Genetic Determinants of Resistance to Fusidic Acid among Clinical Bacteremia Isolates of *Staphylococcus aureus*[▽]

Jonas Lannergård,[†] Tobias Norström,[†] and Diarmaid Hughes*

Microbiology Programme, Department of Cell and Molecular Biology, Box 596, Biomedical Center,
Uppsala University, S-75124 Uppsala, Sweden

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Resistance to fusidic acid in *Staphylococcus aureus* is caused by mutation of the elongation factor G (EF-G) drug target (FusA class) or by expression of a protein that protects the drug target (FusB and FusC classes). Recently, two novel genetic classes of small-colony variants (SCVs) were identified among fusidic acid-resistant mutants selected in vitro (FusA-SCV and FusE classes). We analyzed a phylogenetically diverse collection of fusidic acid-resistant bacteremia isolates to determine which resistance classes were prevalent and whether these were associated with particular phylogenetic lineages. Each isolate was shown by DNA sequencing and plasmid curing to carry only one determinant of fusidic acid resistance, with approximately equal frequencies of the FusA, FusB, and FusC genetic classes. The FusA class (mutations in *fusA*) were distributed among different phylogenetic types. Two distinct variants of the FusC class (chromosomal *fusC* gene) were identified, and FusC was also distributed among different phylogenetic types. In contrast, the FusB class (carrying *fusB* on a plasmid) was found in closely related types. No FusE-class mutants (carrying mutations in *rplF*) were found. However, one FusA-class isolate had multiple mutations in the *fusA* gene, including one altering a codon associated with the FusA-SCV class. SCVs are frequently unstable and may undergo compensatory evolution to a normal growth phenotype after their initial occurrence. Accordingly, this normal-growth isolate might have evolved from a fusidic acid-resistant SCV. We conclude that at least three different resistance classes are prevalent among fusidic acid-resistant bacteremia isolates of *S. aureus*.

Staphylococcus aureus is an important hospital and community pathogen, and fusidic acid is one of several antibiotics used in its management (1, 9, 29, 36, 37, 42, 43). Fusidic acid interacts with translation elongation factor G (EF-G) on the ribosome, where it inhibits protein synthesis by preventing the release of EF-G · GDP (7, 44). There are two mechanisms known to cause resistance to fusidic acid in *S. aureus*: (i) alteration of the drug target (EF-G in complex with the ribosome) by mutation (5, 22) and (ii) protection of the drug target by FusB-family proteins (25). Each of these mechanisms has multiple genetic causes, some of which have only recently been discovered (23, 28).

Mutations altering the drug target (FusA, FusA-SCV, and FusE classes). The first mechanism of fusidic acid resistance to be identified was target alteration caused by mutations in *fusA*, the chromosomal gene encoding EF-G (8). More than 30 different amino acid substitution mutations causing fusidic acid resistance in *S. aureus* have now been identified in EF-G (5, 22, 23). The resistance mutations occur mostly in structural domain III of EF-G, but some also occur in domains I and V (17). In a few clinical isolates, multiple resistance-associated mutations have been found in *fusA*, suggestive of multiple rounds of selection (6, 22). Data from in vitro translation experiments

(12, 32) show that *fusA* mutations reduce the affinity of fusidic acid for the EF-G-ribosome complex. We refer to resistance due to *fusA* mutations as FusA-class resistance. A subset of the mutations in *fusA* resulting in resistance to fusidic acid, most of which are located in domain V of EF-G, cause the small-colony variant (SCV) phenotype. In addition to being resistant to fusidic acid, these mutants have reduced susceptibility to aminoglycosides and are auxotrophic for hemin. We refer to this group of mutants as the FusA-SCV class (23). Another group of fusidic acid-resistant mutants that display an SCV phenotype are referred to as FusE and carry mutations in *rplF*, the gene encoding ribosomal protein L6 (23). Protein L6 maps in the part of the ribosome where EF-G interacts (3, 35).

Protection of the drug target (FusB and FusC [FusB family]). The FusB protein prevents fusidic acid from interacting with EF-G, thus protecting the translation apparatus from inhibition by the antibiotic (25). Several decades ago it was recognized that resistance to fusidic acid could be associated with the presence of a penicillinase plasmid in *S. aureus* (15, 16). An example of this plasmid, pUB101, was sequenced and shown to carry genes coding for cadmium resistance and a gene designated *fusB* (also known as *far*), responsible for fusidic acid resistance mediated by target protection (24–26). The *fusB* gene has also been found to be integrated into the chromosome of *S. aureus* in an epidemic fusidic acid-resistant impetigo clone (27). In addition, chromosomal genes encoding proteins with ~45% amino acid similarity to FusB have been identified (28). These genes, *fusC* (found in *S. aureus* and *Staphylococcus intermedius*) and *fusD* (found in *Staphylococcus saprophyticus*), confer resistance to fusidic acid on *S. aureus*

* Corresponding author. Mailing address: Microbiology Programme, Department of Cell and Molecular Biology, Box 596, Biomedical Center, Uppsala University, S-75124 Uppsala, Sweden. Phone: 46-18-4714354. Fax: 46-18-530396. E-mail: diarmaid.hughes@icm.uu.se.

[†] These authors contributed equally to the manuscript.

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and are presumed to encode proteins that, like FusB, protect EF-G from the antibiotic (28). The *fusB* and *fusC* genes have also been identified in fusidic acid-resistant isolates of *Staphylococcus epidermidis* (20).

Previous studies of the genetic basis of fusidic acid resistance in clinical isolates of *S. aureus* have been limited to an examination of strains recovered from patients with impetigo (26–28). In addition, previous studies could not have looked for evidence of the FusE genotype (mutation in *rplF*), as it has only recently been described and is associated with an SCV phenotype (23). SCVs are of special interest in *S. aureus* infections because they have reduced susceptibility to aminoglycosides and have been isolated from patients with chronic, persistent, and/or recurrent infections (13, 30, 31, 38, 39). This distribution of SCVs is probably a consequence of their ability to persist intracellularly and thus to be shielded from the host immune response (2, 38, 40). The SCV phenotype in *S. aureus* is often unstable, and many strains revert or evolve at a high frequency to a normal colony phenotype (4, 41). For practical reasons (very slow growth, phenotypic instability), SCVs are only rarely identified in clinical laboratory isolates unless special efforts are made to look specifically for them. However, if the phenotypic reversion of the SCV to a normal growth phenotype involves secondary compensatory mutations, then genetic traces of the original SCV mutation could remain in the genotypes of resistant isolates with a normal growth phenotype. It is therefore of interest to determine which fusidic acid resistance mechanisms are associated with invasive *S. aureus* infections. Here, we examined the genetic basis of fusidic acid resistance in a phylogenetically diverse set of clinical bacteremia isolates of *S. aureus*.

MATERIALS AND METHODS

Bacterial strains. Twenty fusidic acid-resistant *S. aureus* bacteremia isolates, originally from the Statens Serum Institut (Copenhagen, Denmark), were described previously in terms of fusidic acid MICs (22). The isolates were collected from patients in 18 Danish hospitals during 1996. In the two cases where two isolates were collected from the same hospital, both *spa* typing and *fusA* analysis showed that the isolates were not clonal (see Results and Fig. 1).

Media and growth conditions. Standard liquid growth medium was LB, and solid growth medium was LA (LB plus 1% agar; Merck, Darmstadt, Germany). B2 broth (1% casein hydrolysate [Merck, Darmstadt, Germany], 2.5% yeast extract [Oxoid, Hampshire, England], 0.1% K₂HPO₄, 0.5% glucose, 2.5% NaCl) was used when *S. aureus* cells were made electrocompetent and as an outgrowth medium after electroporation. NYE agar plates (1% casein hydrolysate, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) were used for plating electroporated cells. Difco Mueller-Hinton agar (Becton, Dickinson and Company, Sparks, MD) was used for all MIC tests. Liquid cultures were grown at 37°C in flasks or tubes on a shaker set at 200 rpm.

Antimicrobials and MIC tests. The antibiotics ampicillin (Sigma Aldrich, Stockholm, Sweden) and fusidic acid (Leo Pharma, Ballerup, Denmark) were used for selection and screening in solid medium, each at 1 µg/ml. Cadmium acetate (Sigma Aldrich) was used at 10^{−4} M (final concentration) in solid medium. MICs of antibiotics were measured on Mueller-Hinton agar using an Etest (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions.

Plasmid DNA preparation. We examined the clinical *S. aureus* strains for the presence of an ~21-kb pUB101-like plasmid (24) coding for ampicillin resistance, cadmium resistance, and fusidic acid resistance. Clinical isolates were grown overnight without shaking and without antibiotic selection in LB supplemented with 0.5% glycine (Sigma-Aldrich, Stockholm, Sweden). Cells from 15 ml of overnight culture were pelleted by centrifugation at 3,000 × g for 15 min. The cell pellet was resuspended in 100 µl TE buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA [pH 8.0]), to which was added 100 µl of lysozyme (L7386; Sigma-Aldrich; 300 µg/ml), 100 µl lysozyme (Sigma-Aldrich; 100 mg/ml) and 45 µl proteinase K (Sigma-Aldrich; 20 mg/ml). This mixture was incubated at 37°C for

30 to 60 min, after which 300 µl of 0.2 M NaOH, 1% sodium dodecyl sulfate was added and incubation continued at room temperature (18°C) for a further 5 to 10 min. Potassium acetate (300 µl, 3 M) was added, and the tube was inverted several times and then kept on ice for 15 min. The solution was centrifuged at 14,000 × g for 30 min at 4°C. The clear supernatant was removed, extracted with phenol-chloroform followed by chloroform, loaded onto a GenElute plasmid miniprep kit (PLN-70; Sigma-Aldrich), washed, and eluted according to the manufacturer's instructions. The eluate was mixed with 0.7 volume isopropanol, mixed, and centrifuged at 14,000 × g in a microcentrifuge at 4°C. The plasmid DNA pellet was washed once with 500 µl 70% ice-cold ethanol, dissolved in 50 µl distilled water, and stored at −20°C. Plasmid DNA was visualized on agarose gels, and the presence of cadmium resistance (*cadXD*) and fusidic acid resistance (*fusB*) sequences was assayed by PCR and DNA sequencing. Plasmids from clinical isolates were transformed into the restriction-negative *S. aureus* strain RN4220 by electroporation.

Electrocompetent cells. *S. aureus* RN4220 (generously supplied by Molly Schmid and Bret M. Benton, formerly of Microcide Pharmaceuticals, Mountain View, CA) was grown overnight in B2 broth at 37°C, diluted 25 times in fresh B2 in an E-flask (total volume, 25 ml), and grown at 37°C to an optical density at 600 nm of ~0.5. Cells were washed three times in 1 volume of filter-sterilized deionized water, followed by two washes with 10% glycerol in 1/2 and 1/5 volumes, respectively. Washing was by centrifugation in SS-34 tubes at 5,000 × g. Before the last centrifugation, the cells were left at room temperature for 15 min. All steps were carried out at room temperature. The final cell pellet was dispersed in 800 µl 10% glycerol and if not used immediately was stored at −80°C in 80-µl aliquots for up to 1 month.

Electroporation. Plasmids purified from clinical *S. aureus* were transformed into RN4220 by electroporation, essentially as described previously (33). All steps were at room temperature. Seventy microliters of electrocompetent cells was mixed with 0.5 to 1 µg of plasmid DNA (50 to 100 ng/µl). Sixty microliters of this mixture was transferred to a 1-mm electroporation cuvette (ECU101; EquiBio, Ashford, Middlesex, United Kingdom) and electroporated using a Bio-Rad gene pulser (Bio-Rad Laboratories AB, Sundbyberg, Sweden). The gene pulser was set at 100 Ω resistance, 25 µF capacitance (2.5 ms optimal time constant), and 2.3 kV. After electroporation, cells were resuspended in 400 µl B2 broth, transferred to a microcentrifuge tube and incubated at 37°C for 24 h to allow recovery. Cells were spread on NYE agar containing ampicillin at 1 µg/ml and incubated at 37°C for 24 to 48 h. Typically, ~200 transformants were recovered.

PCR. To extract template DNA for PCR, a fresh bacterial colony was suspended in 100 µl sterile water to which was added 100 µl acid-washed glass beads (catalog no. G1277; Sigma-Aldrich, Stockholm, Sweden). The bacterium-bead mixture was vortexed at maximum speed on a bench vortex device for 45 to 60 s to disrupt the cells. A 0.5-µl portion of this solution was used as the DNA template to initiate a PCR. PCRs were carried out using a Ready-2-Go PCR bead kit (GE Health, Uppsala, Sweden) in a PTC-200 Peltier thermal cycler (SDS Diagnostics, Falkenberg, Sweden) with oligonucleotides from Sigma (Sigma Genosys Ltd, Sigma-Aldrich House, Haverhill, United Kingdom). PCRs were initiated with 5 min denaturation at 95°C, followed by 30 cycles of 20 s at 95°C, 20 s at 50°C, and either 1 min (*fusB*, *fusC*, *rplF*, and *cadXD*) or 2.5 min (*fusA*) at 72°C. PCR products were visualized by agarose gel electrophoresis, purified prior to sequencing using a QIAquick PCR purification kit (Qiagen, VWR International AB, Stockholm, Sweden) according to the manufacturer's instructions, and quantified after purification using an NO-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The oligonucleotides used for PCR and DNA sequencing are listed in Table 1.

Single-primer semirandom PCR. The genome location of *fusC* in the different strains was determined using a single-primer semirandom PCR method (10). The PCR amplification was performed with one primer per reaction (Fus3F for the downstream region and FusC3R for the upstream region), with the annealing temperature set to 45°C to allow for unspecific reverse priming outside of *fusC*. The resulting mixture of PCR products was sequenced with the nested primers FusC4F (downstream) and FusC1R (upstream).

DNA sequencing. DNA sequencing was performed at Macrogen Inc., Seoul, South Korea, and at the DNA Sequencing Facility (Rudbeck Laboratory, Uppsala University) on an ABI Prism 3700 capillary sequencer using a BigDye terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Each sequencing reaction mixture contained 8 ± 4 ng purified PCR product, 1.6 pmol sequencing primer, 4 µl Terminator Ready Reaction mix, and water to bring the total volume to 10 µl.

Plasmid curing. Clinical isolates carrying pUB101-like plasmids were cured of the plasmid by inoculating ~100 cells into 100 ml LB and growing overnight at 43°C with shaking for ~30 generations and then screening for loss of cadmium

TABLE 1. Oligonucleotides used for PCR and DNA sequencing^a

Primer	Sequence (5'→3')
Forward <i>cadX</i>	CGCAACAGCACAGCCACTGATGA
Reverse <i>cadD</i>	CGATAGCAAGTTGTGGTGCTGAT
Forward <i>fusB</i>	CGCCACTCAATGAGTGACGCT
Reverse <i>fusB</i>	CGGGAGGTGATGATGTTATGT
Forward EF-G 1.....	GTTCATGCTTAGGGCATCG
Forward EF-G 2.....	TGATCGTTTACAAGCTAACGC
Forward EF-G 3.....	ATTCTCCGTGTGTATTCAGG
Forward EF-G 4.....	TGGTCAATACGGTGATGTTCA
Reverse EF-G.....	AGAAATTATTTATAGCGATGC
FusC2F.....	ATGAATAAAATAGAAGTGTATAAGTTTGTAAAA
FusC3F.....	TCATCAAGTCAACTGGA
FusC4F.....	AAGGTATATATGGCGAAA
FusC1R.....	CAATCGATGTAATAAAGTCCGAGA
FusC2R.....	CTATTTTATTTTAAACAATAAATTCGTAAAGATT
FusC3R.....	AAATTTTCGCCATATATACCT
FusDown3.....	GCCTTCACCGTCTTTCTTT
FusDown4.....	GGTGTCACATCATATTTCTCAAGAA
L6F.....	AACACGAGCATGTCTTTTAAACG
L6R.....	CAAAGAAGCAAGAAAACGTAATGTT
spaF.....	GCCAAAGCGCTAACCTTTTA
spaR.....	TCCAGCTAATAACGCTGCAC
spaF2.....	GCCTAAAAGATGACCCAAGC
spaR2.....	CGCGACGACGTCCAG

^a Oligonucleotide sequences to amplify *fusB* and *cadXD* were based on the sequence of pUB101 (GenBank AY373761). The *fusA* sequence is from GenBank AJ237696. The spaF2-spaR2 combination was used for three isolates where the standard spaF and spaR primers did not work.

resistance by replica plating at 37°C (15). Cured strains were obtained at a frequency of $\leq 0.1\%$ and purified on LA plates. Loss of the plasmid was verified by loss of unselected phenotypic traits (ampicillin resistance and fusidic acid resistance) and by PCR of *cadXD*.

RESULTS

Phylogenetic diversity of the clinical isolates. The *spa* typing method (14, 34) was used to assess the phylogenetic diversity of

the 20 *Fus*^r bacteremia isolates examined here. The *spa* sequences were analyzed using the RIDOM StaphType 1.5 program (Ridom, GmbH, Würzburg, Germany; <http://spaserver.ridom.de/>). The 20 isolates belong to 13 different *spa* groups. No more than three isolates belonged to any one *spa* group. A phylogenetic tree based on these *spa* sequences (Fig. 1) was built using the BURP (Based Upon Repeat Patterns) algorithm (21). To validate the strain diversity apparent from the *spa* typing, we also made a phylogenetic tree based on the *fusA* sequences of the 20 isolates. We aligned and compared the *fusA* sequences from 14 *S. aureus* strains for which complete genome sequences were available (www.ncbi.nlm.nih.gov) and from the 20 *Fus*^r bacteremia isolates examined here. We found a total of 24 base pair differences and used these to classify the *fusA* genes into 11 phylogenetic groups. The phylogenetic grouping based on *fusA* is in good agreement with the *spa* typing (data not shown) and with the grouping previously made by multilocus sequence typing for a subset of these genomes (18). The phylogenetic information can be used to evaluate the likelihood of clonal relationships between different clinical isolates and thus the significance of the distribution of different resistance classes. We concluded that this set of 20 bacteremia isolates is genetically diverse.

FusA class resistance. In a previous study, we showed that a number of these clinical isolates had mutations in *fusA* causing fusidic acid resistance (22), and here we found *fusA* mutations in 6 of the 20 clinical strains. The phylogenetic classification made using *spa* typing (Fig. 1) shows that none of these six *fusA* genes belong to the same group (Table 2). The isolates IN441 and IN448 carry an identical resistance mutation in *fusA* (L461S), but because the isolates belong to different phylogenetic groups (t167 and t230, respectively), the mutations must

FIG. 1. Phylogenetic tree based on the *spa* sequences of the 20 bacteremia isolates studied here, shown as a UPGMA dendrogram with the sum of branch lengths being 1.49. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Clustering was performed using the BURP algorithm (21), with the Ridom StaphType 1.5 program (Ridom GmbH, Würzburg, Germany). Isolate identification numbers (INXXX), each with its *Fus* resistance class, follow the *spa* type. C and C* indicate the two different genome contexts of the *fusC* gene in the chromosome.

TABLE 2. Classification of resistance type in clinical *Fus*^r isolates

Isolate ^a		<i>spa</i> type ^b	MIC (μg/ml) ^c	Presence of plasmid ^d	<i>cadX</i> classification ^e	Presence of <i>fusB</i> ^f	Fus class ^g
SSI	IN						
27731	440	t674	8	+	pMW2	—	FusA ^h
27844	442	t189	256	+	pMW2	—	FusA ^h
27768	441	t167	8	+	pMW2 (Δ) ⁱ	—	FusA ^h
27962	444	t353	4	+	pMW2 (Δ)	—	FusA ^h
28362	452	t3000	8	—	—	—	FusA ^h
28130	448	t230	6	+	pMW2	—	FusA ^h
28332	450	t3000	12	+	pUB101	+	FusB
28444	457	t065	12	+	pUB101	+	FusB
27624	438	t065	6	+	pUB101	+	FusB
27856	443	t362	8	+	pUB101	+	FusB
27971	445	t065	8	+	pUB101	+	FusB
27980	446	t715	12	+	pUB101	+	FusB
27657	439	t008	8	(+) ^j	—	—	FusC
28416	456	t008	8	—	—	—	FusC
28150	449	t127	8	+	pMW2	—	FusC
28335	451	t015	8	+	pMW2	—	FusC ^{k,l}
28400	453	t630	6	+	pMW2	—	FusC ^{k,l}
28410	454	t630	6	+	pMW2	—	FusC ^{k,l}
28414	455	t015	6	+	pMW2	—	FusC ^{k,l}
28084	447	t015	12	+	pMW2	—	FusC ^l

^a SSI and IN refer to the Statens Serum Institut, Copenhagen, Denmark, and our stock collection (22), respectively.

^b Phylogenetic group based on *spa* typing in RIDOM nomenclature (Fig. 1).

^c MIC for fusidic acid measured using Etest strips on Mueller-Hinton agar.

^d Presence or absence of an ~21-kb plasmid (visible on an agarose gel after a standard plasmid preparation).

^e Plasmid classification based on the sequence of the *cadX* region (NCBI accession numbers: pUB101, NC_005127; pMW2, NC_005011; p21, NC_002517).

^f Determined by PCR and sequencing.

^g Mechanism of fusidic acid resistance. FusA, resistance-associated mutations in *fusA* (22); FusB, *fusB* gene on a plasmid; FusC, *fusC* gene on the chromosome. No strain has more than one resistance mechanism.

^h Missense mutations in *fusA*: IN440 Ala71Val, Asp189Gly, Pro406Leu; IN442 Ala376Val, Leu461Phe, Asp463Gly, Ala655Pro; IN441 Leu461Ser; IN444: Met453Ile; IN452 Val90Ile; IN448 Leu461Ser.

ⁱ A variant of pMW2 with a 9-bp deletion downstream of the *cadX* coding sequence.

^j A plasmid of 3 to 4 kb is present (no cadmium resistance or β-lactamase phenotype).

^k Silent mutation (TCC to TCT) present at nucleotide position 90 in *fusC*.

^l Sequence from 70 bp downstream of *fusC* differs from that in MSSA476.

have arisen independently. Interestingly, one isolate, IN442, carried four amino acid substitution mutations in *fusA*, including one in codon 655. Mutations in this region of EF-G, codons 655 to 666, are closely associated with the FusA-SCV phenotype (23), suggesting that this isolate might have evolved by compensatory evolution from an SCV.

FusB class resistance. A second source of fusidic acid resistance is the *fusB* gene, frequently borne on the ~21-kb plasmid pUB101 (24). We assayed the 20 clinical isolates for the presence of plasmids and found that 17 carried an ~21-kb plasmid, 1 carried a plasmid of 3 to 4 kb (IN439), and 2 had no detectable plasmids (Table 2). The complete sequences of 50 different plasmids from strains of *S. aureus* are available in public genome databases (www.ncbi.nlm.nih.gov). Three of these plasmids, pUB101, pMW2, and p21, are ~21 kb in size, while most others are either much larger (>38 kb) or smaller (≤8 kb). Each of the sequenced ~21-kb plasmids carries a *cadDX* operon, encoding cadmium resistance, and a *bla* operon, encoding β-lactamase activity, but only pUB101 carries *fusB*, encoding resistance to fusidic acid. Each of the 17 clinical isolates carrying an ~21-kb plasmid was resistant to cadmium and to ampicillin, whereas the remaining three isolates were susceptible to both. PCR was used to assay the 20 isolates for the presence of *cadXD* and *fusB*, using both purified plasmid preparations and total cell lysates. The *cadXD* sequence was found in all 17 clinical isolates that carry an ~21-kb plasmid but not

in any of the remaining three isolates. In contrast, *fusB* could be amplified from only 6 of the 20 isolates (Table 2). The *fusB* sequence was determined from each of these six plasmids and was identical to the published sequence on pUB101 (24). None of these six isolates carried a fusidic acid resistance mutation in *fusA*. The six FusB-class mutants belong to four closely related *spa* types, t065, t715, t3000, and t362 (Table 2 and Fig. 1). Three of these were *spa* type t065, suggesting a possible clonal relationship between these isolates.

We assigned the 17 ~21-kb plasmids to three groups based on the sequence of *cadX* and its surrounding sequences. One group, consisting of the six plasmids with *fusB*, is identical in sequence to pUB101. A second group, consisting of ten plasmids, is identical in sequence to pMW2. The third group, consisting of two plasmids designated pMW2(Δ), is identical to pMW2 in the *cadX* coding sequence and for at least 60 nucleotides downstream, except for a deletion of 9 bp downstream of the *cadX* termination codon (TGAAACGAGTGAAACG AGTTT → TGAAACGAGTTT; the termination codon is underlined). An identical 9-bp deletion is found in p21. The deletion occurs where there is a direct repeat of nine nucleotides, suggesting that it is a result of slippage and mispairing (19) during replication. Because p21 differs from the pMW2(Δ) plasmids at a large number of other nucleotide positions, including some within and downstream of *cadX*, we

TABLE 3. FusB class resistance is plasmid borne

Strain ^a	Fusidic acid MIC (μ g/ml) for:		RN4220 ^c
	Original isolate	Cured isolate ^b	
28332/pIN450	12	0.125	8
28444/pIN457	12	0.064	8
27624/pIN438	6	0.094	8
27856/pIN443	8	0.094	8
27971/pIN445	8	0.094	8
27980/pIN446	12	0.094	8

^a Strain numbers are the Statens Serum Institut (Copenhagen) numbers; plasmid designations are from Uppsala University.

^b MIC for the clinical isolate after plasmid curing.

^c MIC after electroporation of the plasmid into *S. aureus* RN4220.

suggest that pMW2(Δ) is a derivative of pMW2 that has independently acquired a deletion identical to that found in p21.

FusB plasmid curing and transfer. The MIC of fusidic acid for each of the six isolates carrying pUB101 (*fusB*) was measured before and after curing of the plasmid (Table 3). In each case, the original isolates had similar MICs (within one step), and each became susceptible to fusidic acid after curing, showing that the only fusidic acid resistance determinant in these six isolates was carried on the plasmid. Plasmids purified from each of the six isolates were also transformed into RN4220, and the fusidic acid MIC was measured again. The MICs for the transformed RN4220 were similar with all six plasmids and were within one step of the MICs measured in the clinical strains. On this basis, we conclude that these clinical isolates do not have any fusidic acid resistance determinants other than the plasmid-borne *fusB* gene.

FusC class resistance. The remaining eight isolates that lack a *fusA* mutation and have no *fusB* gene were each shown by PCR and DNA sequencing to carry the *fusC* gene on the chromosome and were thus classified as the FusC resistance class (Table 2). In four out of eight FusC isolates, a synonymous mutation (TCC to TCT) was present at nucleotide position 90 in *fusC*, and since these strains also belong to two closely related *spa* types, t630 and t015, it is likely that these four are clonally related. However, the remaining four FusC isolates belong to three different *spa* types (t015, t127, and t008). Thus, the eight *fusC* isolates are distributed among four *spa* types. The *fusC* gene was originally identified in the genome sequence of *S. aureus* MSSA476, where it was found inside the staphylococcal chromosome cassette SCC₄₇₆ (11). Using single-primer semirandom PCR, we amplified and sequenced the up- and downstream regions adjacent to *fusC* in the eight strains carrying this gene. An alignment of the obtained sequences upstream of *fusC* showed 99 to 100% identity to *S. aureus* MSSA476 in all eight isolates, indicating that the *fusC* gene has been incorporated in the genome of these strains as part of an SCC element, independently or in a common ancestor. Strains IN439, IN456, and IN449 (*spa* types t008 and t127) also displayed complete sequence identity to MSSA476 in the noncoding region downstream to *fusC*, in contrast to the remaining five strains (belonging to *spa* types t630 and t015), where the sequence similarity ends 70 bases downstream from the stop codon. We conclude that there are at least two variants of the *fusC* region present in clinical *S. aureus* isolates.

FusE class resistance. No FusE-class mutations were found in any of the 20 strains, as DNA sequencing showed that they all carried wild-type *rplF* genes.

DISCUSSION

Resistance to fusidic acid is associated with fitness costs for *S. aureus* both in vitro and in vivo (6, 22). It would not be surprising if the magnitude or nature of these resistance-associated fitness costs was influenced by genomic context, resulting in a skewed distribution of resistance determinants as a function of genotype. Indeed, of recent *S. aureus* impetigo isolates in Europe, the great majority belong to the same phylogenetic group and carry the same resistance determinant (26, 27), suggesting that the particular combination may be associated with a high fitness.

A priori, one would expect fusidic acid resistance by mutation (FusA class) to be phylogenetically widespread unless it was counterselected in some genotypes. Regarding plasmid-borne fusidic acid resistance (FusB class), its phylogenetic distribution is expected to reflect the complex dynamics of plasmid transmission between strains and the clonal selection of resistant strains. Finally, the distribution of intrinsic fusidic acid resistance associated with the presence of a resistance gene on the chromosome (FusC class) might be expected to be phylogenetically restricted, depending on the probability of independent acquisition of the element into one or more lineages and the time for subsequent genetic diversification of a lineage after acquisition.

Here, we addressed the genetic causes of fusidic acid resistance in a phylogenetically diverse collection of *S. aureus* bacteremia isolates. The resistance determinants we found in these isolates belonged to the FusA class (mutations in *fusA*), the FusB class (presence of pUB101 carrying the *fusB* gene), and the FusC class (*fusC* gene inserted in the chromosome as part of an SCC island). No representatives of the FusE SCV class (mutation in *rplF*) were found. Each isolate carried a single determinant for resistance, and there were approximately equal distributions of the FusA, FusB, and FusC classes among the 20 isolates studied (6:6:8, respectively). Based on phylogenetic relationships (*spa* typing), we could conclude that fusidic acid resistance in all six of the FusA-class isolates most likely arose independently. This is not unexpected for a resistance that arises by mutation, but it does indicate that this resistance class is not closely associated with a particular genetic background (Fig. 1). In contrast, the FusB-class isolates are closely related and possibly clonal based on their *spa* types and their *fusA* and *cadXD* sequences. Likewise, four of eight FusC strains are closely related and possibly clonal based on their identical *fusA* and *fusC* sequences and the identical genomic context of the *fusC* gene, while the remaining four strains (IN439, IN456, IN447, and IN449) are genetically dissimilar. In the genome sequence of *S. aureus* MSSA476, *fusC* is located inside the staphylococcal chromosome cassette SCC₄₇₆ (11). Even though all FusC strains have the *fusC* gene inserted at the same site as MSSA476, the sequence downstream of *fusC* shows genetic rearrangements that divides the strains into two groups. This could mean either that the FusC strains belong to lineages that acquired different SCC elements in at least two independent events or that the element is inherited

from an ancestor common to all strains and has since then gone through genetic rearrangements.

No fusidic acid-resistant isolates of the FusE class could be identified among the strains studied, and as this class of mutants has so far been identified only in vitro, it could be taken as evidence that mutants of this genotype are unlikely to thrive in the clinical setting. In a set of bacteremia isolates, however, this result is expected, as strains with a small-colony phenotype are unlikely to be collected from patients with blood infections.

Interestingly, the FusA isolate IN442 carries a mutation in codon 655, where a different mutation has previously been associated with the FusA-SCV class (23), in addition to two other substitutions (Leu461Phe and Asp463Gly) associated with the FusA class and one substitution associated with growth fitness compensation (Ala376Val) (22). This raises the possibility that IN442 might have initially been a FusA-SCV isolate and evolved to a normal growth phenotype by acquiring intragenic compensatory mutations in *fusA*. Further studies of clinical isolates are required to determine the extent to which SCVs are involved in the development of fusidic acid resistance. Our overall conclusion from this study is that three different genetic determinants of fusidic acid resistance (FusA, FusB, and FusC) are common among *S. aureus* bacteremia isolates. Furthermore, FusA and FusC resistance is widely distributed among different phylogenetic groups of *S. aureus*.

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