ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2007, p. 1737–1740 Vol. 51, No. 5
0066-4804/07/$08.00+0 doi:10.1128/AAC.01542-06

Genetic Basis of Resistance to Fusidic Acid in Staphylococci▽

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Received 11 December 2006/Returned for modification 26 January 2007/Accepted 21 February 2007

Resistance to fusidic acid in *Staphylococcus aureus* often results from acquisition of the *fusB* determinant or from mutations in the gene (*fusA*) that encodes the drug target (elongation factor G). We now report further studies on the genetic basis of resistance to this antibiotic in the staphylococci. Two staphylococcal genes that encode proteins exhibiting ca. 45% identity with FusB conferred resistance to fusidic acid in *S. aureus*. One of these genes (designated *fusC*) was subsequently detected in all fusidic acid-resistant clinical strains of *S. aureus* tested that did not carry *fusB* or mutations in *fusA*, and in strains of *S. intermedius*. The other gene (designated *fusD*) is carried by *S. saprophyticus*, explaining the inherent resistance of this species to fusidic acid. Fusidic acid-resistant strains of *S. lugdunensis* harbored *fusB*. Thus, resistance to fusidic acid in clinical isolates of *S. aureus* and other staphylococcal species frequently results from expression of FusB-type proteins.

Fusidic acid inhibits bacterial protein synthesis by preventing release of elongation factor G (EF-G) from the ribosome (4). It is used both topically and systemically for the treatment of staphylococcal disease (6).

Resistance to fusidic acid in *Staphylococcus aureus* occurs by horizontal acquisition of the *fusB* determinant, which encodes an EF-G-binding protein that protects the staphylococcal translation apparatus from inhibition by fusidic acid (13), or by spontaneous mutation in the gene encoding EF-G (*fusA*) (14). However, some fusidic acid-resistant *S. aureus* strains lack these mechanisms (14), and therefore additional, uncharacterized determinants of resistance to fusidic acid exist. In addition, little is known about the genetic basis of resistance to fusidic acid in staphylococci other than *S. aureus*.

Here we report on the basis of resistance to fusidic acid in strains of four different staphylococcal species. Two of these strains (*S. saprophyticus* ATCC 15305, *S. aureus* MSSA476) were chosen because their genomes encode homologues of the FusB protein (8, 10). Strains of *S. lugdunensis* and *S. intermedius* were examined since they exhibit phenotypic resistance to fusidic acid, the genetic basis of which is unknown. A collection of clinical *S. aureus* strains exhibiting resistance to fusidic acid, but not carrying *fusB* or resistance polymorphisms in *fusA*, was also characterized.

MATERIALS AND METHODS

Bacterial strains and culture. Table 1 lists staphylococcal strains used and generated in the present study. *S. lugdunensis* strains were isolated from patients in Sweden and were identified on the basis of typical morphology and/or smell, resistance to desferrioxamine, and ornithine decarboxylase and pyrrolidonyl arylamidase activity (1). *Escherichia coli* XL-10 Gold and XL-1 Blue (Stratagene, Amsterdam, The Netherlands) were used as cloning hosts.

Unless otherwise stated, strains were cultured in Luria-Bertani broth with aeration or on Luria-Bertani agar at 37°C. Susceptibility testing and determination of bacterial doubling times were performed as described previously (9).

DNA manipulation. Recombinant DNA methods were standard (16). Routine PCR amplification was performed with Platinum *Pfx* (Invitrogen, Paisley, United Kingdom), while the FailSafe PCR System (Epicenter, Madison, WI) was used for long PCR. Southern hybridization for detection of *fusB* (14) was performed on EcoRI-digested DNA at 60°C. Detection of *fusC* was performed in an identical fashion but used a probe generated by PCR with the oligonucleotide primers fusCU and fusCL (Table 2).

Recombinant DNA constructs were routinely created by using plasmid pCU1 (2). Constructs for tetracycline-regulated gene expression were generated by using plasmid pAJ96, a derivative of pALC2073 (3) that carries a transcriptional terminator downstream of the cloning site. For the former, PCR amplicons were introduced into the BamHI restriction site of pCU1 by virtue of engineered BamHI restriction sites at the *5′* end of oligonucleotide primers (Table 2), while engineered KpnI/SacI restriction sites were used for ligation into pAJ96. Constructs were propagated in *E. coli* before recovery and introduction into *S. aureus* RN4220 by electroporation. Tetracycline (100 ng/ml) was used to induce expression from the *xyl*/*tetO* promoter on pAJ96.

GenBank accession numbers. The *fusA* gene of *S. intermedius* (NCTC 11048) has been assigned GenBank accession number AY776250, and that from *S. lugdunensis* was assigned GenBank accession number DQ866810.

RESULTS

FusB homologues mediate resistance to fusidic acid in *S. aureus* and *S. saprophyticus*. A whole-genome sequence analysis of *S. aureus* strain MSSA476 was recently reported (8). This strain is resistant to fusidic acid and carries a gene (SAS0043) encoding a homologue (YP_042173) of the FusB protein. The gene and the fusidic acid resistance phenotype may be associated (8), although this has not been confirmed. We also identified a staphylococcal gene (SSP2165) encoding a FusB homologue (YP_302255) in the genome of *S. saprophyticus* ATCC 15305 (10). Although most staphylococcal species are inherently susceptible to fusidic acid, *S. saprophyticus* is intrinsically resistant to the antibiotic (6). These two staphylococcal FusB homologues exhibit 44% (YP_042173) and 47% (YP_302255) identity to FusB and 41% identity to each other (Fig. 1).

To establish whether these FusB-like proteins represent functional homologues of FusB (i.e., confer resistance to fusidic acid), the genes encoding them were PCR amplified, along

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▽ Published ahead of print on 26 February 2007.

TABLE 1. Staphylococcal strains used and generated in the present study and their susceptibilities to fusidic acid

Strain	Fusidic acid MIC (μg/ml)[a]	Source or reference
S. aureus		
RN4220	0.125	5
CS957	3*	14
74136 (EEFIC)	4	This study
MSSA476	8	8
649(pUB101)	16	13
RN4220(pCU1:*fusB*)	16	13
S. aureus clinical isolates with unknown mechanisms of resistance to fusidic acid		
CS992	2	14
CS730	3*	14
CS808	3*	14
CS866	3*	14
CS602	4	This study
CS851	4	This study
CS979	4	This study
CS1083	4	This study
CS1128	4	This study
CS805	8	This study
CS860	8	This study
CS1076	8	This study
S. aureus recombinant strains expressing FusB or FusB homologues		
RN4220(pCU1)	0.125	This study
RN4220(pCU1:*fusB*)	16	This study
RN4220(pCU1:SAS0043) (*fusC*)	16	This study
RN4220(pCU1:SSP2165) (*fusD*)	2	This study
RN4220(pAJ96)	0.125	This study
RN4220(pAJ96:*fusB*)	32	This study
RN4220(pAJ96:SAS0043) (*fusC*)	16	This study
RN4220(pAJ96:SSP2165) (*fusD*)	4	This study
S. intermedius		
CL1	4	7
CL2	4	7
NCTC 11048	0.064	ATCC (no. 29663)
S. lugdunensis		
16641	4	This study
16496	4	This study
31440	4	This study
40869	0.064	This study
S. saprophyticus		
ATCC 15305	2	10

[a] *, The MIC was determined by Etest in a previous study (14).

TABLE 2. Oligonucleotide primers used in the present study

Designation	Sequence (5′–3′)[a]
fusU	TAAGCGGCCGCAAGATTCTTCAATATCGTCATCTA
groL	GCAGCGGCCGCAGCTCAAGCAATGATTCAAGAAGG
rpsU	ATGGCTGGTACCAACAAAGCATTTGCTCACTA
tufL	GCTGTGAGCTCTGTTTTACCATGGTCAACGTG
sas0043U	GTAGGATCCATTGGGAATGATAAATAGTGA
sas0043L	TTTGGATCCATCGATTAAGAGTGAGGTACA
ssp2165U	ATCGGATCCTGCTTTGTCTGTCACATCTAA
ssp2165L	ACGGATCCAGGTGGGGTTGTCTATA
fusBU	AATGGTACCACTTGTGAAAGGTTGAAAACAATGAA AACAATGATTTATCC
fusBL	AACAGAGCTCATTCCTTAATCTAGTTTATC
sapU	AAAATGGTACCACTTGTGAAAGGTTGAAAACAATG GAAAAACAACTTTAC
sapL	AATAGAGCTCGTTTTTGGTTTATTGAATCT
mssaU	AATGGTACCACTTGTGAAAGGTTGAAAACAATGAA TAAAATAGAAGTGTA
mssaL	CAGGGAGCTCTGGATCTATTTTATTTTAAC
fusCU	GAGGAATATCATATGAATAAAATAGAAGTGTA
fusCL	AGAGTGGATCCCAAAATATAACAACCCTGATC

[a] Engineered regions are underlined.

with their upstream expression signals, using oligonucleotide primers SAS0043U/L and SSP2165U/L for SAS0043 and SSP2165, respectively (Table 2) and introduced into *S. aureus* RN4220 on plasmid pCU1. Both genes conferred resistance to fusidic acid (Table 1). Consequently, these proteins appear to form part of a FusB protein family that confer resistance to fusidic acid in staphylococci. Based on the precedent of ≤80% amino acid identity to represent the dividing line between one resistance determinant and a related one (12), we have designated these proteins FusC (YP_042173 from MSSA476) and FusD (YP_302255 from ATCC 15305), and their corresponding genes were designated *fusC* and *fusD*.

To establish the relative abilities of the FusB, FusC, and FusD proteins to confer resistance in *S. aureus*, constructs were generated enabling identical expression of the three genes. Thus, *fusC* and *fusD* were PCR amplified with oligonucleotide primers MSSAkpn/MSSAsac and SAPkpn/SAPsac (Table 2), resulting in upstream regions and ribosome binding sites identical to those of *fusB*. The *fusB* gene was amplified by using primers fusBkpn/fusBsac (Table 2). All three genes were expressed under identical induction conditions from the *xyl/tetO* promoter on plasmid pAJ96 in *S. aureus* RN4220. Under these conditions, FusB demonstrated a greater ability to protect *S. aureus* from fusidic acid compared to FusC and FusD (Table 1).

Carriage of antibiotic resistance determinants can impose a fitness cost in bacteria. Since constructs enabling equivalent expression of all three resistance genes had been constructed, it was possible to examine whether expression of *fusB*, *fusC*, or *fusD* was associated with a fitness cost in *S. aureus*. However, strains expressing these genes did not exhibit doubling times significantly different from RN4220 carrying pAJ96 (data not shown).

Resistance to fusidic acid mediated by FusB homologues in clinical strains of *S. aureus*. A proportion of fusidic acid-resistant *S. aureus* clinical strains carry neither *fusB* nor mutations in *fusA* (14). Such strains might be resistant to fusidic acid through expression of FusB homologues. Relevant strains (CS strains; Table 1) were screened for the presence of genes

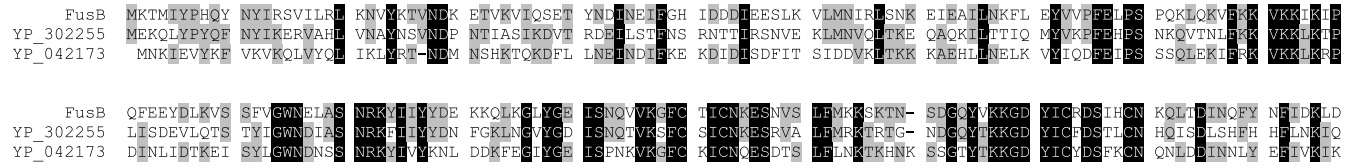

FIG. 1. Protein alignment between FusB, YP_042173 (FusC from *S. aureus* MSSA476), and YP_302255 (FusD from *S. saprophyticus* ATCC 15305). Gray shading indicates identity in two sequences; black shading indicates identity in all three sequences.

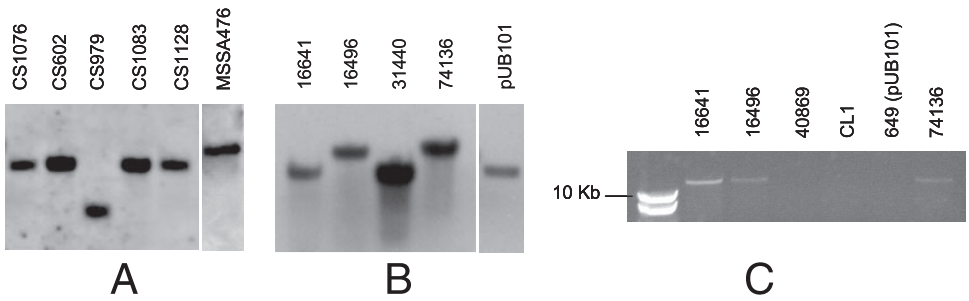

FIG. 2. Detection of *fusB* and *fusC* in staphylococci. (A) Detection of *fusC* by Southern hybridization in a representative set of fusidic acid-resistant clinical strains of *S. aureus* that harbor neither *fusB* nor resistance polymorphisms in *fusA*. MSSA476 is the positive control. (B) Detection of *fusB* by Southern hybridization in clinical strains of *S. lugdunensis*. pUB101 is the positive control, while *S. aureus* 74136 is an EEFIC strain (see the text). (C) PCR analysis maps *fusB* downstream of *groEL* in *S. lugdunensis* 16641 and 16496, the same location as that in EEFIC strain, *S. aureus* 74136. The other three strains are negative controls.

encoding FusB homologues by Southern hybridization. All strains tested positive for *fusC* (Fig. 2). To establish whether these strains harbored *fusC* or polymorphic variants cross-hybridizing with *fusC*, the *fusC* genes from these strains were PCR amplified and sequenced (primers SAS0043U and SAS0043L; Table 2). Since these primers correspond to the start and end of the *fusC* gene, this analysis could not provide DNA sequence information for the gene termini. However, a DNA sequence for >95% of the gene was obtained. The *fusC* genes encoded protein products identical (in the sequenced portion) to that encoded by *fusC* from MSSA476. However, *fusC* from strain CS979 carried a silent mutation (S_{162} was encoded by TCC rather than TCT).

Resistance to fusidic acid in *S. intermedius* and *S. lugdunensis*. Resistant strains of *S. intermedius* (7) and *S. lugdunensis* were ~64-fold less susceptible to fusidic acid than wild-type, sensitive strains of the same species (Table 1). These resistant strains were screened initially for nucleotide substitutions in *fusA*. Since the DNA sequence of *fusA* from these species was not available, oligonucleotide primers for PCR amplification and sequencing of this locus were designed against regions of the upstream (*rpsG*) and downstream genes (*tufA*) that are conserved between *S. aureus* and *Bacillus subtilis.* Thus, the primers rpsU and tufL (Table 2) were used to PCR amplify the entire *fusA* gene from both fusidic acid-sensitive and resistant strains, which were then sequenced in toto.

The *fusA* genes of *S. lugdunensis* and *S. intermedius* encode proteins with 95 and 92% identity, respectively, to EF-G from *S. aureus* N315. No nucleotide polymorphisms were identified in the *fusA* genes of the fusidic acid-resistant *S. lugdunensis* strains relative to the sensitive strains. However, six coding polymorphisms in *fusA* from *S. intermedius* CL1 and CL2 were identified compared to *S. intermedius* NCTC 11048 (NCTC 11048 residue shown first); $N_{187}T$, $V_{197}T$, $D_{231}E$, $N_{294}D$, $S_{368}T$, and $T_{488}S$. To examine whether these polymorphisms were responsible for the observed fusidic acid-resistance phenotype in CL1/CL2, the *fusA* genes of CL1, CL2, NCTC 11048 (negative control) and *S. aureus* CS957 (positive control; Table 1) were expressed in *trans* from pAJ96 in *S. aureus* RN4220. Expression of *fusA* from CS957 conferred resistance to fusidic acid, although expression of the *S. intermedius fusA* genes did not (data not shown).

Subsequently, representative fusidic acid-sensitive and -resistant strains of *S. lugdunensis* and *S. intermedius* were tested for the presence of *fusB* and *fusC* by Southern hybridization. Fusidic acid-resistant *S. intermedius* carried *fusC* (data not shown). The sequences of the *fusC* genes from *S. intermedius* CL1 and CL2 were identical to those found in MSSA476, with the exception of the same silent mutation identified in strain CS979.

Fusidic acid-resistant *S. lugdunensis* strains carried *fusB* (Fig. 2), a finding that was confirmed by PCR amplification and sequencing. We recently determined that *fusB* in the epidemic European fusidic acid-resistant impetigo clone (EEFIC) of *S. aureus* is located on a genomic island called $SaRI_{fusB}$, integrated into the chromosome downstream of the *groEL* gene (14a). Since *fusB* is present on a similar-sized EcoRI fragment in fusidic acid-resistant *S. lugdunensis* and in representatives of the EEFIC such as *S. aureus* 74136 (Fig. 2), we postulated that *fusB* may reside at the same chromosomal locus in *S. lugdunensis*. Southern hybridization established that the *fusB*-positive *S. lugdunensis* strains carried this resistance gene on the chromosome, since no hybridization of the *fusB* probe was detected with plasmid preparations (data not shown). Furthermore, using oligonucleotide primers specific for *fusB* (fusU; Table 2) and conserved regions of staphylococcal *groEL* (groL; Table 2), a PCR product (~15 kb) was generated from *S. aureus* 74136 and the fusidic acid-resistant *S. lugdunensis* strains but not from fusidic acid-sensitive strains or from those that carry plasmid-borne *fusB* (Fig. 2).

DISCUSSION

Early work on fusidic acid resistance in *S. aureus* (11) suggested that horizontally acquired (FusB-type) resistance was the most prevalent mechanism among clinical strains. This suggestion has recently been strengthened by the finding that the major fusidic acid-resistant clone of *S. aureus* (i.e., EEFIC), which is present in several European countries, carries the *fusB* determinant (14). The present study further underlines the importance of this resistance determinant in staphylococci, both for the prototypical *fusB* gene and for genes encoding functional homologues.

Two staphylococcal genes encoding functional homologues of FusB were identified in the present study: one from *S. aureus* MSSA476 (*fusC*) and the other from *S. saprophyticus*

ATCC 15305 (*fusD*). In the latter species, *fusD* appears to be a normal component of the genome, explaining the inherent resistance of this species to fusidic acid (6). Maintenance of a fusidic acid resistance determinant by *S. saprophyticus*, which provides protection against an antibiotic the organism is unlikely to encounter, supports previous arguments that the FusB family of proteins originally evolved to provide a housekeeping function unrelated to fusidic acid resistance (13). Furthermore, expression of FusB and the homologues did not impose a fitness cost, suggesting that maintenance of these determinants in the absence of antibiotic selection pressure does not disadvantage the organisms in which they occur naturally.

The FusB-like proteins of the staphylococci are more closely related to each other than to FusB homologues found in other gram-positive bacteria (13; data not shown). This suggests that these proteins are all descended from a single, ancestral, staphylococcal FusB. Based on the substantial sequence divergence of the three staphylococcal proteins (Fig. 1), the original recruitment of the ancestral *fusB* to the staphylococci probably occurred a long time ago and not as a consequence of the clinical introduction of fusidic acid in the 1960s.

Of the three determinants, *fusD*, found in *S. saprophyticus*, is the only gene encoding a FusB-like protein for which there is currently no evidence for spread between staphylococcal species. In contrast, we detected *fusC* in fusidic acid-resistant strains of *S. intermedius* and in 12 fusidic acid-resistant strains of *S. aureus*. Indeed, *fusC* is responsible for resistance to fusidic acid in all *S. aureus* strains that we have examined that do not carry *fusB* or resistance mutations in *fusA*, including four previously described strains (14). Thus, to date, all examples of fusidic acid resistance in clinical strains of *S. aureus* are the result of expression of a FusB-type protein (encoded by *fusB* or *fusC*) or mutations in *fusA*. For those seeking to detect *fusB* and *fusC* in clinical strains, it is important to note that the level of nucleotide sequence homology between these genes is ~60%, which explains why we did not previously observe hybridization of a *fusB* probe to DNA from strains we now know to contain *fusC* (14).

Fusidic acid-resistant strains of *S. lugdunensis* carried the *fusB* determinant on the chromosome, downstream of *groEL* (Fig. 2), the locus occupied by the $SaRI_{fusB}$ genomic island in the EEFIC strain of *S. aureus*. The emergence of fusidic acid resistance in strains of *S. lugdunensis* in Sweden has occurred after clonal expansion of the EEFIC in Sweden (15) and in a geographical area with a high prevalence of EEFIC strains. This suggests the possibility that *fusB* in *S. lugdunensis* has been acquired from *S. aureus*, although further studies will be required to support this hypothesis.

The *fusB* gene has also recently been reported in bovine isolates of coagulase negative staphylococci (17). That report, together with our own studies, therefore illustrates the importance of FusB and FusB homologues for resistance to fusidic acid in several staphylococcal species.

ACKNOWLEDGMENTS

This study was supported by a research grant to I.C. from LEO Pharma and a BBSRC-CASE PhD studentship in conjunction with LEO.

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