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Genetic and Phenotypic Identification of Fusidic Acid-Resistant Mutants with the Small-Colony-Variant Phenotype in *Staphylococcus aureus*▽

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Small-colony variants (SCVs) of *Staphylococcus aureus* are a slow-growing subpopulation whose phenotypes can include resistance to aminoglycosides, defects in electron transport, and enhanced persistence in mammalian cells. Here we show that a subset of mutants selected as SCVs by reduced susceptibility to aminoglycosides are resistant to the antibiotic fusidic acid (FA) and conversely that a subset of mutants selected for resistance to FA are SCVs. Mutation analysis reveals different genetic classes of FA-resistant SCVs. One class, FusA-SCVs, have amino acid substitution mutations in the ribosomal translocase EF-G different from those found in classic FusA mutants. Most of these mutations are located in structural domain V of EF-G, but some are in domain I or III. FusA-SCVs are auxotrophic for hemin. A second class of FA-resistant SCVs carry mutations in *rplF*, coding for ribosomal protein L6, and are designated as FusE mutants. FusE mutants fall into two phenotypic groups: one auxotrophic for hemin and the other auxotrophic for menadione. Accordingly, we have identified new genetic and phenotypic classes of FA-resistant mutants and clarified the genetic basis of a subset of *S. aureus* SCV mutants. A clinical implication of these data is that FA resistance could be selected by antimicrobial agents other than FA.

Fusidic acid (FA) is an antibiotic used in the treatment of staphylococcal infections (6, 23, 30, 36, 55, 60, 61). The target of FA is the translation elongation factor EF-G in complex with the ribosome (13, 32). FA acts by preventing the release of EF-G from the ribosome after translocation, thus inhibiting further protein synthesis (53, 62). Although it was launched more than 4 decades ago, FA remains a useful antibiotic, not least because it is not cross-resistant with other antibiotics used to treat staphylococci. Although frequencies of resistance to FA have remained generally low, emerging resistance is a problem that could limit the therapeutic options available for treatment of staphylococcal infections (25, 39, 44, 47, 48). To maintain the usefulness of antibiotics, it is important to understand the mechanisms that cause relevant bacterial pathogens to become resistant. We have addressed this question with respect to FA resistance and *Staphylococcus aureus*.

Two mechanistic classes of resistance to FA have so far been described genetically (19). One class, FusA, is associated with mutations in *fusA* (11, 38), the gene encoding the ribosomal translocase, translation elongation factor EF-G. These mutations reduce the affinity of FA for its target, EF-G, on the ribosome. The second class of resistance mechanism, FusB, is usually associated with a 21-kb plasmid, pUB101, carrying the gene *fusB* (40), but is also found in the chromosome (43). The *fusB* gene encodes an inducible protein shown in vitro to protect EF-G against the inhibitory action of FA (42). Recently two homologues of *fusB*, designated *fusC* and *fusD*, have been identified in the chromosome of clinical isolates of *S. aureus* and *Staphylococcus saprophyticus* respectively (41).

We have previously reported the existence of another FA resistance class in *S. aureus* (38), here referred to as FusE. In this report, we show that mutants of the FusE class and some mutants of the FusA class have the classic characteristics of small-colony variants (SCVs) of *S. aureus*.

Bacteria with an SCV phenotype have been described for many species, including human pathogens such as *Staphylococcus aureus* (46). *S. aureus* SCVs are clinically important because they are associated with intracellular growth, increased persistence, and recurrent infections (1, 14, 45, 46, 51, 56, 58). In addition to their small colony size on solid media, SCVs can typically exhibit a variety of other characteristic phenotypes. These include reduced susceptibility to aminoglycosides; auxotrophy for any one of the compounds hemin, menadione, thiamine, or thymidine; reduced hemolytic activity; and reduced colony pigmentation. The phenotypes associated with *S. aureus* SCVs have been rationalized as consequences of a dysfunctional electron transport system (7, 27, 45, 46, 57, 58) or a defect in thymidine biosynthesis (18, 28, 29). The SCV phenotype is reported to be phenotypically unstable, with strains frequently reverting to a normal-colony phenotype (10, 59). The rate of emergence of selected SCVs is increased in a mutator strain, strongly suggesting a genetic rather than a regulatory basis for the phenotype (50). The actual genetic basis for the SCV phenotype in clinical isolates is in most cases unknown. However, selected or constructed mutations in *hemB* (9, 56), *hemH* (50), *menD* (9), and *ctaA* (20) produce the electron transport deficiency-associated phenotypes of SCVs. Recently, it was reported that the thymidine-auxotrophic SCVs

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TABLE 1. Mutants selected in 8325-4 as FA resistant and then screened for SCV phenotypes

Strain and stock	Identified mutation[a]		MIC (μg/ml) of:			Doubling time (min)[b]	Auxotrophy[c]	*n*[d]
	fusA (EF-G)	*rplF* (L6)	FA	STR	KAN			
8325-4	None	None	0.125	3	1.5	24	−	
FusA classic								
AH207	Thr385Asn	None	4	12	3	29	−	
AH194	Pro404Leu	None	4	8	2	25	−	2
AH196	Pro406Leu	None	6	8	3	31	−	
AH198	Gly452Ser	None	32	6	4	38	−	2
AH201	Gly452Cys	None	12	8	6	28	−	
AH197	Leu456Phe	None	4	8	6	34	−	
AH187	His457Tyr	None	96	6	3	29	−	9
AH206	Arg464Ser	None	6	8	6	37	−	
AH190	Arg464His	None	6	8	4	31	−	3
FusA-SCV								
AH188	Gly664Ser	None	4	16	24	46	Hemin	2
FusE								
AH193	None	TAA stop at nt 229	4	24	8	54	Hemin	
AH199	None	TAA stop at nt 241	4	16	8	57	Hemin	
AH202	None	CTG start at nt 1	4	16	8	58	Hemin	
AH204	None	Δ of nt 203–231	4	24	8	57	Hemin	
AH209	None	Δ of nt 404–427	4	16	8	60	Hemin	

[a] None, wild type. Underlined letters represent the mutated residues.
[b] Doubling time (generation time) in minutes during exponential growth in LB.
[c] Auxotrophy tested for hemin, menadione, thymine, and thymidine. −, nonauxotrophic.
[d] Number of independent isolates with the same genotype and phenotype.

from cystic fibrosis patients carry mutations in the thymidylate synthase gene, *thyA* (12).

Here we report that all FusE mutants and a subset of FusA mutants selected in *S. aureus* are SCVs. We identify particular mutations in EF-G and ribosomal protein L6 that separately are associated with the SCV phenotype. We also show that a subset of mutants selected in *S. aureus* as SCVs on the basis of resistance to aminoglycosides belong to either the FusA-SCV or FusE resistance class.

MATERIALS AND METHODS

Strains and antibiotics. *S. aureus* 8325-4 was used as the standard drug-susceptible laboratory wild-type strain. Clinical *S. aureus* isolates from bacteremia patients (20 FA resistant and 10 FA susceptible) were supplied by Statens Serum Institut, Copenhagen, Denmark. Strains were grown in liquid Luria broth (LB) or on Luria agar plates (LA) unless otherwise stated. Liquid cultures were incubated overnight at 37°C on a shaker at 255 rpm. Strain stocks were stored frozen in LB plus dimethyl sulfoxide (7%) at −80°C. Antibiotics were dissolved in methanol (FA sodium salt at 5 mg/ml; Leo Pharma, Ballerup, Denmark) or double-distilled water (kanamycin [KAN] at 5 mg/ml; Sigma-Aldrich AB, Stockholm, Sweden) and stored at −20°C until used.

Selection of drug-resistant mutants. A fresh overnight culture in LB was diluted, and approximately 100 CFU were inoculated into each of 20 tubes, each containing 2 ml LB, and grown into stationary phase. One hundred microliters from each overnight culture was plated on selective media. FA resistance was selected at 2 μg/ml. KAN resistance was selected at 8, 16, and 24 μg/ml. Plates were incubated 48 h at 37°C before picking colonies and purifying on selective media.

MIC. The MIC was measured using Etest strips (AB Biodisk, Solna, Sweden) in accordance with the manufacturers' description. A single colony was dissolved in 0.9% NaCl to a density of 0.5 McFarland unit. A cotton swab was used to spread the bacterial suspension over the surface of a Mueller-Hinton plate (Difco Becton Dickinson, MD). After application of the Etest strip, plates were incubated at 37°C for 18 to 24 h until growth of the SCV lawn was evident.

Growth rate. Bacterial doubling times in LB were measured using the BioscreenC machine (Oy Growth Curves Ab Ltd., Helsinki, Finland). Cells were inoculated in 200 μl LB at a density of 10^4 to 10^5 cells/ml in 100-well honeycomb plates. Measurements of the optical density at 600 nm were taken each 10 min over a 24-h period at 37°C.

Auxotrophy complementation tests. Auxotrophy was assayed by complementation with hemin, menadione sodium bisulfite, thiamine pyrophosphate, and thymidine (all from Sigma-Aldrich AB, Stockholm, Sweden). Five-millimeter-diameter filter discs (3MM paper; Whatman International, Maidstone, United Kingdom) were soaked in each solution at a concentration of 1,000 μg/ml (except menadione, which was used at 200 μg/ml) and placed onto LA plates spread with ~10^5 CFU of the strain to be tested. Plates were incubated overnight at 37°C. An increase in colony size proximal to the cellulose disc was interpreted as a positive result (complementation of auxotrophy).

PCR and sequencing. Template DNA was prepared by dissolving a bacterial colony in 100 μl double-distilled water, adding ~100 μl of 0.25-mm acid-washed glass beads (Sigma-Aldrich AB, Stockholm, Sweden), and vortexing for 10 s to disrupt the cells. Primers for amplifying and sequencing *S. aureus* genes were designed based on the publicly available genome sequences. After an initial denaturation step of 5 min at 95°C, 30 cycles of 15 s at 95°C, 15 s at 50°C, and ~1 min/kb at 72°C were run. The last elongation step was prolonged to 5 min. Sequencing reactions were carried out at the DNA Sequencing Facility at Rudbeck Laboratory, Uppsala, Sweden, and at Macrogen Inc., Seoul, South Korea. Sequences were analyzed using BioEdit (version 7.0.1).

RESULTS

Isolation and classification of FA-resistant mutants in *S. aureus* 8325-4. To isolate non-*fusA* chromosomal mutants, 20 independent cultures of *S. aureus* 8325-4 were grown into stationary phase and 10^8 CFU from each were spread on LA plates containing 2 μg/ml of FA. On average, 10 colonies appeared on each plate after an overnight incubation at 37°C. Twenty-eight FA-resistant colonies, including 7 small colonies from different cultures, were picked and purified. DNA sequencing revealed that the great majority of the mutants (n = 23) had point mutations in the *fusA* gene. Twenty-one of these were found in domain III (Table 1), in agreement with pub-

lished data on the importance of this region of EF-G for FA resistance mutations (26, 38). The remaining two *fusA* mutations both had the substitution G664S in domain V of EF-G (Table 1). Only one FA-resistant mutation, A655P, has previously been identified in domain V of *S. aureus* EF-G, and that was in a clinical isolate that carried three additional mutations in domain III of EF-G (38). The remaining five FA-resistant mutants had no mutations in *fusA* and thus were classified as FusE. These five FusE mutants and the two FusA domain V mutants differ significantly from the 25 FusA domain III mutants in that they have a small-colony phenotype, requiring ≥48 h at 37°C to form 1-mm-diameter colonies on LA. The slow growth of the FusE and FusA domain V mutants is evident also in liquid culture (Table 1). We asked whether these slow-growing FA-resistant mutants might be similar to the previously described SCV type in *S. aureus* (46).

Characterization of slow-growing FA-resistant mutants isolated in 8325-4. The classic SCV phenotypes described in *S. aureus* include, in various combinations, enhanced resistance to aminoglycosides; auxotrophy for thymidine; and auxotrophy for components of the electron transport system such as hemin, menadione, and thiamine (46). We assayed all 28 FA-resistant mutants for each of these phenotypes (Table 1). The 21 FusA domain III mutants vary widely in resistance to FA (MIC, 4 to 96 μg/ml), as expected, but are relatively uniform with respect to the other phenotypes tested (Table 1). Thus, they display relatively small increases in generation time (1.1- to 1.6-fold longer); small increases in MIC for the aminoglycosides KAN and streptomycin (STR) (1.3- to 4-fold increases); no significant change in MIC for the aminoglycoside gentamicin (not shown); and no auxotrophy for hemin, menadione, thiamine, or thymidine.

In contrast, the FusA domain V mutants and the FusE mutants have markedly different phenotypes (Table 1). Their FA MIC is uniform and at a medium level (MIC, 4 μg/ml), their generation times are significantly longer (2.3- to 3.6-fold longer), and their susceptibility to aminoglycosides is greatly reduced (KAN MIC, 5- to 16-fold increase; STR MIC, 5- to 8-fold increase; gentamicin MIC, 3- to 6-fold increase). In addition, the FusA domain V mutants and each of the members of the FusE class have an auxotrophy on LA that is complemented by hemin (Fig. 1 and Table 1). Each of these specific traits has been reported previously as a characteristic of the SCV phenotype (46). We concluded that these FA-resistant mutants (FusA domain V and FusE) should be classified within the SCV group of *S. aureus*.

Selection of Kan^r SCV mutants of 8325-4. To test the robustness of our classification of some FA-resistant mutants as SCVs, we reversed the selection process. Thus, we selected Kan^r mutants, screened for SCVs, and asked whether any displayed an FA-resistant phenotype. In a preliminary experiment, SCVs were selected both in a rifampin-resistant (Rif^r) derivative of 8325-4 and in the original Rif^s 8325-4. The use of a marked strain is an extra control that the selected SCVs originate from the strain plated. In those selections, 21/524 Rif^r SCVs were FA resistant, while 51/3,300 Rif^s SCVs were FA resistant. The low percentage of FA-resistant SCVs among the SCVs selected with KAN may explain why the FA-resistant class has not been identified previously by others. We concluded that some mutants selected with KAN as SCVs have the FA-resistant phenotype and proceeded to make a larger-scale experiment to obtain mutants for genetic and phenotypic analysis. Twenty independent cultures of *S. aureus* 8325-4 Rif^r were grown overnight into the stationary phase. A total of 10^8 cells from each culture were spread on LA containing 8, 16, or 24 μg/ml of KAN. The plates were incubated 48 h at 37°C to allow slow-growing variants to form visible colonies. At 8 μg/ml of KAN, ~10^3 colonies were visible, while at 16 and 24 μg/ml, the numbers of Kan^r colonies were ~200 and ~30, respectively. Eight Kan^r clones (two medium/large and six small) were picked from each culture and selection level, purified on selective plates, and then screened for FA resistance (LA plus FA, 1 μg/ml). Of the 360 SCV Kan^r clones checked, 64 (18%) were resistant to FA. No FA-resistant mutants were found among the 120 medium/large colonies tested. The *fusA* gene (EF-G) was completely sequenced in each of these 64 FA-resistant SCV mutants. Mutations causing single-amino-acid substitutions in EF-G were identified in 38/64 mutants. When multiple identical isolates from the same culture were excluded, 27 independently selected mutations (12 different amino acid substitutions) were identified, distributed between structural domains I, III, and V of EF-G (Table 2). The majority of SCV mutations in EF-G occurred in domain V (20 independent, 6 different), followed by domain III (5 independent, 4 different), and finally domain I (2 different). The remaining 25 FA-resistant SCVs carried no mutations in *fusA* and were thus classified as FusE mutants.

FIG. 1. Complementation of hemin auxotrophy (8325-4 FusE-SCV).

Phenotypes of Kan^r-selected SCVs of 8325-4. Each of the 64 FA-resistant SCV strains was assayed for MIC, doubling time, and complementation of auxotrophy by hemin, menadione (Fig. 2), thiamine, and thymidine. The results are summarized in Table 2, showing a single representative of each of the 12 different FusA mutants and all 25 FusE mutants. All of the mutants had increased MICs for KAN (3- to 170-fold wild type), STR (3- to 80-fold wild type), and FA (32 to 384-fold

TABLE 2. Mutants selected in 8325-4 as Kan^r SCVs and then screened for FA resistance

Strain and stock (SCV)	Identified mutation[a]		MIC (μg/ml) of:			Doubling time (min)[b]	Auxotrophy[c]	n[d]
	fusA (EF-G)	*rplF* (L6)	KAN	STR	FA			
8325-4								
AH001			1.5	3	0.125	24	−	
FusA-SCV								
AH366 (SCV-38)	Pro114His	None	12	24	12	77	Hemin	
AH369 (SCV-41)	Gln115Leu	None	6	12	48	52	Hemin	
AH364 (SCV-36)	Asp434Asn	None	8	24	256	64	Hemin	
AH334 (SCV-6)	Thr436Ile	None	128	192	48	54	Hemin + menadione	
AH336 (SCV-8)	His438Asn	None	16	12	8	65	Hemin	
AH338 (SCV-10)	Arg464Cys	None	48	24	2	67	Hemin	2
AH340 (SCV-12)	Gly617Asp	None	12	24	4	52	Hemin	7
AH332 (SCV-4)	Ala655Glu	None	12	12	12	68	Hemin	2
AH329 (SCV-1)	Arg659Cys	None	12	24	12	63	Hemin	5
AH331 (SCV-3)	Arg659His	None	16	24	8	49	Hemin	3
AH347 (SCV-19)	Arg659Ser	None	12	32	12	91	Hemin	
AH367 (SCV-39)	Gly664Ser	None	12	16	12	46	Hemin	2
FusE								
AH380 (SCV-52)	None	−1 FS at nt 158	4	24	6	52	Hemin	
AH352 (SCV-24)	None	−1 FS at nt 106	6	24	4	53	Hemin	
AH377 (SCV-49)	None	+1 FS at nt 23	6	24	4	49	Hemin	
AH348 (SCV-20)	None	TGA stop at nt 244	8	16	8	62	Hemin	
AH370 (SCV-42)[e]	None	TAA stop at nt 229	8	24	8	46	Hemin	
AH346 (SCV-18)	None	−1 FS at nt 238	8	24	8	77	Hemin	
AH333 (SCV-5)	None	TAA stop at nt 249	8	24	12	56	Hemin	
AH392 (SCV-64)	None	−1 FS at nt 383	12	16	4	57	Hemin	
AH379 (SCV-51)	None	TAA stop at nt 418	12	16	6	45	Hemin	2
AH349 (SCV-21)	None	−1 FS at nt 404	16	64	4	67	Hemin	
AH374 (SCV-46)	None	−5 FS at nt 236–240	32	96	8	59	Hemin	
AH358 (SCV-30)[f]	None	−1 FS at nt 423	48	64	12	100	Hemin	
AH339 (SCV-11)	None	−1 FS at nt 144	64	128	6	120	Hemin	
AH337 (SCV-9)[e]	None	−1 FS at nt 220	96	256	12	130	Hemin	
AH357 (SCV-29)	None	TAA stop at nt 301	96	256	12	79	Hemin	
AH384 (SCV-56)[g]	None	−1 FS at nt 221	128	256	24	140	Hemin	
AH378 (SCV-50)[e,g]	None	−1 FS at nt 221	16	256	8	200	Menadione	
AH351 (SCV-23)	None	−4 FS at nt 45–48	64	256	16	180	Menadione	
AH360 (SCV-32)	None	ATA start at nt 3	96	192	24	140	Menadione	
AH383 (SCV-55)	None	TAA stop at nt 139	96	192	16	200	Menadione	
AH362 (SCV-34)	None	TAA stop at nt 282	128	256	12	170	Menadione	
AH330 (SCV-2)[f]	None	−1 FS at nt 423	128	96	12	200	Menadione	
AH390 (SCV-62)	None	+1 FS at nt 84	256	64	16	170	Menadione	
AH386 (SCV-58)[e]	None	−1 FS at nt 90	256	96	24	180	Menadione	

[a] None, wild type. Underlined letters represent the mutated residue. FS, frameshift mutation.
[b] Doubling time (generation time) in minutes during exponential growth in LB.
[c] Auxotrophy tested for hemin, menadione, thymine, and thymidine. −, nonauxotrophic.
[d] Number of independent isolates with the same genotype and phenotype.
[e] SCV-42, -9, -50, and -58 were sequenced for all ribosomal proteins and rRNA operons to determine whether any other mutations in the ribosome could distinguish between the hemin- and menadione-auxotrophic classes.
[f] One of a pair of mutants with the same mutation in *rplF* but different phenotypes.
[g] One of a pair of mutants with the same mutation in *rplF* but different phenotypes.

wild type). In addition, the MIC for the aminoglycoside gentamicin was increased by 4- to 48-fold (data not shown). Doubling times in liquid media were increased significantly for all SCV mutants, as expected. Among the 12 different FusA SCVs, 11/12 were complemented by hemin alone, while 1 was complemented by both hemin and menadione. Among the 25 FusE SCVs, 17 were complemented by hemin, while the remaining 8 were complemented by menadione (Table 2). None of the 37 SCV mutants was complemented by thiamine or thymidine.

We also tested 19 SCVs selected independently in 8325-4 as Kan^r but not showing the FA resistance phenotype on plates. Each of these had a MIC for KAN in the range of 24 to 256 μg/ml and a MIC for FA that was unchanged from the wild type (0.125 μg/ml). Thus, only a specific subgroup of aminoglycoside-resistant SCVs have an increased MIC for FA.

We asked whether FA-resistant SCVs might be mutator strains: i.e., whether the two phenotypes FA resistance and SCV might have arisen independently because of a hypermutator phenotype. Eleven FA-resistant SCVs, independently isolated in 8325-4, were tested for mutation frequency by screening five independent cultures of each strain for spontaneous Rif^r mutants on LA with 100 μg/ml RIF. The parental strain 8325-4 was used as a control on mutation frequency. The Rif^r mutation frequencies ranged from 10^{-8} to 6×10^{-7}. In addition, we measured the mutation frequency to Str^r for the

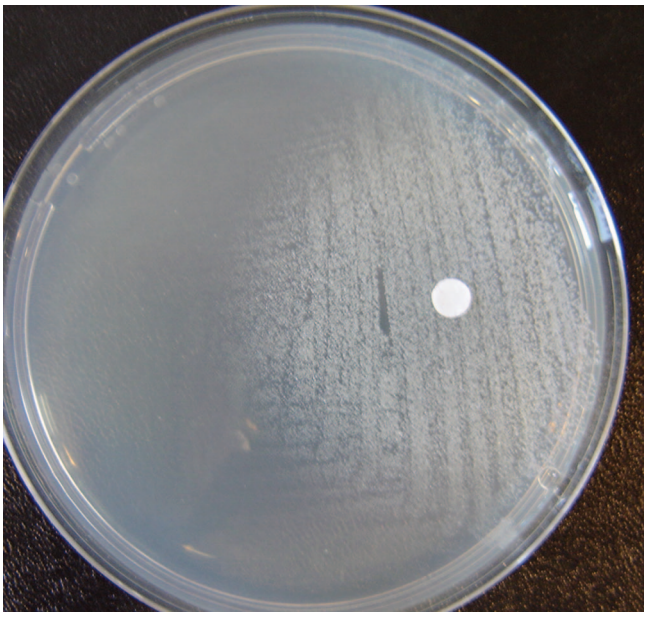

FIG. 2. Complementation of menadione auxotrophy (8325-4 FusE-SCV).

FA-resistant SCVs listed in Table 2. (Str^r was assayed because these mutants are already RIF resistant). No significant increase in mutation frequency relative to the wild type was observed for any of the mutants. The normal range of mutation frequencies argues against hypermutation as the origin of FA resistance among SCVs.

Identification of mutations in ribosomal protein L6 specific to FusE mutants. We rationalized that the FA-resistant phenotype of the FusE mutants might be caused by a mutation affecting the ribosome. Accordingly we choose two hemin-auxotrophic (SCV-9 and SCV-42) and two menadione-auxotrophic (SCV-50 and SCV-58) FusE mutants and determined, by PCR and DNA sequencing, their genotypes for each of the rRNA operons and for all 56 annotated ribosomal protein genes, including those that have been documented to interact with EF-G (8). In all four strains, we identified mutations in only one of the sequenced genes, *rplF*, the gene coding for ribosomal protein L6. We then sequenced *rplF* in all SCVs analyzed in this paper. We found that all FusE mutants had mutations in *rplF* (Tables 1, 2, and 3), whereas this gene was wild type in all FusA-SCVs. The mutations identified in *rplF* are predicted to knock out the function of the gene. They each mutate the coding sequence and include nucleotide substitutions altering the start codon, insertion, or deletion mutations that alter the reading frame and nucleotide substitution mutations that introduce premature termination codons. Mutations were located throughout *rplF*, from as early as the start codon to as late as nucleotide (nt) 418 in the 534-nt coding sequence. We also sequenced *rplF* in 15 of the FA-resistant SCVs isolated in the preliminary selections in 8325-4 and the 8325-4 Rif^r variant and identified L6 mutations in both sets of strains (seven different frameshift mutations in *rplF* [data not shown]). Finally, we sequenced *fusA* and *rplF* in 19 independently isolated FA-susceptible SCVs (selected in 8325-4 for KAN resistance) and found no mutations in either gene. We conclude that knockout mutations in *rplF* are specific to the FusE mutants and are not a general characteristic of all SCVs. We also predict that one or both of the FusE subgroups, either the hemin auxotrophs or the menadione auxotrophs, must carry an additional mutation, to explain this difference in auxotrophy. From our sequence analysis we conclude that the additional mutation is not in a ribosomal protein or rRNA gene.

Selection of Kan^r SCVs in FA-susceptible clinical isolates of *S. aureus*. Two independent cultures of each of 10 different FA-susceptible (MIC, $\leq$0.25 μg/ml) clinical bacteremia isolates were grown; used to select for Kan^r at 8, 16, or 24 μg/ml; and then screened for SCVs. Similar numbers of Kan^r colonies were selected at each level as previously found with strain 8325-4. Eight small colonies were picked from each selection plate (480 colonies total), purified at the same level of KAN, and then screened for FA resistance (LA plus FA, 1 μg/ml). Fifteen FA-resistant mutants were isolated from 8 of the 10 clinical isolates (Table 3). DNA sequencing of *fusA* showed that 10 mutants belonged to the FusA-SCV class (mutant *fusA*, EF-G) with the remaining 5 being FusE (mutant *rplF*, L6). Among the FusA-SCV mutants, 9 of 10 were in domain V, while 1 mutation was mapped in domain III (Pro404Arg, which has not been described before). Four of the FusA-SCVs were auxotrophic for hemin, while the remaining six showed no auxotrophy. These six FusA SCVs were the fastest growing of those isolated, and the lack of obvious complementation probably reflects their intrinsically faster growth. All five of the FusE mutants were complemented by menadione. All 15 FA-resistant SCVs selected from the clinical isolates also showed loss of pigmentation. We concluded that SCVs with an FA-resistant phenotype, of both the FusA-SCV and FusE classes, can be selected from clinical isolates of *S. aureus*.

DISCUSSION

Genetics and phenotypes of FA-resistant SCVs. Recently the genetic basis of the SCV phenotype in thymidine-auxotrophic clinical isolates of *S. aureus* was identified as knockout mutations in *thyA*, the gene coding for thymidylate synthase (12). The genetic causes of other clinical or spontaneous SCVs are still unknown, but the hemin- and menadione-auxotrophic SCV phenotypes have been reproduced in laboratory strains constructed by disrupting *hemB* (56) and *menD* (9), genes essential for cytochrome biosynthesis.

Here we have identified a novel subset of *S. aureus* SCVs that are resistant to FA. Thus, we have shown that some mutants selected in *S. aureus* for resistance to the antibiotic FA are also SCV mutants. Using the reverse approach, we have shown that some mutants selected in *S. aureus* as SCVs, on the basis of resistance to aminoglycosides, are resistant to FA. The FA-resistant SCVs fall into distinct genetic and phenotypic classes and carry mutations in *fusA* or in *rplF*. (i) FusA-SCV mutants have amino acid substitutions in EF-G (most in structural domain V, but some in domain I or III) and are auxotrophic for hemin. (ii) FusE mutants have knockout mutations in *rplF* (ribosomal protein L6) and are auxotrophic for hemin or for menadione. The different auxotrophies associated with the FusE class (some of which have identical mutations in *rplF* [see Table 2]) suggest that one or the both of these subgroups must carry an additional mutation. This interrelatedness of the

TABLE 3. Mutants selected as Kan^r SCVs in FA-susceptible clinical isolates and then screened for FA resistance

Strain and stock	Source[a]	Identified mutation[b]		Antibiotic MIC (μg/ml) of:			Doubling time (min)[c]	Auxotrophy[d]	*n*[e]
		fusA (EF-G)	*rplF* (L6)	KAN	STR	FA			
	IN476–IN494	None	None	2	6	0.047	24	−	
FusA-SCV									
AH421	IN488	Pro404Arg	None	8	12	3	36	−	
AH288	IN476	Gly617Asp	None	16	32	6	53	Hemin	2
AH294	IN490	Gly628Val	None	16	8	2	40	−	
AH287	IN476	Ala655Glu	None	16	48	12	64	Hemin	
AH291	IN484	Arg659His	None	24	24	4	43	−	
AH297	IN492	Ser660Pro	None	24		2	53	Hemin	
AH296	IN490	Gly664Ala	None	16	8	3	39	−	
AH292	IN484	Gly666Val	None	32	16	6	36	−	
AH298	IN494	Gly666Val	None	32	6	6	37	−	
FusE									
AH422	IN488	None	+2 FS at nt 222	128	32	12	81	Menadione	
AH290	IN478	None	Duplicate nt 202–293	256	64	8	200	Menadione	
AH420	IN484	None	Δ of nt 72–80	256	256	12	150	Menadione	
AH417	IN480	None	−1 FS at nt 422	256	256	24	180	Menadione	
AH416	IN476	None	Δ of nt 395–398	256	256	48	170	Menadione	

[a] Each of the original FA-susceptible clinical isolates had similar MICs for KAN, STR, and FA and similar doubling times in LB.
[b] None, wild type. FS, frameshift mutation.
[c] Doubling time (generation time) in minutes during exponential growth in LB.
[d] Auxotrophy tested for hemin, menadione, thymine, and thymidine. −, nonauxotrophic.
[e] Number of independent isolates with same genotype and phenotype.

FA resistance and SCV phenotypes was demonstrated both in the standard laboratory wild-type strain, 8325-4, and in a variety of clinical *S. aureus* isolates.

Clinical relevance of FA-resistant SCVs. FA-resistant SCV mutants have several features that make them interesting from a clinical perspective. First, they arise at a high spontaneous frequency relative to other FA-resistant mutants. When FA resistance is selected at ~1 μg/ml of the drug, approximately 50% of the resistant mutants belong to the SCV classes (Table 1) (38). Second, the MICs of the resulting FA-resistant SCVs are in the same range as the great majority of classical FusA mutants and of FusB strains. Third, although their characteristic slow growth might be expected to result in a strong Darwinian selection against them in the absence of antibiotic selective pressure, there is evidence to suggest that this interpretation is too simplistic. Thus, *S. aureus* SCVs have been associated with an ability to establish and maintain chronic infections as shown directly in animal models and indirectly by the isolation of SCVs from patients suffering chronic infections (12, 46). This feature has not been shown directly for FA-resistant SCVs as they have not previously been described. However, we earlier described an FA-resistant clinical isolate that carries multiple mutations in *fusA* causing amino acid substitutions in structural domains III and V of EF-G (38). The domain V residue mutated in that clinical isolate, A655P, is also mutated in FusA-SCVs selected in this study in 8325-4 (Table 2) and in a susceptible clinical isolate (Table 3), although the substitution, A655E, is different. The multiply mutated FA-resistant clinical isolate carried, in addition to A655P, two substitutions in domain III (L461F and D463G), in a region previously associated with mutations causing FA resistance (38). One attractive possibility to explain this genotype is that that the domain V mutation arose as an SCV and that the two mutations in domain III were subsequently selected for improved growth fitness and/or increased FA resistance. Because FA-resistant SCVs arise spontaneously at a high frequency, they might be a significant source of FA-resistant mutants in clinical settings. This remains to be studied.

FA resistance mutations in EF-G and L6 in terms of structure and position. The major function of EF-G is to promote the translocation, in a coordinated movement, of two tRNA molecules and the mRNA, through the central cavity formed between the two subunits of the ribosome. The distribution of FA resistance mutations in EF-G is strikingly nonrandom. All of the mutations, both classical FusA and FusA-SCV, map in one of the EF-G structural domains I, III, or V. The domain boundaries of EF-G have been defined for the structure of the homologous protein from *Thermus thermophilus* (2) transposed to *S. aureus* (32). Structural domain I (the guanine nucleotide-binding domain) extends from residues 1 to 280, domain III from residues 404 to 483, and domain V from residues 606 to 693. Classical FA resistance mutations selected in *S. aureus* in this study and those selected previously (38) map overwhelmingly in structural domain III (80 independent isolates), with a few found in domain I (5 independent isolates). In contrast, the FusA-SCV mutations map mostly in structural domain V (31 independent isolates), with fewer in domains III (5 independent isolates) and I (2 independent isolates). Clearly, the FusA-SCV mutants are strongly associated with domain V, whereas the classical FusA mutants are predominantly associated with alterations in domain III. The interaction of EF-G with the ribosome has been mapped in the post-translocational state by site-directed hydroxyl radical probing, including three residues in domain V (64). This showed that amino acid residues in domain V of EF-G are in very close proximity to specific regions of 23S rRNA on the large ribosomal subunit, in particular to helices 43, 44, 89, and 95 (known also as the sarcin-ricin loop). This unambiguously positions

domain V of EF-G relative to 23S rRNA. When additional hydroxyl radical probing data, together with the available structural data (3–5), were used to dock EF-G onto the ribosome, it supported this positioning of domain V and further suggested that structural domain III of EF-G lies in close proximity to ribosomal protein S12 on the 30S ribosomal subunit (63). Additional structural studies of EF-G and its interactions with the ribosome may provide information on the functional significance of these mutational preferences and their association with the different phenotypes associated with classical FusA and FusA-SCV mutants.

Mutants selected in *Escherichia coli* for reduced susceptibility to the aminoglycoside gentamicin have been shown to be mutated in ribosomal protein L6 (15). These mutants also have reduced susceptibility to other aminoglycosides. The L6 mutations restrict the errors of translation (31). Two of these L6 mutations have been characterized genetically. They carry a 7-nt duplication or an 11-nt deletion, respectively, in each case predicted to result in a reading frame shift and premature termination in the C-terminal half of the protein (21). These truncated mutant proteins have been shown to be present within the ribosomes of gentamicin-resistant *E. coli* (15). The increased accuracy of translation by these mutant ribosomes may be caused by increased proofreading of ternary complexes, because the L6 mutations are predicted to distort elongation factor binding on the large subunit (21). L6 is situated at the subunit interface close to the GTPase center in the region of the L7/L12 stalk (8) and has been cross-linked to EF-G (54). L6 may modulate the RNA structure that forms the elongation factor binding site (37). The L6 mutants isolated in the present study in *S. aureus* are distributed throughout the *rplF* coding sequence and are also predicted to cause premature termination of L6 synthesis. In two extreme cases, we have identified mutations altering the initiation codon (Table 1 and Table 2). Our conclusion from the nature and distribution of the mutations in *rplF* is that the SCV phenotype of FusE mutants can be associated with a ribosome completely lacking L6 protein. This does not rule out the possibility that for some of the FusE mutants a truncated L6 is bound to the ribosome. An unusual feature of protein L6 in the ribosome is that, unlike most ribosomal proteins, it interacts significantly with several other ribosomal proteins. The members of this protein cluster include L3, L6, L13, and L14, and all are found close to the elongation factor binding site (8). The existence of this protein cluster motivated us to ask whether a second mutation in one of these proteins might explain the two different phenotypes, hemin or menadione auxotrophy, associated with FusE mutants. Accordingly, we sequenced the genes for each of these ribosomal proteins from two hemin-auxotrophic and two menadione-auxotrophic FusE mutants. We extended this analysis by sequencing the genes for all 56 annotated ribosomal proteins based on sequences in the *S. aureus* genome and for each of the rRNA genes. We found no additional mutations in any of the four mutants and thus cannot yet explain the basis for the two phenotypic variants of FusE mutants.

Our tentative conclusion from the genetic analysis of FusA-SCV and FusE mutants is that *fusA* and *rplF* mutations cause FA resistance by altering, directly or indirectly, the structural conformations of EF-G on the ribosome and thus its sensitivity to inhibition by FA. The documented aminoglycoside resistance caused by L6 mutations in *E. coli* (15) and the interactions of L6 and EF-G on the ribosome (8) make it plausible that the reduced aminoglycoside susceptibility we have measured in FusA-SCV and FusE mutants is also, at least in part, caused at the level of antibiotic binding to the ribosome.

Similarities between FA-resistant SCVs and *fusA1* in *Salmonella enterica* serovar Typhimurium. An FA-resistant mutant in *Salmonella enterica* serovar Typhimurium shares some phenotypic characteristics with the FA-resistant SCVs described here. The *fusA1* allele has the mutation P414L in domain III of EF-G (26). *fusA1* causes slow growth both on rich solid medium and in liquid medium. The mutant P414L EF-G has an increased K_m for GTP and is inhibited in translation in vitro by ppGpp (35). ppGpp is a global transcriptional regulator molecule produced in a RelA-dependent reaction at the factor binding site of the ribosome (17). ppGpp is normally produced at a low basal level in fast-growing cells and is strongly induced when cells enter starvation or stationary phase (17). However, strains with the mutant P414L EF-G make only one-third the normal basal level of ppGpp and have a very low level of induction upon starvation (35). ppGpp is a positive regulator of the RNA polymerase sigma factor RpoS (22), itself a global regulator of stress response genes (24). Strains with the mutant P414L EF-G are defective in RpoS induction (34) and as a consequence produce reduced levels of heme, have increased sensitivity to oxidative stress, and have a reduced rate of aerobic respiration (33). Addition to the growth medium of precursors of heme biosynthesis alleviates the sensitivity to oxidative stress (33). Thus, this FA-resistant mutant in *S. enterica* serovar Typhimurium shares with FA-resistant SCV mutants in *S. aureus* an auxotrophy in the heme biosynthesis pathway. It was earlier shown that mutations that directly affect heme biosynthesis, *hemL* in *S. enterica* serovar Typhimurium (16) or *hemB* in *E. coli* (49), result in SCVs that cause persistent and recurrent infections. In the case of *fusA1*, it is clear that the SCV-like phenotypes are indirect consequences of alterations in gene expression resulting from an aberrant EF-G–ribosome interaction. Accordingly, the FA-resistant SCVs in *S. aureus* may also have their SCV phenotypes as a result of altered gene expression patterns resulting from aberrant ribosome activities, mediated through either mutant EF-G or mutant L6. We have also shown that mutations altering EF-G or ribosomal protein L6 are specific to FA-resistant SCVs, and they were not found in any of 19 independently isolated FA-susceptible SCVs.

Potential for coselection of FA resistance in *S. aureus*. SCVs of *S. aureus* frequently show increased resistance to other antibiotics (46) and can even be selected by triclosan (52). A clinically relevant conclusion from our data is that FA resistance in *S. aureus* can be selected by aminoglycosides. If this is also so in the clinical setting, then the dynamics of antimicrobial drug use driving the selection and spread of FA resistance will have to be considered in a wider context.

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