Journal of Dairy Research (2006) **73** 322–327. *© Proprietors of Journal of Dairy Research 2006*
doi:10.1017/S002202990600183X *Printed in the United Kingdom*

Comparative sequence analysis of *spa* gene of *Staphylococcus aureus* isolated from bovine mastitis: characterization of an unusual *spa* gene variant

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Received 28 October 2005 and accepted for publication 5 January 2006

The protein A encoding gene *spa* of four *Staphylococcus aureus* strains isolated from bovine clinical mastitis was amplified by PCR and sequenced. The four strains were selected after an initial screening of *spa* gene of 41 strains isolated from mastitic cows and were subjected to detailed investigations. According to the sequencing results the *spa* gene of three strains (M1, M2, M3) appeared with gene segments encoding five (E, D, A, B and C) and four (E, A, B and C) IgG binding domains for two (M1, M3) and one (M2) strain, respectively and with gene segments encoding four, two and two repeats of the octapeptide Xr-repeats for the strains M1, M2 and M3, respectively. For the remaining *Staph. aureus* strain (M4) gene segments encoding IgG binding domains E, D and A and a new domain BC with a size of 219 bp could be observed. The BC domain appears, with a deletion of a 123 bp segment from the border region between both domains, as fused domain of both previously characterized domains. The Xr-region of this strain had 11 octapeptide repeats.

Keywords: Cattle, mastitis, *Staphylococcus aureus*, *spa* gene, protein A, sequence variation.

Staphylococcus aureus is a well known pathogen causing human and animal infections. These bacteria produce many potential virulence factors also including cell wall-anchored surface proteins with binding properties for different host proteins (Navarre & Schneewind, 1999). Among these, protein A, a 42-kDa molecule existing in a secreted and membrane-associated form, interacts with a variety of human and animal imunoglobulins. It consists in turn of five highly homologous extracellular Ig-binding domains, designated as domains E, D, A, B, and C. Structural studies have shown that 11 amino acids of the IgG binding region which are mostly located in two α helical regions are essential for immunoglobulin binding. The IgG binding domains are followed by the X region responsible for the binding to the peptidoglycan. This region consists, in turn, of two structurally different domains, a highly repetitive polymorphic region with a variable number of repeats (Xr) with an octapeptide structure, followed by the second region (Xc) which encodes for the 81 amino acids constant C-terminal cell wall attachment sequence (Guss et al. 1984; Uhlen et al. 1984).

Protein A, expressed by most strains of *Staph. aureus*, possesses two distinct Ig-binding activities: each domain can bind Fcγ, the constant region of IgG involved in effector functions, and Fab responsible for antigen recognition in a non-antigen specific manner (Boyle, 1990a).

The role of protein A as a potential virulence factor of *Staph. aureus* has been pointed out by various authors (Marone et al. 1987; Genovese et al. 2000; Hartleib et al. 2000; Palmqvist et al. 2002).

However, protein A has unique binding properties and is widely used for various laboratory techniques such as affinity purification of polyclonal and monoclonal antibodies, immunoprecipitation, ELISA, dot blot and in western immunoblotting (Boyle, 1990b). In addition, it is well known that protein A has antitumour and

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https://doi.org/10.1017/S002202990600183X Published online by Cambridge University Press

immunostimulatory properties (Das et al. 2002). Indications for a protein A-based extracorporal immunoadsorption had been summarized by Matic et al. (2001).

In the present study the protein A encoding gene *spa* of *Staph. aureus* strains isolated from bovine mastitis was amplified by PCR, sequenced and compared with already available sequences of the GenBank.

Materials and Methods

Bacterial isolates, identification and molecular characterization

The initially investigated 41 *Staph. aureus* strains isolated from mastitic cows in Mexico had been characterized genotypically as described by El-Sayed et al. (2006). The protein A encoding gene *spa* of these strains was characterized by molecular analysis amplifying *spa* gene and the gene segments encoding the IgG binding domains and the Xr-encoding region. Amplification of the gene was performed using the oligonucleotide primer Spa 1 (5′- CAC CTG CTG CAA ATG CTG CG-3′) and Spa 4 (5′- CAC CAG GTT TAA CGA CAT-3′) and the following thermocycler programme: 1 × denaturation at 94 °C for 180 s, followed by 30 times 94 °C for 60 s, 58 °C for 60 s, 72 °C for 60 s, and a final cycle at 72 °C for 5 min. This programme was also used, as described by Seki et al. (1998), with the oligonucleotide primer Spa 1 and Spa 2 (5′- GGC TTG TTG TTG TCT TCC TC-3′) for amplification of the gene segment encoding the IgG binding domains. To amplify the Xr encoding part of *spa* gene, the oligonucleotide primer Spa 3 (5′- CAA GCA CCA AAA GAG GAA-3′) and Spa 4, according to Frénay et al. (1996), were used, with the thermocycler programme: 1 × denaturation at 94 °C for 180 s, followed by 30 times 94 °C for 60 s, 60 °C for 60 s, 72 °C for 60 s, and a final cycle at 72 °C for 5 min.

Isolation of genomic DNA was carried out with 3–5 colonies of freshly subcultured *Staph. aureus* strains. The colonies were homogenized in 50 µl TE buffer (10 mmol of Tris HCl/l, 1 mmol of EDTA/l, pH 8·0), followed by the addition of 1 µl lysostaphin (1·8 U/µl; Sigma, Deisenhofen, Germany). After incubation for 1 h at 37 °C, 1 µl proteinase K (15·1 mg/ml, Boehringer, Mannheim, Germany) was added and the suspension was reincubated for 2 h at 56 °C. Proteinase K was finally inactivated by boiling the mixture for 10 min. After centrifugation at 10 000 ***g*** for 5 min the supernatant was cooled on ice before use in PCR.

For PCR amplification, the reaction mixture (20 µl) contained 0·7 µl of primer 1 (10 pmol/µl), 0·7 µl of primer 2 (10 pmol/µl), 0·4 µl of deoxynucleoside triphosphate (10 mmol/l; MBI Fermentas, St. Leon-Rot, Germany), 2·0 µl of 10 × thermophilic buffer (Promega, Mannheim, Germany), 1·2 µl of $MgCl_2$ (25 mmol/l; Promega), 0·1 µl of *Taq* DNA polymerase (5 U/µl, Promega), and 12·9 µl of distilled water. Finally, 2·0 µl of DNA preparation was added to each 0·2-ml reaction tube.

The tubes were subjected to thermal cycling (T3 thermocycler, Biometra, Göttingen, Germany) with the programmes described above. The presence of PCR products was determined by electrophoresis of 10 µl of the reaction product in a 1·5 % agarose gel with 1 × TAE buffer (40 mM-Tris–HCl, 1 mM-EDTA, 1·14 ml glacial acetic acid/l, pH 7·8).

For sequencing the *spa* gene of four selected *Staph. aureus* strains (M1, M2, M3 and M4) was amplified by PCR. The amplicons were eluted from the gel using QIAEX®II (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Sequencing was performed by Sequence Laboratories (Göttingen, Germany).

A sequence blast was carried out using the data base of the National Centre for Biotechnology Information (NCBI) available under http://www.ncbi.nlm.nih.gov/BLAST.

Results

Amplification of *spa* gene of the 41 investigated strains yielded amplicon sizes of approximately 1150 bp (*n*=3), 1120 bp (*n*=1), 1100 bp (*n*=6), 1080 bp (*n*=5), 1050 bp (*n*=2), 1020 bp (*n*=7), 1000 bp (*n*=8), 950 bp (*n*=1), 820 bp (*n*=7) and 800 bp (*n*=1). With the exception of strain M4 with an amplicon size of 800 bp, all amplicons of the gene segment encoding the IgG binding region revealed a size of 900 bp (*n*=30) or 700 bp (*n*=10). Amplification of the gene segments encoding the Xr-region yielded amplicons of approximately 320 bp (*n*=2), 250 bp (*n*=4), 200 bp (*n*=6), 180 bp (*n*=5), 150 bp (*n*=2), 120 bp (*n*=13) and 100 bp (*n*=9).

Amplification of *spa* gene of the four selected strains yielded sizes of 1050, 800, 1000 and 1120 bp for the strains M1, M2, M3 and M4, respectively. Investigating the gene segments encoding the IgG binding region and the Xr-region with the oligonucleotide primer Spa 1 and Spa 2 and Spa 3 and Spa 4, respectively revealed amplicon sizes of 900 bp (M1 and M3), 800 bp (M4) and 700 bp (M2) for the IgG binding domain encoding segments (Fig. 1) and 320 bp (M4), 150 bp (M1) and 100 bp (M2 and M3) for the Xr-region encoding segments, respectively. Amplicon sizes of 900 and 700 bp corresponded, according to Uhlen et al. (1984), to 5 and 4 IgG binding repeats, amplicon sizes of 320, 150 and 100 bp, according to Frénay et al. (1996), to 11, 4 and 2 Xr-repeats, respectively. According to Uhlen et al. (1984), the size of 800 bp did not correspond to any of the known *spa* gene variants. Sequencing the gene segments encoding the IgG binding domains yielded segments encoding the domains E, D, A, B and C for the *Staph. aureus* strains M1 and M3 and E, A, B and C for strain M2. Sequencing the gene segment encoding the IgG binding domains of strain M4 yielded segments encoding the domains E, D and A and a fused domain BC (Fig. 2). Sequencing the Xr-region encoding gene segments revealed, according to Ridom SpaServer-Nomenclature (http://www.ridom.de/spaserver/nomenclature.shtml;

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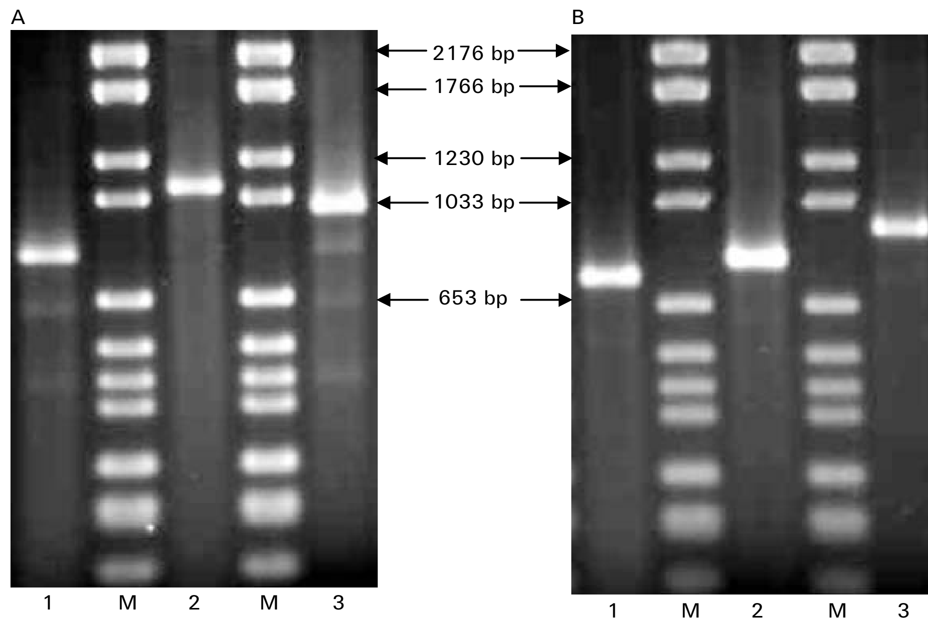

Fig. 1. Typical PCR products of *spa* gene of the *Staph. aureus* strains M2 (1), M4 (2) and M3 (3) using the oligonucleotide primer Spa 1 and Spa 4 with sizes of approximately 800 bp, 1120 bp and 1000 bp, respectively (A) and by using the oligonucleotide primer Spa 1 and Spa 2 with sizes of 700 bp, 800 bp and 900 bp, respectively (B). M=DNA molecular weight marker VI (Roche Diagnostics GmbH, Mannheim, Germany).

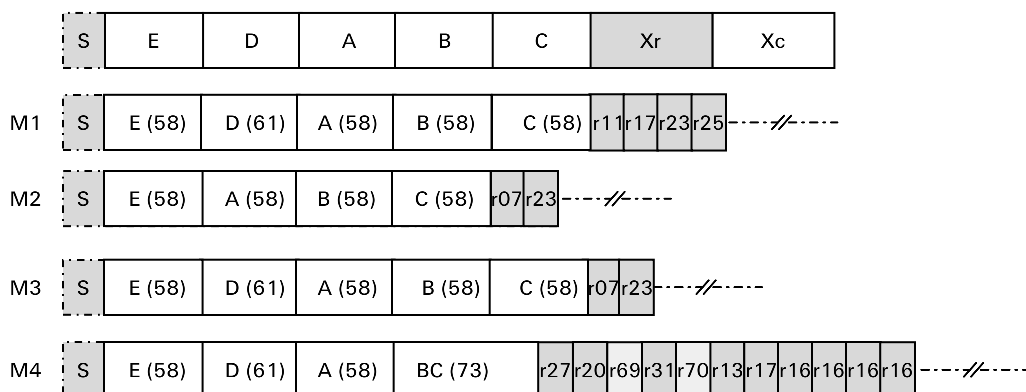

Fig. 2. Schematic structure of protein A of the four selected *Staph. aureus* strains following the pattern described by Guss et al. (1990); S is the signal sequence, E, D, A, B, C, BC are the five IgG-binding subunits and the fused domain BC and their length in amino acids. Xr is the repetitive part followed by Xc in the C-terminal part of the X region. The classification of the octapeptides of Xr-region was performed according to Ridom SpaServer-Nomenclature. In the present work two novel octapeptides are firstly reported (r69 and r70).

Harmsen et al. (2003)), the octapeptide classes shown in Fig. 2. With the exception of the fused domain BC, sequence analysis of all investigated domains corresponded to those reported by Tashiro & Montelione (1995) showing the presence of the three helices I, II and III, which are characteristic for the protein A domains. Sequencing of domain BC yielded the presence of the helical bundles of helix I of domain B and helix I, II and III of domain C, respectively (Fig. 3). The sequences were given to GenBank under the following accession numbers: AJ606379, AJ606380, AJ606381 and AJ829762 for strain M1, M2, M3 and M4, respectively.

Discussion

In the present study the protein A encoding gene *spa* of four selected *Staph. aureus* strains was further characterized by PCR amplification and sequencing. The four strains were isolated together with 37 additional *Staph.*

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Strain		H 1		H 2	H 3
8325/B	ADNKFNK	EQQNAFYEIL	HLPNL	NEEQRNGFIQSLKD	DPSQSANLLAEAKKLNDAQAPK
MW2/B	ADNKFNK	EQQNAFYEIL	HLPNL	NEEQRNGFIQSLKD	DPSQSANLLAEAKKLNDAQAPK
Cow /B	ADNKFNK	EQQNAFYEIL	HLPNL	NEEQRNGFIQSLKD	DPSQSANLLAEAKKLNDAQAPK
M1 /B	ADNNFNK	EQQNAFYEIL	HLPNL	NEEQRNGFIQSLKD	DPSVSTDILAEAKKLNDAQAPK
M2 /B	ADNKFNK	EQQNAFYEIL	HLPNL	NEEQRNGFIQSLKD	DPSQSANLLAEAKKLNDAQAPK
M3 /B	ADNKFNK	EQQNAFYEIL	HLPNL	NEEQRNGFIQSLKD	DPSQSANLLAEAKKLKNAQAPK
M4 /B	ADNKFNK	EQQNAFYEIL	HLPNL	NE------------------------------	←Δ 41 amino acids (123 bp) → ----
M4 /C	----------------	EQQNAFYEIL	HLPNL	TEEQRNGFIQSLKD	DPSVSKEILAEAKKLNDAQAPK
8325/ C	ADNKFNK	EQQNAFYEIL	HLPNL	TEEQRNGFIQSLKD	DPSVSKEILAEAKKLNDAQAPK
Mu50/C	ADNKFNK	EQQNAFYEIL	HLPNL	NEEQRNGFIQSLKD	DPSVSKEILAEAKKLNDAQAPK
MW2/C	ADNKFNK	EQQNAFYEIL	HLPNL	TEEQRNGFIQSLKD	DPSVSKEILAEAKKLNDAQAPK
N315/C	ADNKFNK	EQQNAFYEIL	HLPNL	NEEQRNGFIQSLKD	DPSVSKEILAEAKKLNDAQAPK
Cow /C	ADNKFNK	EQQNAFYEIL	HLPNL	TEEQRNGFIQSLKD	DPSVSKEILAEAKKLNDAQAPK
M1 /C	ADNKFNK	EQQNAFYEIL	HLPNL	TEEQRNGFIQSLKD	DPSVSKEILAEAKKLNDAQAPK
M2 /C	ADNKFNK	EQQNAFYEIL	HLPNL	TEEQRNGFIQSLKD	DPSVSKEILAEAKKLNDAQAPK
M3 /C	ADNKFNK	EQQNAFYEIL	HLPNL	TEEQRNGFIQSLKD	DPSVSKEILAEAKKLNDAQAPK

Fig. 3. Amino acid sequence of domain B and C of the *Staph. aureus* strains 8325-4 (J01786), MW2 (NC_003923), Mu50 (NC_002758), Cowan 1 (M18264) and the four *Staph. aureus* strains characterized in the present study. The deleted fragment of fused domain BC of strain M4 (Δ 123 bp) corresponds to 41 amino acids leading to the conservation of the open reading frame. The two α-helices, according to Guss et al. (1990), essential for binding to IgG are indicated as boxes. The arrows represent, according to Darwin & Daggett (2000), the helices H1, H2 and H3, respectively.

aureus strains from mastitic cows in Mexico and were characterized previously for various genotypic properties (El-Sayed et al. 2006). Amplification of *spa* gene of the four strains with the oligonucleotide primer Spa 1 and Spa 4 yielded amplicon sizes of approximately 1050, 800, 1000 and 1120 bp for the strains M1, M2, M3 and M4, respectively, indicating size and sequence polymorphisms among these four strains. The two gene segments encoding the IgG binding domains and the Xr-region could be further analysed by the use of the oligonucleotide primer Spa 1 and Spa 2 and Spa 3 and Spa 4, respectively. According to the published *spa* gene sequences, the gene segment encoding the IgG binding domains usually consists of five IgG binding segments as described for the *Staph. aureus* strains MW2 (NC_003923), 8325-4 (J01786) and Cowan 1 (M18264). However, the absence of one or more of the five IgG binding domains was reported for the *Staph. aureus* strain N315 (NC_002745) and Mu50 (NC_002758) and for some *Staph. aureus* strains described previously by Schwarzkopf et al. (1993), Seki et al. (1998), Akineden et al. (2001) and Stephan et al. (2001). Amplification of the *spa* gene segment encoding the IgG binding region of the four strains of the present study revealed that strain M1 and M3 had five IgG binding domains, while strain M2 consisted of four domains owing to the absence of domain D. Although all five domains are highly homologous in sequence and each domain possesses Ig-binding capacity, previous investigations demonstrated that the D domain plays a major role in IgG binding because of its larger size or because of its ability to bind both VH3 Fab- and Fcy fragments (Roben et al. 1995). Jansson et al. (1998) reported that all five domains of Spa were able to bind Fc and Fab. However, according to Graille et al. (2000), helix II and III of domain D interact with the variable region of Fab heavy chain while helix I of domain B interacts with Fcγ with lesser involvement of helix II. The importance of the lack of domain D for IgG binding activity of strain M2 or for the virulence of this strain in bovine mastitis is not known at present. However, the *Staph. aureus* strains N315 and Mu50 with four IgG binding domains lack domain C.

PCR amplification and sequencing of the gene segment encoding the IgG binding region of strain M4 revealed the IgG binding domains E, D, A and a fused domain BC. The domains B and C, each represented by 58 amino acids appeared, through the deletion of 41 amino acids from the border region, as a novel 75 amino acid domain BC. The deleted fragment with a size of 123 bp, which caused the fusion of both domains, leads in turn to the conservation of the open reading frame of this segment of protein A. In addition, the deleted fragment did not affect the sequences of the helical structure. Sequence analysis of domain BC of strain M4 revealed the presence of four helices, namely I, I, II and III. Fusion of domain B and C occurred at a 9 bp direct repeat (AAG AAC AAC) which normally exists at the beginning of helix I and II.

However, a mutated *spa* gene described by Guss et al. (1985) showed a point mutation leading to the induction of a stop codon in the gene (X03286), which in turn terminates the gene translation.

Sequence analysis of the Xr-encoding region detected 4, 2, 2 and 11 repeats for the strains M1, M2, M3 and M4, respectively. Sequence variations of this region were reported by numerous authors and had already been used for epidemiological studies (Frénay et al. 1996; Walker et al. 1998; Shopsin et al. 1999; Stephan et al. 2001;

https://doi.org/10.1017/S002202990600183X Published online by Cambridge University Press

Montesinos et al. 2002; Cabral et al. 2004). According to the Ridom SpaServer-Nomenclature, 94 different Xr-types are known at present. Sequencing of the Xr-encoding region of strain M4 characterized eight Xr-types. It was of interest that this strain possessed the repeat r16 four times at the end of the Xr-region. The diversity of the short sequence repeat region seems to arise from deletion and duplication of the repetitive units and also by point mutation. While the biological function is not known, the Xr-region may serve to extend the N-terminal immunoglobulin G binding portion of the protein through the cell wall. However, the importance of these 11 repeats for the virulence of *Staph. aureus* strain M4 and for colonization of udder tissue is not known. Frénay et al. (1994) reported *Staph. aureus* strains harbouring more than seven repeats. These strains were found to be epidemic MRSA. This was supported by Montesinos et al. (2002) who described *Staph. aureus* with 11 repeats as the most common type involved in an epidemic human outbreak caused by methicillin-resistant *Staph. aureus*. However, the three other investigated *Staph. aureus* strains investigated in the present study, also isolated from cases of clinical mastitis, had repeat numbers of four, two and two.

According to the results of the present study the *spa* gene of *Staph. aureus* isolated from bovine mastitis appeared with a high diversity in size and sequence which could be used in epidemiological studies. However, the importance of these sequence variations, also including the natural variant with the fused domain BC, for Ig binding activity or for virulence of these strains remains to be elucidated.

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