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Non-*spa*-Typeable Clinical *Staphylococcus aureus* Strains Are Naturally Occurring Protein A Mutants▽

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Staphylococcus aureus **is a major human pathogen responsible for increasing the prevalence of community- and hospital-acquired infections. Protein A (SpA) is a key virulence factor of *S. aureus* and is highly conserved. Sequencing of the variable-number tandem-repeat region of SpA (*spa* typing) provides a rapid and reliable method for epidemiological studies. Rarely, non-*spa*-typeable *S. aureus* strains are encountered. The reason for this is not known. In this study, we characterized eight non-*spa*-typeable bacteremia isolates. Sequencing of the entire *spa* locus was successful for five strains and revealed various mutations of *spa*, all of which included a deletion of immunoglobulin G binding domain C, in which the upper primer for *spa* typing is located, while two strains were truly *spa* negative. This is the first report demonstrating that nontypeability of *S. aureus* by *spa* sequencing is due either to mutation or to a true deficiency of *spa*.**

Staphylococcus aureus is an important human pathogen responsible for many community- and hospital-acquired infections (22). *S. aureus* can cause various diseases, ranging from superficial skin infections to severe and life-threatening diseases, such as pneumonia, osteomyelitis, endocarditis, and sepsis (36). Recently, the prevalence of community- and hospital-acquired methicillin (meticillin)-resistant *S. aureus* (MRSA) infections has increased (2). MRSA infections, especially, are responsible for enhanced mortality and significant increases in the length of hospitalization and hospital charges (4).

Protein A (SpA) represents an important virulence factor for *S. aureus* (7, 8, 29) and has recently been shown to be involved in the pathogenesis of *S. aureus* pneumonia (10, 11), to be expressed in invasive diseases (21), and to play a role in proteinaceous biofilm formation (25). SpA is a protein of 42 kDa and comprises several regions with different functions: The signal sequence (S region) in the N-terminal part is followed by four or five highly homologous immunoglobulin G (IgG)-binding domains in tandem (the E, D, A, B, and C regions) (20). The C-terminal region, or X region, is divided into two domains: (i) the repeat region X_R, consisting of variable repeats with mostly octapeptide structures, which are used for *spa* typing, and (ii) the X_C region, consisting of a conserved sequence, which confers anchoring to the cell wall via an LPXTG-binding motif (31, 32). The best-studied function of SpA is the interaction with human IgG by binding to the F_c part, thereby compromising the host immune system (6, 13). Furthermore, SpA can bind to various host structures, such as the von Willebrand factor and the receptor gC1qR/p33 on platelets (15, 27), which promote adhesion to platelets.

In recent years, *spa* (*S. aureus* protein A) typing has been used frequently as a typing method. As a single-locus sequence-based typing method, it combines a number of technical benefits, such as rapidity, reproducibility, and portability (33), thereby allowing easy interlaboratory comparability via the Internet and synchronization to a central server (http://www.ridom.de/spaserver/) (1, 14). *spa* typing uses the sequence of the polymorphic X region, which consists of a variable number of tandem repeats of 24 bp, as a genetic marker (9). This region has been shown to be rather stable and allows distinguishing of strains to a degree comparable to that of pulsed-field gel electrophoresis (PFGE) and whole-genome DNA microarray (19). Interestingly, mutations of the repeat region, including insertions, deletions, and point mutations, have been observed only after long-term persistence of *S. aureus* in vivo in the airways of cystic fibrosis patients, allowing calculation of the clock speed of this region (18) and to establish an algorithm for the analysis of this region (24).

So far, no *spa*-deficient clinical strains have been described. Although some studies demonstrate 100% *spa* typeability (12), non-*spa*-typeable strains have been detected recently (23, 34). The reason for this is not known. Therefore, this study was performed to investigate the underlying mechanism of the nontypeability of eight invasive *S. aureus* strains, which were isolated from bacteremic patients with different infections. We sequenced the whole *spa* locus and investigated the expression of SpA by Western blot analysis and real-time PCR.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Seven of 148 methicillin-susceptible *S. aureus* (MSSA) strains (4.7%) and 1 particular MRSA strain (9 of 1,300 MRSA strains [0.7%] were nontypeable), which were cultured in the Department of Clinical Microbiology, Hvidovre Hospital, in 2004, were non-*spa* typeable and were further analyzed. The strains were isolated from blood cultures of patients with invasive infections (Table 1). For cultivation of *S. aureus*, tryptic soy broth (Difco, now Becton Dickinson GmbH, Heidelberg, Germany) and brain heart broth (Merck, Darmstadt, Germany) were used. For the growth curve analysis, cultures were grown at 37°C on a rotary shaker. For determination of

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TABLE 1. Patient and strain information

Strain	Susceptibility	Phage group	Age (yr)/sex[a]	Disease	MLST result
NT935	MSSA	II (3C/71)	72/W	Endocarditis (mitral valve)	NT[b]
NT936	MSSA	II (3A/3C/55/71)	52/W	Soft tissue infection (neutropenia caused by chemotherapy)	NT
NT937	MSSA	III (53/93)	43/M	Osteomyelitis	NT
NT938	MSSA	III (6/53/54/75/83A/84/89)	58/M	Osteomyelitis (Th10 to L5 plus abscessus epiduralis and subduralis)	NT
NT939	MSSA	II (55)	40/M	Spondylitis (Th12)	NT
NT940	MSSA	NT	5/M	Osteomyelitis (left femur)	NT
NT941	MSSA	I (80 complex, a subtype of group I)	12/M	Osteomyelitis (right clavicular-sternal joint)	NT
NT942	MRSA	NA[c]	67/M	Urinary tact infection	ST8

[a] W, woman; M, male.
[b] NT, nontypeable.
[c] NA, not analyzed.

the optical density at 578 nm beyond a value of 2, the optical density value was calculated from readings of diluted cultures.

Molecular analysis. Amplification and sequencing of the whole *spa* locus were performed with chromosomal DNA from each strain as a template. Chromosomal DNA was purified with the PrestoSpin D kit (Molzym GmbH & Co., KG, Bremen, Germany) after cell lysis with lysostaphin (WAK Chemie Medical GmbH, Steinbach/Ts, Germany). The primer sites for PCR amplifications were designed according to a consensus sequence of the sequenced strains N315, Mu50, MW2, MSSA 476, MRSA 252, and 8325-4. The oligonucleotide primers used for PCR are listed in Table 2. Sequencing analysis was performed at Eurofins MWG Operon (Martinsried, Germany).

Southern blot hybridization analysis. Southern blot hybridization analysis of genomic DNA digested by SmaI (Roche Diagnostics GmbH, Mannheim, Germany) was performed using standard procedures. The Southern blot was probed with a 252-bp fragment of the *spa* gene including the S and E regions (Table 2). The probe was labeled with the PCR DIG Probe Synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany).

Molecular typing analysis of non-*spa*-typeable isolates. PFGE was performed as described recently (17). Similarity of strains was evaluated using the Dice coefficient and the criterion of a difference of fewer than 6 bands as described by Tenover et al. (35). Multilocus sequence typing (MLST) was performed as described previously (5). Phage typing was performed by the method of Blair and Williams with the contemporary international set of typing phages and two Danish experimental phages, which have been used since 1969 to differentiate among isolates of group III and the 83A complex (16).

RNA extraction and real-time PCR. For RNA extraction, a combination of the FastRNA Pro Blue kit (RNApro solution and Lysing Matrix B; Qbiogene [part of MP Biomedicals], Heidelberg, Germany), the RNAprotect Bacteria reagent (Qiagen, Hilden, Germany), and the RNeasy minikit (Qiagen) was used. Cells were mechanically disrupted by a Fast Prep FP120 instrument (Qbiogene). The remaining DNA was eliminated by DNase (Qiagen).

For the real-time PCR, cDNA was synthesized from 25 ng RNA by using the Quantitect reverse transcription kit (Qiagen) according to the manufacturer's recommendations. Real-time amplification was conducted using specific primers (Table 2) and was carried out on an iCycler iQ real-time PCR-system (Bio-Rad, Hercules, CA) using the iQ SYBR green Supermix (Bio-Rad). The levels of mRNA expression of *spa* were normalized against the expression of the internal control gene *gyrA* (DNA gyrase subunit A). The transcript quantities are expressed as changes (*n*-fold) relative to the values of the internal control (3).

Protein A extraction. Cell wall-bound protein A was extracted from 5-h cultures. After centrifugation, the pellet was washed twice with 25 mM Tris-HCl buffer. Cell lysis was done with lysostaphin (WAK Chemie Medical GmbH), in combination with DNA degradation with DNase (Roche Diagnostics GmbH). After centrifugation, the pellet was used for further investigations.

Secreted protein A from the medium was precipitated from sterile-filtered supernatants (0.2-μm-pore-size filter; Sartorius Biotech GmbH, Göttingen, Germany) of 5-h cultures by trichloroacetic acid. The protein pellets were washed with acetone, air dried, and dissolved in 25 mM Tris-HCl buffer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% [wt/vol] total acrylamide) and were subsequently electrotransferred (Transblot SD; Bio-Rad) to nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany) using established protocols. To detect SpA, affinity-purified immunoglobulins from rabbit serum were used as primary antibodies, and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins were used as secondary antibodies (Bio-Rad). Protein A P3838 (Sigma-Aldrich Chemie GmbH, Munich, Germany) was used as a positive control, and the PageRuler Plus prestained protein ladder from Fermentas GmbH (St. Leon-Rot, Germany) was used as a marker.

Nucleotide sequence accession numbers. The sequences of the non-*spa*-typeable strains NT935, NT937, NT938, NT939, and NT941 have been deposited in GenBank under accession numbers FJ491257 to FJ491261.

TABLE 2. Primers used in this study

Primer	Sequence (5′→3′)
spa-1F[a]	ATATGGATCCGATGACTTTACAAATACATAC AGGGGGT[b]
spa-1R[c]	ATATCCCGGGTGAGGCGTTTCAGAAGTTGTT TAGA[d]
spa-2F[a]	GATGACTTTACAAATACATACAGGGGGT
spa-2R[e]	AAAATGCACTGAGCAACAAAAGATG
spa-3F[f]	ATAGCGTGATTTTGCGGTT
spa-3R[g]	CTAAATATAAATAATGTTGTCACTTGGA
rt-*spa* F	TATCTGGTGGCGTAACA
rt-*spa* R	TAGGCATATTTAACACTTGAT
*gyr*A F	CAACATTACGTCCTTTAGGC
*gyr*A R	GCTACATCAAGCCCTACAAC
spa-probe F	CAATTCGTAAACTAGGTGTAGG
spa-probe R	TTGGAGCTTGAGAGTCATTA

[a] Located 34 to 7 bp upstream of the S region.
[b] The SmaI restriction site is underlined.
[c] Located 180 to 156 bp downstream of the stop codon of *spa*.
[d] The BamHI restriction site is underlined.
[e] Located 139 to 115 bp downstream of the stop codon.
[f] Located 178 to 160 bp upstream of the S region.
[g] Located 332 to 305 bp downstream of the stop codon.

RESULTS

Genetic analysis of *spa* of non-*spa*-typeable strains. All strains were responsible for invasive *S. aureus* infections (Table 1). *spa* was detected in five of eight non-*spa*-typeable strains by amplifying the entire gene with flanking primers (Fig. 1A).

Sequence analysis of the amplicons of the non-*spa*-typeable strains revealed deletions, upstream of the repeat region X_R, of one to four IgG-binding domains, including region C, the region where the upstream primer for *spa* typing is located. In strain NT935, a deletion from the middle part of IgG-binding domain C up to the beginning of the repeat region X_R resulted in a frameshift mutation with a premature stop codon (Fig.

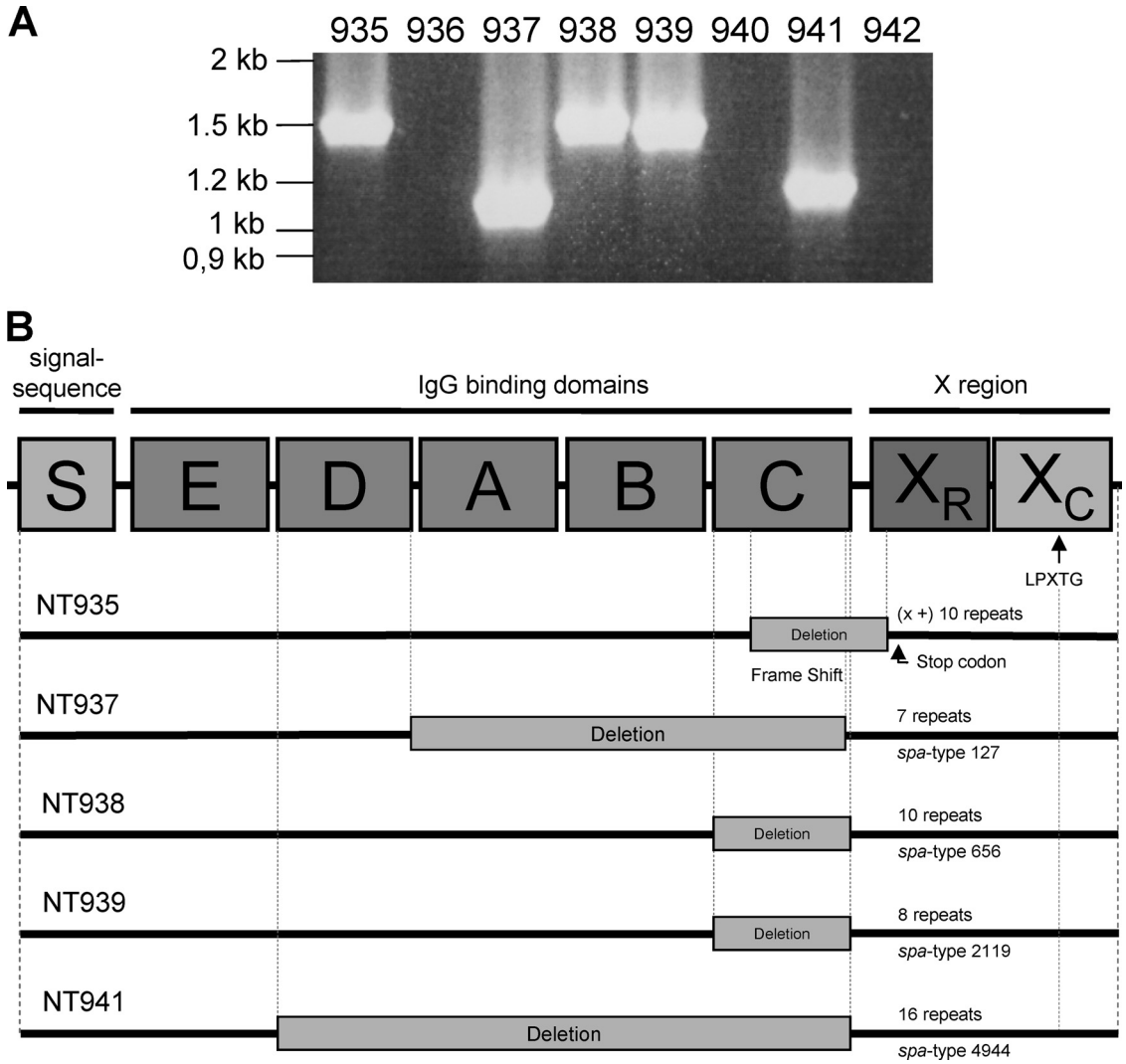

FIG. 1. (A) Amplification of the entire *spa* locus. By use of primers flanking the entire *spa* locus, *spa* could be amplified in five of eight non-*spa*-typeable strains: NT935, NT937, NT938, NT939, and NT941. (B) Results of sequencing of the *spa* locus for five non-*spa*-typeable *S. aureus* strains: NT935, NT937, NT938, NT939, and NT941. The deletions are indicated by gray rectangles. Strain NT935 revealed a deletion from the middle part of IgG-binding domain C up to the beginning of the repeat region X_R, resulting in a frameshift mutation, presumably leading to a premature stop codon. In strain NT937, a deletion of the three IgG-binding domains A, B, and C occurred; in strains NT938 and NT939, a deletion of IgG-binding domain C was observed; and in strain NT941, a deletion of the four IgG-binding domains D, A, B, and C was demonstrated.

1B). Southern blot analysis revealed the presence of *spa* in one additional strain, NT940 (Fig. 2A).

Expression of protein A by real-time PCR and Western blot analysis. *spa* transcription could be detected by RT-PCR, though with different values, in all *spa*-positive strains (Table 3). The typical pattern of *spa* transcription, with the highest transcription in the early- or late-logarithmic growth phase, was observed. Interestingly, the strains with three to four deletions of IgG-binding domains displayed the highest *spa* transcription levels (strains NT937 and NT941). However, transcription of these mutated genes resulted in detection of only low levels of protein or almost no detectable proteins as assessed by Western blot analysis (Fig. 2B and C). In contrast, low levels of *spa* transcription yielded large amounts of cell wall-associated protein and secreted protein A, as demonstrated for strain NT938 (Fig. 2B and C; Table 3).

Epidemiologic relationships of non-*spa*-typeable strains. PFGE allowed all non-*spa*-typeable strains to be distinguished (Fig. 3). The fragment patterns of the strains revealed that three of the eight strains analyzed were closely related (strains NT936, NT935, and NT939), while the other strains were genetically unrelated (Fig. 3).

DISCUSSION

This is the first report demonstrating the existence of clinical *S. aureus* strains in which either protein A is missing or deletions of one or several IgG-binding domains have occurred. SpA is a versatile molecule of ca. 42 kDa that exists mostly in a cell wall-associated form and has the ability to interact with several host components (7). Its polymorphic region is used as a single-locus sequence-typing method due to its discrimina-

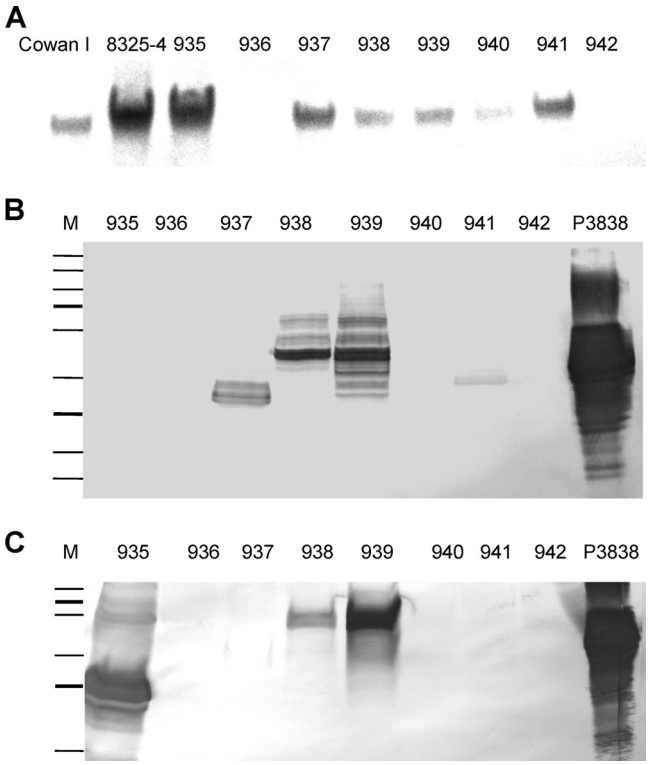

FIG. 2. (A) Southern blot hybridization analysis. SmaI-digested chromosomal DNAs of non-*spa*-typeable strains were probed with a 252-bp fragment of *spa*, which detects the S and E regions. As positive controls, DNAs of the *S. aureus* strains Cowan I and 8325-4 were used. (B and C) Western blot analyses. Cell wall extracts (B) and secreted proteins (C) of the non-*spa*-typeable strains were incubated with affinity-purified immunoglobulins from rabbit serum as primary antibodies and with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins as secondary antibodies. As a marker, the PageRuler Plus prestained protein ladder from Fermentas GmbH was used. Protein A P3838 (Sigma-Aldrich Chemie GmbH) was used as a positive control.

tory power (9). Recently, the development of an open Web-based database (www.ridom.de/spaserver/) (14) in which data of more than 83,000 strains from 63 countries are collected (March 2009) has resulted in a rapid increase in *spa* typing for molecular analyses and epidemiological studies. Most *S. aureus* strains are typeable by using standard *spa* primers for PCR and sequencing (12). Rarely, strains that are nontypeable by this method are encountered (23, 34). To find the underlying reasons for this nontypeability, we performed a thorough analysis of eight non-*spa*-typeable strains, which were collected from blood cultures of patients with invasive *S. aureus* infections in Denmark.

Sequencing of the entire *spa* locus revealed the presence of various deletions in all strains, all of which included a deletion of the IgG-binding domain C, in which the upstream primer used for *spa* typing is located. Only two strains actually failed to give a positive result, indicating that these strains were true *spa*-deficient strains. Although *spa* has been shown to be an important virulence factor in the pathogenesis of *S. aureus* infections (29), the two *spa*-deficient strains were isolated from patients with severe *S. aureus*-related infections, indicating that the strains were still virulent and invasive in spite of being *spa* deficient. Peacock et al., in their study of 334 *S. aureus* carriage and invasive isolates, detected the presence of *spa* in 90 and 94% of strains, respectively, by PCR analysis (30). However, their analyses also depended on a PCR approach that might have missed *spa*-positive *S. aureus* strains with mutations in the primer binding region. Southern blot analysis may have detected a higher number of *spa*-positive strains.

In addition to strain NT935, which exclusively secreted SpA into the medium due to a loss of the LPXTG region, two more strains (NT938 and NT939) also released SpA into the medium in measurable amounts as detected by Western blot analysis. Usually, most of the protein is bound to the cell wall. However, in some strains, SpA has been shown to be released from the cell wall into the medium, presumably due to autolysis during stationary phase (26).

spa expression is tightly regulated, and a number of different regulators that either repress (*agr*, *sarA*) or stimulate (*mgrA*,

TABLE 3. Overview of the results for non-*spa*-typeable *S. aureus* strains

Strain	PCR/sequencing result	Deletion	*spa* type	Result(s) by:		
				Southern blotting	Real-time PCR[a]	Western blotting[b]
NT 935	+/+	C domain+ frameshift + stop codon	Unknown; CCTGGT + 10 repeats	+	EL, 0.44; LL, 0.47; SP, 0.08	Ex
NT 936	−/−			−	ND	
NT 937	+/+	A, B, and C domains	t127	+	EL, 59.71; LL, 19.70; SP, 7.73	CW
NT 938	+/+	C domain	t656	+	EL, 0.01; LL, ND; SP, 0.01	Ex + CW
NT 939	+/+	C domain	t2119	+	EL, 0.57; LL, 0.09; SP, 0.08	Ex + CW
NT 940	−/−			+	EL, 0.02; LL, 0.05; SP, 0.01	
NT 941	+/+	D, A, B, and C domains	A4944	+	EL, 168.9; LL, 6.28; SP, 0.71	CW
NT 942	−/−			−	ND	

[a] EL, early-log phase; LL, late-log phase; SP, stationary phase; ND, nondetectable.
[b] Ex, extracellular; CW, cell wall associated.

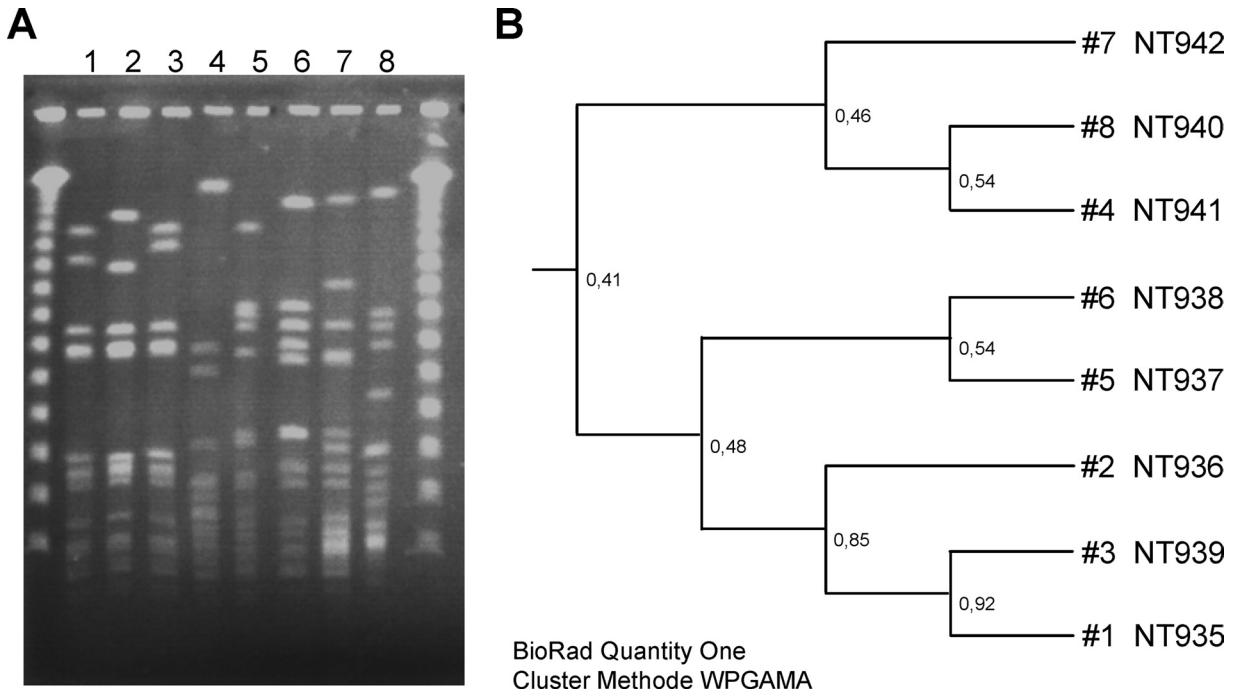

FIG. 3. Phylogenetic tree of the non-*spa*-typeable *S. aureus* strains. (A) PFGE results. The molecular weight marker used consists of concatemers of lambda phage DNA. (B) Deduced phylogenetic tree after SmaI digestion of chromosomal DNA from the non-*spa*-typeable strains.

sarS, *sarT*, *arl*, and *rot*) the transcription of *spa* are involved in its regulation (28). The fact that the transcription of *spa* in all strains followed the pattern usually observed, with the highest levels of transcripts in the early- and late-logarithmic phases, suggests that no major deletions of upstream regulators occurred in the strains investigated.

PFGE analysis revealed that only three of eight strains were genetically related, indicating that (i) there was no outbreak of a specific clone with non-*spa* typeability in Denmark at the time of strain collection and (ii) mutations within the *spa* region can occur in different genetic backgrounds.

In summary, in this study we showed that *spa* DNA and RNA were present in six out of eight non-*spa*-typeable strains. However, sequencing was successful for only five of these strains. The *spa* gene revealed deletions between 161 and 705 bp, all of which included the region of the upstream primer binding site usually used for *spa* typing, thus explaining the impossibility of typing. Expression analysis of SpA revealed that four of five strains covalently bound SpA to the cell wall, while one strain exclusively secreted the protein into the medium due to a premature stop codon and a missing LPXTG region. Although in all non-*spa*-typeable strains investigated, either *spa* mutations occurred or *spa* was absent, these strains were still able to induce invasive disease, demonstrating the redundancy of virulence factors in *S. aureus*.

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