Staphylococcus aureus mutants lacking cell wall-bound protein A found in isolates from bacteraemia, MRSA infection and a healthy nasal carrier

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Truncated SpA, not anchored to the cell wall, can still be found during carriage or infection, underlining the fact that there is no single virulence factor in Staphylococcus aureus that is required for generating a specific type of infection.

Keywords
staphylococcal protein A mutants; non-typeable spa; deviating spa repeat length.

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Introduction

Staphylococcus aureus is a major human pathogen, producing a multitude of virulence factors making it able to cause several types of infection, from superficial lesions to life-threatening systemic conditions such as endocarditis, osteomyelitis, pneumonia, meningitis and sepsis. Despite the extensive number of virulence factors, S. aureus is also a commensal, persistently colonizing the anterior nares of 20–30% within a healthy population (Wertheim et al., 2005; Olsen et al., 2013). Virulence factors include both surface proteins (exponential growth phase) and secreted proteins (stationary phase). Surface proteins are involved in attachment (MSCRAMMs) and evasion of the host immune response [e.g. leukocidins, staphylococcal protein A (SpA), CHIPS and Eap] (Gordon & Lowy, 2008). SpA contributes to S. aureus pathogenesis by interfering with the immune responses (Foster, 2005) and activating inflammation (Gomez et al., 2004).

SpA is a 40–60 kDa protein with an N-terminal signal sequence, followed by an immunoglobulin G (IgG)-binding region and an X region consisting of: (1) a repeat region $X_R$, with a variable number of repeats, each of ~ 8 amino acids (aa) length and (2) a C-terminal region, $X_C$ (Fig. 1, see FPR3757). The signal sequence leads SpA into a protein export pathway, whereas the C-terminal elements, consisting of a LPXTG motif, followed by a hydrophobic domain and a charged tail (Schneewind et al., 1993), are involved in covalent anchoring of the protein to the cell wall. SpA with mutations in the charged tail will be secreted into the medium instead of being anchored to the cell wall, whereas mutations in the LPXTG motif result in proteins retained in the cellular compartments however not anchored to the cell wall (Schneewind et al., 1992). The polymorphic
The X<sub>R</sub> region is the basis for spa typing and contains a variable number of repeats that are generated by deletions, duplications and point mutations (Shopsin et al., 1999). Recently, the X<sub>R</sub> region was found to be required for type I IFN activation in lungs during infection of animals (Martin et al., 2009). The IgG-binding region consists of five highly homologous IgG-binding domains of ~58 aa each, designated E, D, A, B and C (Uhlen et al., 1984). Isolates carrying four repeats of the IgG-binding domains have also been found (Sjödahl, 1977). The IgG-binding domains bind tightly to the Fc region of IgG (Moks et al., 1986), limiting opsonization and hence phagocytosis as the bacterial cells are coated with IgG in a conformation not recognized by neutrophils. Each IgG-binding domain can also bind to the Fab region of the VH3 subclass immunoglobulins (Jansson et al., 1998), resulting in B lymphocyte apoptosis (Goodyear & Silverman, 2003). SpA has also been found to interact with tumour necrosis factor receptor 1 (TNFR1) (Gomez et al., 2006), von Willebrand factor (Hartleib et al., 2000) and platelet gC1qR/p33 (Nguyen et al., 2000) and has recently been shown to promote bacterial aggregation and formation of biofilms (Merino et al., 2009), as well as binding to osteoblasts, thereby triggering signals that weaken bone in osteomyelitis (Claro et al., 2011).

This work was initiated when spa repeats of deviating lengths were identified in S. aureus isolates from five blood cultures as well as one methicillin-resistant S. aureus (MRSA) infection and one carrier isolate of S. aureus. The aims of this study were to investigate the implications of these frameshift mutations in the spa repeat region, to find whether the frameshift mutations would cause truncations of SpA and to determine the localization of the expressed SpA. The putative size of each SpA was calculated from the full spa gene sequence, and the localization of SpA, as well as size estimates, was determined by Western blot using antibodies specific for SpA.

Parts of this study were presented at the 14th International Symposium on Staphylococci and Staphylococcal Infections (ISSSI), Bath, UK, September 6–9, 2010.

**Materials and methods**

**Staphylococcus aureus isolates**

Each year approximately 1500 S. aureus bacteraemia isolates and 800 MRSA isolates are received by the National Staphylococcus Reference Laboratory at Statens Serum Institut, Denmark, as part of the national surveillance of S. aureus bacteraemia and MRSA. All isolates have since 2007 been typed as well as tested for the presence of mecA and pvl as previously described (Larsen et al., 2008). Among 12 000 isolates in the period of 2004–2009, six isolates were found with deviating spa repeats: one MRSA causing infection and five bacteraemia isolates (Table 1). Additionally, one deviating spa repeat was identified in two different isolates in 2008 in a large Norwegian S. aureus collection (n = 2459) from the population-based Tromsø Staph and Skin Study (Sangvik et al., 2011). Both isolates were from the same individual, sampled at two different time points, 32 days apart. In all the seven isolates with deviating spa repeats, the variable X region could be amplified with the spa typing PCR primers spa-1113f and spa-1514r (Strommenger et al., 2006), but due to the deviating repeats, the isolates were designated nontypeable (NT) in the Ridom STAPHTYPE software v2.2.1 (Ridom GmbH, Würzburg, Germany) (Harmsen et al., 2003).

*Fig. 1* Spa of S. aureus FPR3757 (GenBank accession no. NC_007793) demonstrates a full-length protein A, harbouring an N-terminal signal peptide (S), five Ig-binding domains (E, D, A, B and C), a variable repeat region (X<sub>R</sub>, one white box per repeat), and a C-terminal region for anchoring to the cell wall (X<sub>C</sub>). Sequencing results from the spa locus for seven S. aureus isolates with deviating spa repeat lengths: 6101235, 42100, 60558, 62309, 65947, 67702 and 68536 (GenBank accession nos.: KC142149, KC142143, KC142144, KC142145, KC142146, KC142147 and KC142148, respectively); display deviating repeats with frameshift mutations (black box) and the frameshifted translated region upstream of the premature stop codon (grey box). The calculated size includes the signal peptide of 36 aa/3.6 kDa (DeDent et al., 2008). *A faint band of 50 kDa was observed in the cellular fraction (Fig. 2).
spa gene amplification and sequencing

The entire spa coding sequence including the promoter region was amplified using three different primer sets, including the previously described primers for amplification of the variable X region for typing (Strommenger et al., 2006). The novel primers are listed in Table 2. All PCRs were set up with 0.5 μM of each primer, 0.16 μM dNTP, 3 mM MgCl₂, and 1 U of AmpliTaq Gold (Life Technologies, CA) with the following cycling conditions: 95 °C for 7 min; 35 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min; and 72 °C for 10 min. The sequencing was performed using a standard sequencing protocol (Applied Biosystems by Life Technologies) and an ABI 3130XL sequencer (Applied Biosystems), and the sequences were analysed with BIONUMERICS version 6.0 or 6.5. Two additional primers, spa-seq-1 and spa-seq-2, were included for sequencing of the promoter region and the 5′-end of the spa gene.

Multilocus sequence typing (MLST) and eBURST

MLST was performed as described previously (Enright et al., 2000) on the seven isolates with deviating spa repeats. PCR products were sequenced on both strands, and multilocus sequence types were assigned using the S. aureus MLST website. Clonal complexes were assigned using eBURST on the entire public S. aureus MLST database (May 2012). We acknowledge the use of the S. aureus MLST database that is located at Imperial College London and is funded by the Wellcome Trust.

Western blot analyses

Western blot analyses were performed on bacterial extracts and culture supernatants derived from S. aureus cells grown in brain–heart infusion (BHI) broth (Oxoid, Cambridge, UK) at 37 °C, 220 r.p.m. Stationary phase cells were obtained from overnight cultures diluted in BHI to OD₆₀₀ nm ~ 0.3, whereas exponential phase cells were obtained by diluting overnight culture 1 : 100 in BHI and incubating as above to OD₆₀₀ nm ~ 0.3. Exponential- and stationary phase cells were pelleted, and the culture supernatants were collected for analysis. The pelleted cells were washed twice in 5 mL PBS (Biochrom, Berlin, Germany) before resuspension in 3.5 mL or 100 μL PBS (for 35-fold upconcentration compared to the supernatant). Thirteen microlitre supernatant or resuspended cells from pellet were added 2-μL dithiothreitol (DTT; Sigma-Aldrich, St. Louis, MO) and 5-μL NuPAGE LDS sample buffer (Invitrogen by Life Technologies) and incubated at 70 °C for 10 min and stored at −20 °C. Samples were analysed as previously described (Johannessen et al., 2007). Briefly, proteins were separated on NuPAGE Novex Bis-Tris 4–12% gels using MES SDS running buffer, NuPAGE antioxidant and MagicMark Western protein standard, all purchased from Invitrogen. The gel was blotted onto 0.45-μM pore size polyvinylidene difluoride membrane (MerckMillipore, Billerica, MA). SpA was detected by a staphylococcal protein A primary antibody: monoclonal antiprotein A, clone SPA-27 (Sigma-Aldrich) and HRP-conjugated secondary}

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**Table 1** Staphylococcus aureus isolates and deviating repeats

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Methicillin resistance</th>
<th>Repeat succession</th>
<th>Sequence of deviating repeat</th>
<th>Size of DR (bp)</th>
<th>Premature stop codon</th>
<th>MLST type</th>
<th>MLST CC</th>
</tr>
</thead>
<tbody>
<tr>
<td>61011235</td>
<td>Nasal carrier</td>
<td>MSSA</td>
<td>08-16-02-16-DR-13-17-34-16-34</td>
<td>AAAGAAGACAACAAAAACCTGGT</td>
<td>25</td>
<td>TAA</td>
<td>45</td>
<td>45</td>
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<tr>
<td>42100</td>
<td>Infection</td>
<td>MRSA</td>
<td>26-DR-17-20-17-12-16-17-34</td>
<td>AAAGAAGACGGCAACAAA</td>
<td>28</td>
<td>TAA</td>
<td>228</td>
<td>5</td>
</tr>
<tr>
<td>60558</td>
<td>Bacteraemia</td>
<td>MSSA</td>
<td>08-16-02-16-DR-17-34</td>
<td>AAAGAAGACAACAAAACCTGGT</td>
<td>23</td>
<td>TAA</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>62309</td>
<td>Bacteraemia</td>
<td>MSSA</td>
<td>07-23-12-34-34-12-23-DR-12-23</td>
<td>AAAGAAGACAACAAAAACCTGGC</td>
<td>25</td>
<td>TAA</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>65947</td>
<td>Bacteraemia</td>
<td>MSSA</td>
<td>11-19-DR-93-17-34-24-34-22-25</td>
<td>AAAGAAGACAACAA</td>
<td>14</td>
<td>TGA</td>
<td>2384</td>
<td>8</td>
</tr>
<tr>
<td>67702</td>
<td>Bacteraemia</td>
<td>MSSA</td>
<td>11-19-12-21-17-34-24-DR-22-25</td>
<td>AAAGAAGACAACAAAAACCTGGT</td>
<td>25</td>
<td>TAA</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>68536</td>
<td>Bacteraemia</td>
<td>MSSA</td>
<td>07-23-12-34-DR-12-12-23-02-12-23</td>
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<td>25</td>
<td>TAA</td>
<td>58</td>
<td>15</td>
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</table>

DR, deviating repeat.

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**Table 2** Primers designed in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>spa-dns-f</td>
<td>CTTCATCCAAAGCCTTA</td>
</tr>
<tr>
<td>spa-dns-r</td>
<td>GAGTAGAAAAGTGGAG</td>
</tr>
<tr>
<td>spa-ups-f</td>
<td>TGTCTTCCTCTTTTGGTG</td>
</tr>
<tr>
<td>spa-ups-r</td>
<td>GTGTGCTGTATCTAAAGTG</td>
</tr>
<tr>
<td>spa-seq-1</td>
<td>CTTTGGAGCTTGAGAGT</td>
</tr>
<tr>
<td>spa-seq-2</td>
<td>GATGATCCAAGCCAAAGT</td>
</tr>
</tbody>
</table>

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antibody: anti-mouse IgG HRP-linked antibody (Cell Signalling Technology, Beverly, MA). The Western Blotting luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a chemiluminescence substrate. *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228 were included as positive and negative control, respectively, for protein A in the Western blot analyses.

**Results**

**Sequencing revealed frameshift mutations and premature stop codons**

The complete SpA-encoding region, in addition to 300 bp upstream and 130 bp downstream from the spa consensus, was determined for all seven isolates included in this study (Fig. 1). None of the isolates had identical spa repeat successions, although the same deviant repeat was found in three of the isolates. Six isolates had a deviation associated with the span of adenines in the 5th and 6th codons of a regular 24- or 27-bp repeat, with one base insertion or deletion, resulting in repeats of 23, 25 or 28 bp (Table 1). The seventh isolate contained either a short spa repeat of 14 bp followed by a 24-bp repeat (r93) or possibly one 38-bp repeat formed by fusion of two repeats. The seven deviating spa repeats all caused frameshift mutations in the SpA coding sequence, leading to a premature translational stop. The truncations occurred upstream of the cell wall-binding recognition sequence LPXTG, suggesting that the final gene product would be unable to bind to the cell wall. The predicted size of each truncated protein varied between 32 and 47 kDa as indicated in Fig. 1, including the signal sequence of 36 aa/3.6 kDa. The SpA-encoding sequence from five isolates displayed five IgG-binding domains, whereas in isolates 42100 and 61011235, only four IgG-binding domains were observed, with domain C and domain A absent, respectively. The deletions of these domains were in frame, not causing premature translational stops upstream of the repeat region. Six to 12 bp upstream of the translation start site of the spa gene, a putative ribosomal binding site (RBS) was observed, with the sequence 5'-AGGGGG-3' in six of the seven isolates. The exception was isolate 65947, displaying 5'-AAAGGG-3'.

**MLST displayed the epidemiological distance between the seven isolates**

The isolates were assigned to six different MLST types: 8, 15, 45, 58, 228 and the novel ST2384 due to the novel *gfp* allele type 292 (Table 1) and belonged to four different clonal complexes (CCs); CC5, CC8, CC15 and CC45.

**Western blotting confirmed truncation and secretion of SpA**

Analysis of the seven atypical spa gene sequences (Fig. 1) suggested that the isolates would produce a truncated protein A lacking the cell wall-binding ability. To investigate this, a Western blot was performed on both bacterial cells and culture supernatants. A ∼ 50-kDa band, corresponding to a full-length protein A, was detected in the cellular fraction of the wild-type *S. aureus* ATCC 25923 grown to stationary phase (Fig. 2). The culture supernatant from the wild type contained a diffuse band of ∼ 60 kDa that was recognized by the SpA-specific antibodies. From the Western blot analysis, it was found that for six of the seven isolates, SpA was mainly present in the supernatant and that the size of the truncated proteins corresponded well with the sizes predicted from the sequence analyses (Fig. 1). The exception was isolate 65947, for which no SpA could be detected, neither in the bacterial pellet nor in the supernatant. For isolates 67702, 68536 and 61011235, unspecific bands of ca. 50 kDa were detected in the cellular fraction. In general, similar results were obtained using bacterial cells in exponential growth; however, several weak bands of lower molecular weight were detected in the Western blot analysis (data not shown).

**Discussion**

When analysing a large material of ∼ 12 000 isolates from bacteraemia or infection and ∼ 2500 colonization isolates from healthy carriers, seven naturally occurring SpA mutants with frameshift mutations in the *spa* region were found. All the isolates were predicted to be truncated in the C-terminal end of SpA, lacking the elements for covalent binding to the cell wall. SpA is a highly conserved virulence factor of *S. aureus*, and isolates devoid of SpA are rarely observed. The widespread conservation and abundance of SpA in *S. aureus* isolates indicate that the protein plays an important role, and SpA-deficient mutants were phagocytosed more efficiently by neutrophils *in vitro* (Gemmell & O’Dowd, 1983). The contribution of SpA to virulence has previously been studied in animal models, and SpA-deficient mutants were found to have decreased virulence in several animal infection models (Patel *et al.*, 1987; Gemmell *et al.*, 1997; Palmqvist *et al.*, 2002). However, five of the SpA mutants addressed in this study were found in blood.
cultures, and one SpA mutant was isolated from an MRSA infection. This suggests that the isolates are still virulent despite the altered amino acid sequence and SpA not being attached to the cell wall (Fig. 1). One isolate (65947) seemed to completely lack SpA expression, but still had the ability to cause bacteraemia, indicating that despite the multifunctional immune evasion properties of SpA, it is only one of many factors contributing to staphylococcal disease. The SpA mutant strain from the healthy nasal carrier was isolated from the same individual at two occasions, 32 days apart, indicating that cell wall-attached SpA is not crucial for persisting in a healthy carrier. SpA was not attached to the cell wall in any of the studied isolates. However, it was still expressed and secreted into the extracellular environment in six of seven cases, and the IgG-binding region was intact. Merino et al. (2009) observed that SpA could induce biofilm formation without being anchored to the cell wall, and hence, SpA would still be beneficial for \textit{S. aureus}, even in the secreted form. Indeed, a potential immune evasion strategy as a consequence of chemokine-induced SpA release has been suggested (Yung et al., 2011).

The three isolates sharing an identical deviant spa repeat were found in isolates belonging to three different MLST types and three different CCs, indicating that these mutations have occurred at several independent occasions. Six of the seven atypical spa repeats were associated with a run of six adenines, displaying one adenine insertion or deletion compared to a regular length repeat. Frameshift mutations tend to occur at repetitive DNA sequences or nucleotide runs and are described by Streisinger's model of replication slippage, where a misalignment at repetitive DNA sequences can produce either addition or deletion of nucleotides (Lovett, 2004). For isolate 65947, the short deviating repeat was possibly a result of a deletion or a duplication of parts of a repeat, resulting in a short 14-bp fragment between the spa repeats r19 and r93 in the X\textsubscript{R} region. Alternatively, a fusion of two neighbouring repeats could have formed one 38-bp repeat. Actually, the observed sequence could be obtained from spa type 1008, which is common in Denmark, by fusing parts of the r12 and r21 repeats.

The regulation of spa expression is complicated, involving multiple factors, including Agr, SarA, SarS, SarT, Rot and MgrA (Gao & Stewart, 2004). Sequencing of the promoter region of spa did not reveal major differences between isolate 65947 and the other six isolates; however, the two substitutions in the RBS region may affect translation and hence explain the absence of SpA on Western blot for this isolate.

Obtaining a spa type for isolates with a deviating repeat length is currently not possible, and as they are reported NT in the commercially available spa typing softwares, it is difficult to know how widespread these mutants are. In a study by Baum et al. (2009), non-spa-typeable clinical \textit{S. aureus} isolates were described that had a deletion in the IgG-binding domain C, in which the forward primer for spa typing is located. These isolates could however be typed using alternative primers as the X\textsubscript{R} region was intact and complied with the rules for the spa typing nomenclature. The isolates presented from our study, all gave a product in the Ridom spa typing PCR, as the primer binding sites were still intact.

Naturally occurring mutants secreting SpA into the extracellular environment were sporadically observed among isolates involved in disease and carriage, indicating that cell wall-bound SpA is not essential for survival and virulence of \textit{S. aureus} in the host. This reminds us that with the abundance and redundancy of virulence factors in \textit{S. aureus}, including surface proteins, removing just one factor is not enough to leave this bacterium unable to cause disease.

**Acknowledgements**

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**Authors’ contribution**

Marit Sorum and Maria Sangvik contributed equally to the study.

**References**


