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Staphylococcus aureus Fatty Acid Auxotrophs Do Not Proliferate in Mice

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Inactivation of acetyl-coenzyme A (acetyl-CoA) carboxylase confers resistance to fatty acid synthesis inhibitors in *Staphylococcus aureus* on media supplemented with fatty acids. The addition of *anteiso*-fatty acids (1 mM) plus lipoic acid supports normal growth of Δ*accD* strains, but supplementation with mammalian fatty acids was less efficient. Mice infected with strain RN6930 developed bacteremia, but bacteria were not detected in mice infected with its Δ*accD* derivative. *S. aureus* bacteria lacking acetyl-CoA carboxylase can be propagated *in vitro* but were unable to proliferate in mice, suggesting that the acquisition of inactivating mutations in this enzyme is not a mechanism for the evasion of fatty acid synthesis inhibitors.

The steady increase in multidrug-resistant *Staphylococcus aureus* prevalence has spurred the search for novel antibiotics to attack this clinically important organism (1). The bacterial type II fatty acid biosynthesis system (FASII) is a novel target that has engendered considerable attention, and there are multiple natural-product antibiotics that target the pathway (2). Most Gram-negative bacteria are susceptible to FASII inhibitors even when exogenous fatty acids are provided because they lack the ability to activate exogenous fatty acids to produce acyl carrier proteins (ACPs) and synthesize the hydroxyacyl-ACPs to support lipopolysaccharide biosynthesis (2). However, the behavior of Gram-positive bacteria is different. These organisms do not produce hydroxy-fatty acids, and they are capable of incorporating exogenous fatty acids by ligating them to ACPs (3). These acyl-ACPs are either elongated by the FASII system or incorporated into phospholipids via the acyl-PO_4/acyl-ACP-specific PlsX/PlsY/PlsC acyltransferase system (3). Thus, the FASII pathway can be inactivated through genetic deletions or FASII drugs in *S. agalactiae*, and the organism is able to grow if supplied with an exogenous fatty acid supplement (4). In *S. pneumoniae*, endogenous fatty acid synthesis is repressed in the presence of exogenous fatty acids, leading to the replacement of endogenous fatty acids with those provided in the medium even if FASII is not chemically or genetically inactivated (3, 4). Although *S. aureus* also incorporates exogenous fatty acids into membrane phospholipids via acyl-ACP, FASII inhibitors remain effective against this Gram-positive organism even in the presence of exogenous fatty acids (3).

AFN-1252 is a compound in clinical development that blocks the enoyl-ACP reductase step of *S. aureus* FASII (5–7). Two classes of AFN-1252-resistant *S. aureus* mutants were isolated (3). One class consists of missense mutations in the *fabI* gene that lead to the production of a mutant FabI protein that is refractory to AFN-1252. When exogenous fatty acids were supplied in the media during selection, AFN-1252-resistant clones appeared 100 times more frequently (3). Genetic analysis showed that these isolates harbored mutations that completely inactivated one of the four genes required for acetyl-coenzyme A (acetyl-CoA) carboxylase (ACC) activity and were fatty acid auxotrophs (3). ACC produces malonyl-CoA from acetyl-CoA and is essential to support *de novo* fatty acid synthesis (8). If *S. aureus* can circumvent therapy by relying on host fatty acids, the effectiveness of FASII therapies would be compromised through the acquisition of *acc* mutations. The goal of this work was to characterize the utilization of fatty acids in *S. aureus* strains lacking ACC activity and determine if these *acc* mutants are capable of proliferating in mice. The results show that a *S. aureus accD* knockout strain is a fatty acid and lipoic acid auxotroph, grows poorly on mammalian fatty acid structures, and does not cause bacteremia in a mouse infection model.

Strain PS01 (Δ*accD*), a derivative of strain RN4220 (3), was used to examine the growth requirements of strains lacking ACC activity. *S. aureus* produces primarily branched-chain saturated fatty acids. The two principal fatty acids in strain RN4220 are *anteiso* 15:0 and *anteiso* 17:0 (*a*15:0/*a*17:0), with 17:0 occupying position 1 and 15:0 occupying position 2 of its membrane phospholipids (3). Therefore, the first growth experiments were performed with a mixture (2:1) of *anteiso* 15:0 and 17:0 fatty acids. Strain PS01 (Δ*accD*) did not exhibit growth in CY medium (9) in the absence of a fatty acid supplement (Fig. 1A). The inclusion of 1 mM total fatty acid as a complex with 10 mg/ml fatty-acid-free bovine serum albumin (BSA) in CY medium allowed strain PS01 to grow, but growth ceased prior to reaching the stationary phase (Fig. 1A). These data indicated that another nutrient was limiting, and because lipoic acid is produced from the FASII pathway (10), the requirement for lipoate was investigated. The addition of a lipoate supplement (0.1 μg/ml) along with the fatty acids restored normal growth to strain PS01 (Fig. 1A). CY medium contains yeast extract, which was the source of lipoate to support some growth in CY media incorporating fatty acid. The lipoic acid requirement could also be satisfied by the inclusion of 1 mM octanoate to the medium (not shown). This result was expected based on the established pathways for lipoate biosynthesis in *Bacillus subtilis* (11); however, octanoate was not able to supply the fatty acid requirement of the Δ*accD* strain. The concentration of fatty acids required to support normal growth on strain PS01 (Δ*accD*) in CY medium was determined (Fig. 1B). Strain PS01 required a 1

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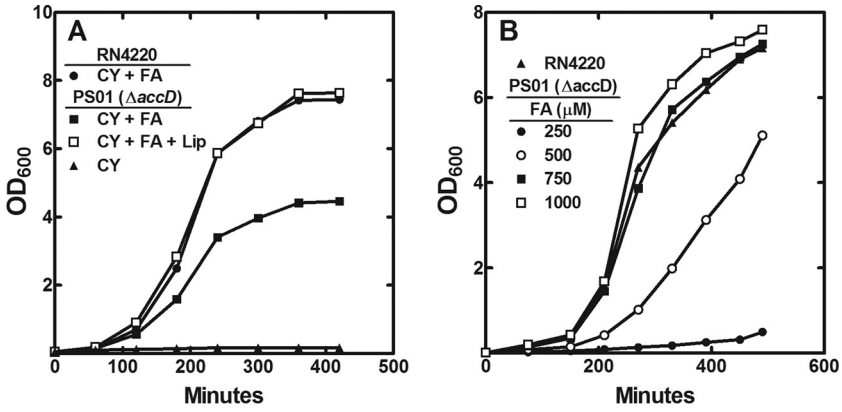

FIG 1 *S. aureus* strain PS01 (Δ*accD*) is a fatty acid and lipoate auxotroph. (A) Growth of wild-type strain RN4220 or strain PS01 (Δ*accD*) in CY medium (9) with or without *S. aureus* fatty acids (FA; a 1 mM concentration of a 2:1 mixture of *anteiso* 15:0/17:0 and 10 mg/ml fatty-acid-free BSA) and/or lipoate (Lip; 0.1 μg/ml). OD_{600}, optical density at 600 nm. (B) Growth of strain PS01 on different concentrations of the FA supplement.

mM fatty acid supplement to reach the same rate of growth observed in the wild-type strain. These data established that strain PS01 is a fatty acid and lipoic acid auxotroph.

Mammalian fatty acids are mixtures of straight-chain saturated, unsaturated, and polyunsaturated fatty acids and are devoid of the branched-chain fatty acids that are produced by *S. aureus*. Strain PS01 was grown overnight in the *a*15:0/*a*17:0 mixture and then subcultured to media supplemented with 1 mM mammalian fatty acids and lipoate. Strain PS01 did not grow on 16:0 alone, illustrating that saturated fatty acids did not support growth (Fig. 2A). The strain was able to grow with 18:1 and with a mixture of fatty acids found in human serum (12) (Fig. 2A). In this experiment, the cells began with a normal fatty acid composition that was diluted out by the fatty acid supplement as the cells grew. Thus, the experiment was repeated using cells grown overnight in their respective fatty acid compositions to completely replace their normal *anteiso* fatty acids (Fig. 2B). Cells adapted to mammalian fatty acids were clearly deficient in growth compared to cells grown with their native fatty acids. The growth rates of strains RN4220 and PS01 were also compared using human serum as the source of the fatty acid/lipoic acid supplement (Fig. 2C). There was a marked defect in the growth of PS01 (Δ*accD*) in serum compared to that of the wild-type strain. These results showed that *S. aureus* ACC mutants were able to proliferate using only mammalian fatty acids for membrane phospholipid synthesis, but these fatty acids could not support the same rate of growth as that seen after supplying the normal fatty acid structures.

Although we were able to propagate the PS01 strain in the laboratory, the growth requirements for this strain suggested that it may be significantly attenuated in animals. The laboratory experiments used the genetically malleable strain RN4220; however, for the mouse experiments, we used strain *S. aureus* RN6930 (13) because this strain has a track record in mouse infection models (14, 15). Its Δ*accD* derivative, strain JP103, was created using a TargeTron gene knockout system (16) to insert a 0.9-kb group II intron at 164 bp into the *accD* gene using the plasmid and primers as described previously (3). The first step was to introduce the plasmid containing the group II intron flanked by *accD* sequences into strain RN6930 by phage-mediated transfer (15). After selection for insertions and curing the plasmid, the insertional inacti-

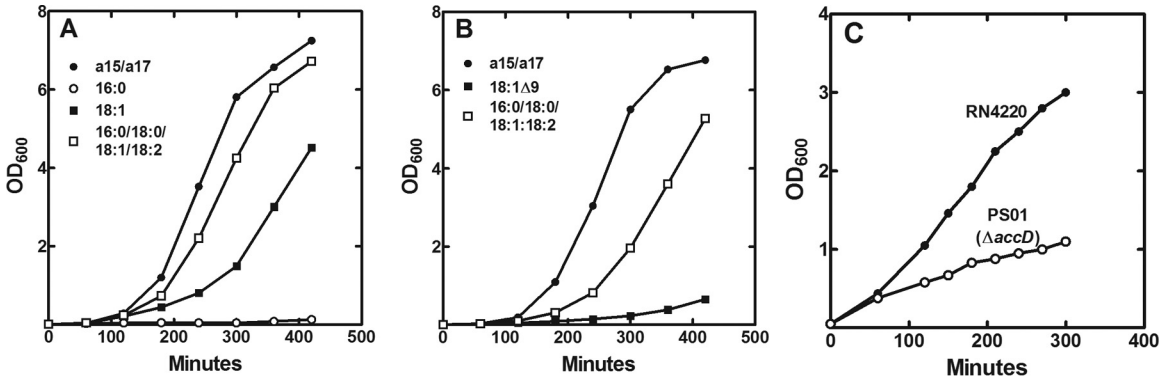

FIG 2 Growth characteristics of strain PS01 (Δ*accD*) in the presence of mammalian fatty acids and serum. (A) Strains were grown overnight on a mixture of *S. aureus anteiso* 15:0/17:0 fatty acids and then subcultured into media containing the indicated fatty acids, and growth was monitored. The final concentration of the fatty acids (16:0 or 18:1), the mixture of *S. aureus* fatty acids (*anteiso* 15:0/17:0 [a15/a17]; 2/1), or a mixture of fatty acids found in human serum (16:0/18:0/18:1/18:2; 2/1/2/3) (12) was 1 mM. Fatty acids were delivered as complexes with 10 mg/ml fatty-acid-free BSA and 0.1 μg/ml lipoate. (B) Strains were grown overnight in the respective fatty acid supplements and then subcultured into the media containing the same fatty acid composition, and growth was monitored. (C) A comparison of the growth of wild-type strain RN4220 and the growth of strain PS01 in human serum. These growth experiments were performed twice on different days with the same results. The data shown are from a representative experiment.

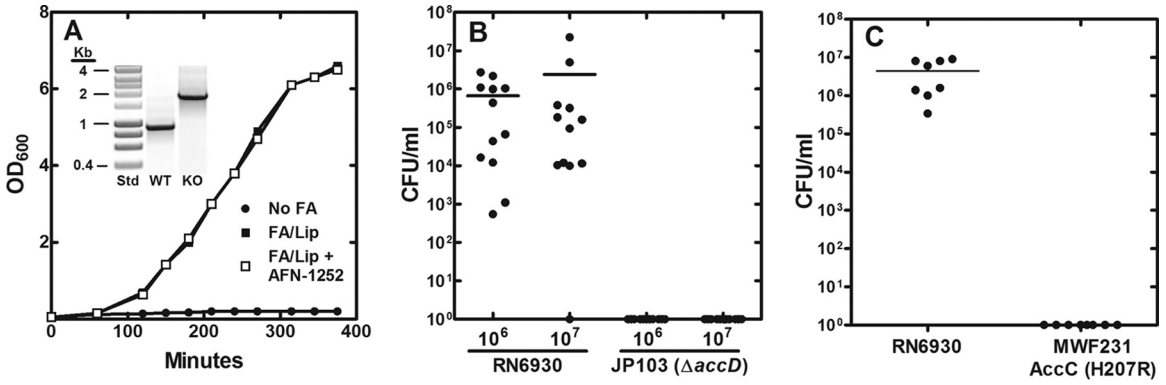

FIG 3 Growth requirements and virulence of strain JP103 (Δ*accD*). (A) Strain JP103 required the presence of extracellular fatty acids and lipoate to achieve a growth rate equivalent to that of wild-type strain RN6930, and no growth was observed in the absence of the fatty acid supplement. Strain JP103 was constructed by the insertion of a 0.9-kb group II intron into the same position (164 bp) as that in the *accD* gene as previously described for strain PS01 (3). Primers located outside the gene were used to confirm the insertion. These primers amplified a 0.9-kb product in strain RN6930 corresponding to the wild-type *accD* gene, whereas a 1.8-kb product was obtained from strain JP103, indicating the insertion of the group II intron into the *accD* gene. FA/Lip means a 1 mM mixture of *anteiso* 15:0/17:0 (2/1) plus 10 mg/ml fatty-acid-free BSA plus 0.1 μg/ml lipoate. Std, standard; WT, wild type; KO, knockout. (B) The results from two independent experiments with six mice in each group are combined in this panel. Mice were inoculated by interperitoneal injection with either 1×10^6 or 1×10^7 CFU of either strain RN6930 or JP103 (Δ*accD*) suspended in 0.1 ml of 5% gastric hog mucin as described previously (27). (C) Mice were challenged with 1×10^7 CFU of strain MWF231, an AFN-1252-resistant, fatty acid auxotroph with a point mutation in the *accC* gene predicted to encode AccC(H207R). After 24 h, blood samples were taken and CFU per ml were determined by plating on LB agar supplemented with FA/Lip. No colonies were detected in mice challenged with strain JP103 or strain MWF231. The lower limit of detection using our dilution series was 500 CFU/ml.

vation of the *accD* gene was confirmed by PCR, which showed a 0.9-kb insert in the *accD* gene in strain JP103 (Fig. 3A, inset). Strain JP103 was confirmed to be a derivative of strain RN6930 by its strong hemolytic phenotype on tryptic soy-blood-agar plates, which was in sharp contrast to the nonhemolytic phenotype of strain RN4220. Like strain PS01, the *accD*-deleted JP103 strain was a fatty acid auxotroph, required both *anteiso* fatty acids and lipoate for maximum growth *in vitro*, and was completely refractory to growth inhibition by AFN-1252 (Fig. 3A).

The ability of strain JP103 (Δ*accD*) to efficiently scavenge extracellular fatty acids and proliferate in a mammalian host was tested in a mouse infection model. Mice were inoculated by interperitoneal injection of different amounts of either strain RN6930 or JP103 (Δ*accD*) to compare the abilities of the two strains to proliferate *in vivo*. After 24 h, blood samples were taken from the animals and the CFU levels per ml of blood were determined. All mice infected with strain RN6930 exhibited significant bacteremia (Fig. 3B). In contrast, no *S. aureus* colonies were recovered from the blood of mice inoculated with strain JP103 (Δ*accD*) (Fig. 3B). Injection of mice with CFU levels of greater than 1×10^7 of either strain caused immediate animal distress requiring euthanasia after a few hours due to acute toxicity attributed to the injection of so many bacteria. These data show that strain JP103 (Δ*accD*) was unable to cause bacteremia in mice.

This work was extended by selecting for an AFN-1252-resistant derivative of strain RN6930 on plates containing 40 ng/ml AFN-1252 and a 1 mM concentration of a 15:0/17:0 fatty acid mixture. Individual colonies were isolated, and as reported previously using strain RN4220 (3), all clones exhibited no growth in the absence of a fatty acid supplement. Acetyl-CoA carboxylase mutations with partial activity were not obtained using this selection method because if there had been even a low level of activity, the pathway would have been blocked at the FabI step, leading to ACP depletion and the inability of the strain to incorporate exogenous fatty acids (3). DNA sequencing identified strain MWF231 as a fatty acid auxotroph with a missense mutation in the *accC* gene predicted to direct the expression of an AccC(H207R) enzyme. His207 is known to be critical for the AccC half-reaction of acetyl-CoA carboxylase (17). Challenging mice with 10^7 CFU of strain MWF231 did not result in bacteremia (Fig. 3C). Thus, an AFN-1252-resistant derivative of strain RN6930 containing a point mutation in acetyl-CoA carboxylase subunit C had an absolute requirement for exogenous fatty acids and did not proliferate in mice.

The importance of fatty acid composition to membrane lipid homeostasis and cell physiology is clear from decades of extensive investigation (for a review, see reference 18). Based on this body of knowledge, it is not surprising that *S. aureus* grows best when supplied with its normal mixture of *anteiso* branched-chain saturated fatty acids as opposed to the straight-chain saturated and unsaturated mammalian fatty acids. *S. aureus* contains hundreds of fatty acid modified proteins (19), and the acylation of these lipoproteins is required for virulence (20). It is unknown whether the functions of these proteins may be altered by modification with unnatural fatty acids. FASII also produces the octanoyl-ACP precursor for the lipoate biosynthetic pathway (10). The importance of lipoate in *S. aureus* intermediary metabolism is apparent from the requirement for a lipoate supplement to support growth of the Δ*accD* strains. Thus, *acc* knockouts must scavenge lipoate as well as fatty acids from the host, and our experiments illustrate that the quality and/or availability of these nutrients in mice attenuates the virulence of the bacteria. Our data provide a mechanistic rationale for the observed efficacy of the ACC inhibitors against *S. aureus* infections (21) and for the lack of *acc* mutations arising in animals treated with AFN-1252 (6, 22) or other FASII inhibitors (23–26).

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