5 domain consists of 128 aa and is present 7 and 5 times in SasG and Aap, respectively [20,21]. Recently, the G5 domains were found to be zinc-dependent adhesion modules and a “zinc zipper” mechanism was suggested for G5 domain-based intercellular adhesion in SasG- or Aap-mediated biofilm accumulation [42]. SasC also contains a repeat region with 17 repeats of 72 aa being similar to the DUF1542 domain (see Fig. 1). The SasC repeats do not share sequence similarities with the B-repeats of SasG and Aap and are not involved in biofilm formation. Instead in SasC, the domain conferring cell aggregation and biofilm formation could be localized to the N-terminal domain by subcloning experiments (see Fig. 1).

Furthermore, SasG and Aap must undergo proteolytic cleavage to become active, while SasC-mediated biofilm formation does not depend on proteolytic cleavage: the formation of SasC-mediated biofilms was unchanged in the presence of the protease inhibitor α2-macroglobulin (data not shown) that blocked SasG and Aap-mediated biofilm formation [20,21]. Moreover, S. carnosus expressing apa only formed a detectable biofilm after treatment with 2 µg/ml of trypsin leading to the production of a truncated version of Aap or when an N-terminally truncated version of apa was cloned in S. carnosus [20]. In contrast, no truncation of SasC was involved in SasC-mediated biofilm formation. Thus, the mechanism of SasC-mediated biofilm accumulation clearly differs from that mediated by SasG/Aap. Because of the lack of sequence similarities, the SasC-mediated mechanism probably is also distinct from that mediated by Bap [22].

Recently, a role for FnBPA and FnBPB in biofilm accumulation, which is independent of their Fn- and Fg-binding activities was reported. Like with SasC, the domain conferring biofilm accumulation is located within the N-terminal domain (A domain), which also includes the domain for Fg-binding. However, biofilm formation seems to be independent of Fg-binding, because a single aa exchange within that region abolished Fg-binding, but did not influence the capability for biofilm formation [40]. Because the FnBPs and SasC do not share significant sequence similarities within their N-terminal domains, the mechanism of biofilm accumulation between the two also seems to differ.

The N-terminal SasC domain shows significant homology to the N-terminal domains of Mrp andFmtB, both of which have been reported to be involved in methicillin resistance [32,33]. However, the mechanism of Mrp andFmtB conferring methicillin resistance is not completely clear and may be indirect. A function of these proteins in biofilm formation has not been proposed so far.

The mechanism of SasC-mediated biofilm accumulation is not known yet and may involve either protein-protein interactions...
conferred by the N-terminal SasC domain or protein-carbohydrate interactions conferred by the FIVAR motif. The FIVAR motif is located at the C-terminus of the N-terminal domain (see Fig. 1) and has been proposed to have a sugar-binding function. Further analyses are necessary to elucidate these possibilities.

SasC not only mediates cell cluster formation and intercellular adhesion, but also slightly increases the attachment of the cells to polystyrene. Initial attachment to a polystyrene surface depends on cell surface hydrophobicity [43,44]. With 25.1% hydrophobic aa, the percentage of hydrophobic aa of SasC is comparable to that of AtlE (26.4%) previously found to mediate initial attachment to polystyrene in *S. epidermidis* [8]. Remarkably, the higher level of initial attachment to polystyrene observed with *S. carnosus* and *S. aureus* producing subclone 1 correlated with a higher percentage of hydrophobic aa (26.6%) and a lower percentage of charged aa (23.7%: 12.3% basic and 11.4% acidic aa) of subclone 1 in comparison to subclone 2, which contains 26.6% hydrophobic aa and 30.1% charged aa (14.7% basic and 15.4% acidic aa).

Although the *S. aureus* wild-type strains Col, SH1000, and 4074 all harbour the *sasC* gene, we were not able to detect SasC in lysostaphin lysates by SDS-PAGE, but only by Western immunoblot analysis indicating very low *sasC* expression in vitro. However, the identification of SasC as an in vivo-expressed antigen during infection delineates its potential clinical significance [45]. Thus, *sasC* expression may be induced in vivo. Several genes are involved in the regulation of *S. aureus* biofilm formation, such as *agr* [46,47], *sarA* [48,49], *sigB* [50], *rvf* [51], *tedR* [52], *arlRS* [53], and *alsSD* [54]. Further experiments are necessary to characterize the expression of *sasC*.

Homologous *sasC* genes were found in the eight sequenced *S. aureus* genomes analyzed and the sequence similarities of the SasC gene products ranged from 89% to 97%. Consistently, we found a very high prevalence of the *sasC* gene among clinical *S. aureus* strains delineating the potential importance of SasC in colonization and infection. In conclusion, we identified and characterized a novel *S. aureus* surface protein, SasC, involved in cell aggregation

**Figure 6. SasC-mediated initial attachment of *S. carnosus* or *S. aureus* strains.** Phase-contrast micrographs of attached cells of *S. carnosus* (TM300) or *S. aureus* (SH1000) strains on polystyrene Petri dishes (A) or on glass slides (B).

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Figure 7. SasC does not bind to extracellular matrix proteins Fg, vWF, and TSP-1 or to platelets. Bars: black, S. aureus 4074; dark grey, S. carnosus (pCX19); light grey, S. carnosus (pSasC4074); white, S. carnosus (pSasCsub2). Solid black line: S. aureus 4074; small dashed line: S. carnosus (pCX19); dashed black line, S. carnosus (pSasC4074); dashed grey line, S. carnosus (pSasCsub2). The results represent the mean of three independent experiments. Standard deviations are indicated. doi:10.1371/journal.pone.0007567.g007
and biofilm formation, which may play an important role in colonization during infection with this important pathogen.

**Materials and Methods**

**Ethics Statement**

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the local ethics committee (Ethikkommission der Ärztekammer Westfalen-Lippe und der Medizinische Fakultät der WWU Münster) (reference number: Sitzung 19.05.1999). All volunteers provided written informed consent for the collection of samples and subsequent analysis.

**Bacterial strains, plasmids, and media**

The *sasC* gene was cloned from the clinical strain *S. aureus* 4074 [55] and from *S. aureus* Col [28]. *S. carnosus* TM300 [56], *S. aureus* 4074 [55], and *S. aureus* SH1000 [35] were used as cloning hosts. The *sasC* Tn917 insertion mutant SMH2035 was isolated by sequencing Tn insertion junctions from a Tn917 mutant library of *S. aureus* SH1000, using direct sequencing from isolated genomic DNA [57]. One of the mutants gave a junction sequence, which mapped to an insertion point at 1812698 in the *S. aureus* N315 genome. This corresponded to inactivation of SasC at aa 934. *E. coli* TG1 was used to construct the plasmid for the production of the 6 x His-DUF1542 fusion protein and its purification.

For the expression of *sasC* in staphylococci, the vector pCX19, a derivative of the xylose-inducible expression vector pCX15 [58] and for the production and purification of the 6 x His-DUF1542 fusion protein, the Qiaexpress vector pQE30Xa (Qiagen) was used.

To determine the prevalence of *sasC*, a 489 bp internal *sasC* fragment was amplified from genomic DNA of 68 clinical *S. aureus* isolates obtained from patients at the University Hospital of Münster (Münster, Germany).

The staphylococci were routinely cultivated in TS broth (TSB). *E. coli* strains were grown in Luria-Bertani (LB) medium. TS and LB agar contained 1.4% agar. TSB and LB agar were supplemented with 1% xylose to induce *sasC* expression. Selection for resistance to antibiotics in *E. coli* was performed with 100 μg/ml ampicillin and in staphylococci with 10 μg/ml chloramphenicol or 5 μg/ml erythromycin, when appropriate. Wild-type strains not harboring a plasmid were grown in the presence of 0.07% ethanol in the respective assays, when compared to the strains harboring a plasmid to rule out an effect of the ethanol.

![Figure 8. *S. aureus* SH1000 *sasC* shows reduced biofilm formation.](image1.png)

Quantitative assay of biofilm formation. Lanes: 1, *S. aureus* SH1000; 2, *S. aureus* SH1000 *sasC*: A, 1% xylose; B, 0.25% glucose; C, no additional carbohydrate source. doi:10.1371/journal.pone.0007567.g008

![Figure 9. Detection of SasC by immunofluorescence microscopy.](image2.png)

Strains grown in TSB were incubated with anti-His-DUF1542 antiserum raised in rabbits. Bound antibodies were detected with fluorescein-conjugated anti-rabbit F(ab′)2 fragment. Cells were viewed with a fluorescence microscope. *S. carnosus* (pSasC4074) cells reacted with the anti-His-DUF1542 antiserum, indicating the surface location of SasC. Magnification, ×400. A, *S. carnosus* (pSasC4074); B, *S. carnosus* (pCX19). doi:10.1371/journal.pone.0007567.g009
the sasC Tn917 insertion mutant SMH2035 is stable, no antibiotic or ethanol was included in the assays comparing the SH1000 strain and its sasC mutant.

Bovine serum albumin (BSA) and α-thrombin (bovine) were purchased from Sigma. Human Fg was purchased from Enzyme Research Labs. The monoclonal antibody against human CD42a (GP IX) (conjugated with phycoerythrin [PE]) was delivered by Exalphi via NatuTec. Syto 13 for labeling of staphylococcal cells was purchased from Molecular Probes via Mobitec.

**DNA manipulations, transformation, PCR, DNA sequencing, websites, and Pfam accession numbers**

DNA manipulations and transformation of *E. coli* were performed according to standard procedures [59]. *S. carnosus* and *S. aureus* strains were transformed with plasmid DNA by protoplast transformation [60]. Plasmid DNA was isolated using the Qiagen Plasmid Kit and chromosomal DNA was isolated using the QIAamp DNA Blood Mini Kit according to the instructions of the manufacturer (Qiagen). PCR was carried out with the PCR Supermix High Fidelity (Invitrogen) or with the Expand Long Template PCR System (Roche, Mannheim, Germany) in accordance with the protocol of the supplier. The primers (see Table 1) were synthesized by MWG-Biotech (Ebersberg, Germany).

The DNA sequence of both strands of the sasC gene of the clinical isolate *S. aureus* 4074 was determined by MWG-Biotech using a LI-COR DNA sequencer.

The DNA and deduced protein sequences were analyzed using the program “JustBio” at http://www.justbio.com. The protein sequences were compared with those of known proteins using the programs BLASTP [61] and FASTA [62]. The alignments were done using the program ClustalW at the European Bioinformatics Institute (EBI, Cambridge, UK). The Pfam accession numbers are: PF04650 for the YSIRK_signal; PF07554 for the FIVAR motif; PF07564 for the DUF1542 domain; PF00746 for the Gram_pos_anchor (LPXTG_anchor) available at: http://pfam.sanger.ac.uk/ [63]. The signal peptide of SasC was predicted by using the program “SignalP” at http://www.cbs.dtu.dk/services/SignalP/.

**Biofilm formation assay**

For quantification of the biofilm-forming capacity, a test for biofilm production was performed essentially as described previously [44]. Briefly, strains were grown in TSB supplemented with 1% xylose for 24 h at 37°C in 96-well polystyrene microtiter plates (cell star; Greiner, Frickenhausen, Germany), the wells were washed with phosphate-buffered saline (PBS) and adherent biofilms were stained with 0.1% safranin (Serva). In some experiments, instead of 1% xylose, 0.25% glucose or no additional carbohydrate source was added. Absorbance was measured with a Micro-ELISA-Autoreader at 492 nm. Strains were tested at least in triplicate. Determination of biofilm formation on a glass surface was carried out essentially in the same way, except that glass tubes were used instead of microtiter plates and 5 ml of TSB were inoculated instead of 200 μl.

**Initial adherence to a polystyrene or a glass surface**

Initial cell attachment was tested as described previously [44]. Briefly, diluted cell suspensions of bacteria in PBS were incubated for 30 min in polystyrene Petri dishes (Sarstedt) at 37°C or on glass slides and after a washing procedure, attached bacteria were evaluated by phase-contrast microscopy.

**Construction and purification of the 6 x His-DUF1542 fusion protein and anti-His-DUF1542 antiserum**

For the construction of the His-tagged fusion protein, the primers CHsasCDUFor and CHsasCDUFrev were used to amplify 8 of the DUF1542-repeats of sasC from genomic DNA

### Table 1. Oligonucleotide primers used in this study.

| Primer name | Oligonucleotide sequence (5′→3′) |
|-------------|---------------------------------|
| CHsasCfor   | GTG AGA TCT CCA GGA GGA AAA CGA AAT GAA TTT G (flgII) | cloning sasC |
| CHsasCrev   | CGT GGG CCC AAT TAT GAT TCT TTT TCG TTT TTA GTA CG C (Smad) | cloning sasC |
| CHsasCDUFor | CAA CAT ATC CGA GAC ATG AAT G | expression |
| CHsasCDUFrev| GTG GGA TCC TTA TTG TTG CTT AAC TGC ATC TCT AGC (BamHI) | expression |
| CHsasSub1rev| CGT ATG TTG CAT TTG ATT AG | subcloning sasC |
| CHsasSub1for| GAT CAT CTT GCA CGC GTC AC | subcloning sasC |
| CHsasSub2rev| CGT AGT TAA GGC TTG TGC ACC | subcloning sasC |
| CHsasSub2for| GAA AAA GCT GTT AAA GAA AAG | subcloning sasC |
| CHsasC1for  | GCA ACG AAT CAA GGA TTG G | prevalence |
| CHsasC1rev  | TGA CAG CAC TTC GGT AGG | prevalence |
| CHsasC1rev  | GCA ACG AAT CAA GGA TTG G | prevalence |
| pCX19for    | CTA AAT CGA TTT CTG GCC C | sequencing |
| SasC60for   | GTATTGAGGAAGTAAAGTGG | sequencing |
| SasC70for   | CCA ACA ACT GAT CCT AAT GCC | sequencing |
| Sub1sasC4620rev | CAT CAG TGG CAT GTT CCC C | sequencing |
| Sub2sasC1380for | GAATATGGAATCTGCTGG | sequencing |
| SasC80rev   | CCATTGATTTGATGAAGCACC | sequencing |
| SasC2100rev | CCT CGA TAA CTT GTA TTG CTG C | sequencing |
| SasC3860for | GGC GAA GGC TAT TGA AGC GG | sequencing |
| SasC5040for | GAT GCA ATC CGA AAT ACG TTG G | sequencing |
of S. aureus Col, introducing a BamHI-site at the 3’ end. The PCR-amplified fragment was cloned into the vector pQE30Xa, so that the gene fragment is in frame with the His-codons. One representative clone expressing the DUF1542-repeat was designated E. coli (pHis-DUF1542) (see Fig. 1). The 6 x His-DUF1542 fusion protein was purified under native conditions via its His-tag using Ni-NTA affinity chromatography (Ni-NTA Spin Kit; Qiagen) according to the protocol of the suppliers (see Fig. 3B). The yield of the 6 x His-DUF1542 fusion protein was in the range of 150 µg per 10 ml culture volume as determined by the Coomassie Blue D250 Protein Assay (PIERCE; Rockford, IL, USA) distributed by Perbio Science, Bonn, Germany). The purified 6 x His-DUF1542 fusion protein was used to immunize rabbits by Eurogentec (Belgium) according to their standard immunization program.

Protein isolation, SDS-PAGE, and Western blot analysis

Staphylococcal surface proteins covalently linked to the peptidoglycan were prepared from cultures that were grown overnight in TSB broth at 37°C by lysostaphin treatment. For this, the staphylococcal cells were harvested by centrifugation, washed and then, the cell pellet was resuspended in 20 ml Tris-buffered saline (TBS) pH 7.4 per g bacteria. After adding 300 µg lysostaphin (Ambi Products LLC, Lawrence, NY, USA; distributed by WAK Chemie, Steinbach, Germany) and 100 µg DNase (Sigma) per g bacteria, the suspension was incubated at 37°C with shaking. Afterwards, the cell debris was removed by centrifugation (45 min, 13,000 rpm, 4°C). Lysostaphin lyses were stored at −20°C. Crude cell lysates of E. coli (pHis-DUF1542) were prepared from non-induced and induced (addition of 1 mM Isopropanol-D-thiogalactoside [IPTG] and continued growth of 4 h) cultures by harvesting the cells, resuspending the cell pellet in sample buffer, and heating the suspension for 5 min at 93°C. Additionally, as a negative control, a crude cell lysate was prepared from an induced culture of E. coli (pQE30). After centrifugation, 7 µl of the cell lysates from E. coli, 9 µl of the staphylococcal cell lysates, or 2 µl of the purified protein (containing 1.5 µg) were subjected to SDS-PAGE (10% separation gel and 4.5% stacking gel). Proteins were stained with Coomassie brilliant blue R250 (0.1%).

For Western immunoblot analysis, staphylococcal surface proteins, crude cell lysates, or purified proteins were prepared and separated by SDS-PAGE as described above and transferred to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The membranes were then blocked in Tris-buffered saline (TBS)/3% BSA (overnight) and washed three times with TBS/0.5% Tween 20 (TBST). Afterwards, the nitrocellulose membranes were incubated for 2 h with the anti-His-DUF1542 antiserum diluted 1:2000 in TBST/3% BSA. As a negative control, incubation was performed in TBST/3% BSA with 1 (1:2000) or without preimmune serum. The reaction of proteins with specific antibodies was detected by incubation (1 h) with anti-rabbit immunoglobulin G (IgG)/alkaline phosphatase (AP) conjugate (Dako GmbH, Hamburg, Germany). The membranes were then incubated with the anti-His-DUF1542 antiserum (Dako GmbH, Hamburg, Germany) diluted 1:5000 in TBS/0.5% Tween 20 and washed three times with TBS/0.5% Tween 20 (TBST). After centrifugation, 7 µl of the cell lysates from E. coli, 9 µl of the staphylococcal cell lysates, or 2 µl of the purified protein (containing 1.5 µg) were subjected to SDS-PAGE (10% separation gel and 4.5% stacking gel). Proteins were stained with Coomassie brilliant blue R250 (0.1%).

Purification and fluorescence-labelling of Fg, thrombospondin-1 (TSP-1), or von Willebrand factor (vWF)

Labelling of Fg was performed as described previously [64]. TSP-1 in an adhesive conformation was purified from freshly isolated human platelets as described before [65]. For labelling TSP-1 with fluorescein-isothiocyanate (FITC) (Calbiochem; La Jolla, CA, USA), FITC was added to TBS/2 mM CaCl2 containing TSP-1 in a molar ratio of 600:1 and incubated for 24 h at 4°C. Unbound label was removed was removed using a Sephadex G-25 PD-10 column equilibrated with TBS. The concentration of FITC-labeled TSP-1 was determined by using the Pierce BCA protein assay (Pierce Europe B.V., BA oud-Beijerland, The Netherlands) according to the manufacturers instructions. vWF was purified and labeled according to Harleib et al [66].

Flow cytometric analysis of Fg-FITC, TSP-1-FITC, or vWF-FITC binding to staphylococci

Measurement of the binding of Fg-FITC, TSP-1-FITC, or vWF-FITC to staphylococcal cells was analyzed as described before for the binding of vWF-FITC to S. aureus [66]. Briefly, bacteria from an overnight culture (120,000 cells/µl) were incubated with Fg-FITC, TSP-1-FITC, or vWF-FITC (final concentrations 0, 50, or 100 µg/ml) in TBS/2 mM CaCl2 for 10 min at room temperature. After washing and sonication, bacteria (5,000 cells/determination) were analyzed in a flow cytometer (Becton Dickinson, FACSCalibur flow cytometer, Heidelberg, Germany) using an excitation wave length of 480 nm at the FACSCalibur standard configuration with a 530 nm bandpass filter. Data were obtained from fluorescence channels in a logarithmic mode.

Preparation of platelets

Blood was taken from healthy adult volunteers who had not taken any medication affecting platelet function for at least 2 weeks before the study. Platelet-rich plasma (PRP) was prepared from anticoagulated blood by centrifugation and the platelets were gel-filtered on a Sephadex Cl-2B column [67]. To inhibit fibrin polymerisation, experiments were performed in the presence of the peptide GPRP (1.25 mM) as described previously [67]. The platelets were labeled by incubation with a monoclonal anti-CD42a (GP IX) antibody conjugated with PE at saturated concentrations for 30 min.

Preparation of bacteria and flow cytometric measurement of Staphylococcus-platelet associate formation

Staphylococcus-platelet associate formation was measured essentially as described before [68]. Briefly, bacteria grown over night were washed in TBS, briefly sonicated, and diluted with TBS/2 mM CaCl2 to 250,000 bacteria/µl. Bacteria were labeled with the fluorescent dye Syto 13 (emission similar to FITC) at a concentration of 2 µM for 10 min, washed in TBS and briefly sonicated again.

Platelets were activated with γ-thrombin at the given concentrations for 4 min and subsequently, labeled bacteria were added. Bacteria and platelets (10:1) were coincubated for 15 min at room temperature and conjugate formation was measured immediately thereafter in a flow cytometer. Associates were identified by double labelling with Syto 13 (FL-1, “FITC like” signal) and anti CD42a-PE (FL-2, PE signal), and given as the rate of bacteria-positive platelets. Given are the mean values of three independent experiments.

Immunofluorescence microscopy

The detection of SasC by immunofluorescence microscopy was performed essentially as described before [17]. Briefly, cultures were grown aerobically in TSB for 16 h at 37°C. After washing, aliquots (30 µl) were applied to glass slides. The slides were air-dried and the bacteria were fixed by heat. The fixed cells were incubated with anti-His-DUF1542 antiserum or preimmune
serum diluted 1:500 in PBS for 2 h at 37°C in a humid chamber, washed 4 times with PBS, and then incubated with FITC-conjugated anti-rabbit F(ab')2 fragment diluted 1:500 for 1 h at 37°C in a humid chamber. The slides were washed twice with PBS and twice with double-distilled water. Then, the slides were dried, covered with fluorescent mounting medium (Dako, Hamburg, Germany), and viewed with a fluorescence microscope (Zeiss, Oberkochem, Germany).

Nucleotide sequence accession number
The EMBL/GenBank/DDBJ accession number of the sasC DNA sequence of strain 4074 is FM202067.

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