Analysis of Mupirocin Resistance and Fitness in *Staphylococcus aureus* by Molecular Genetic and Structural Modeling Techniques

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Chromosomal resistance to mupirocin in clinical isolates of *Staphylococcus aureus* arises from V₅₈₈F or V₆₃₁F mutations in isoleucyl-tRNA synthetase (IRS). Whether these are the only IRS mutations that confer mupirocin resistance or simply those that survive in the clinic is unknown. Mupirocin-resistant mutants of *S. aureus* 8325-4 were therefore generated to examine their *ileS* genotypes and the in vitro and in vivo fitness costs associated with them before and after compensatory evolution. Most spontaneous first-step mupirocin-resistant mutants carried V₅₈₈F or V₆₃₁F mutations in IRS, but a new mutation (G₅₉₃V) was also identified. Second-step mutants carried combinations of previously identified IRS mutations (e.g., V₅₈₈F/V₆₃₁F and G₅₉₃V/V₆₃₁F), but additional combinations also occurred involving novel mutations (R₈₁₆C, H₆₇Q, and F₅₆₃L). First-step mupirocin-resistant mutants were not associated with substantial fitness costs, a finding that is consistent with the occurrence of V₅₈₈F or V₆₃₁F mutations in the IRS of clinical strains. Second-step mutants were unfit, but fitness could be restored by subculture in the absence of mupirocin. In most cases, this was the result of compensatory mutations that also suppressed mupirocin resistance (e.g., A₁₉₆V, E₁₉₀K, and E₁₉₅K), despite retention of the original mutations conferring resistance. Structural explanations for mupirocin resistance and loss of fitness were obtained by molecular modeling of mutated IRS enzymes, which provided data on mupirocin binding and interaction with the isoleucyl-AMP reactive intermediate.

The antibiotic mupirocin (see Fig. 6, structure 1) inhibits bacterial isoleucyl-tRNA synthetase (IRS) (24). It has potent activity against staphylococci and is used as a topical agent for the treatment of skin and postoperative wound infections and for prevention of nasal carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) (4).

The clinical use of mupirocin has resulted in the emergence of resistance in methicillin-susceptible *S. aureus* (MSSA), MRSA, and glycopeptide intermediate *S. aureus* (GISA) (5, 6, 9, 16, 29). Two mupirocin resistance phenotypes are exhibited (10). High-level resistance (MIC $\geq$ 512 μ g/ml) results from acquisition of the *mupA* determinant that encodes a mupirocin-resistant IRS, whereas low-level resistance (MIC = 8 to 256 μ g/ml) results from alteration of the native IRS as a consequence of spontaneous mutations in the *ileS* gene (2, 4, 8). Until recently, chromosomal mupirocin resistance arising through point mutations in *ileS* was considered clinically unimportant (4, 13). However, low-level mupirocin resistance appears to be more prevalent in clinical isolates than high-level resistance (6, 9, 25), and the emergence of low-level mupirocin resistance is thought to increase failure rates for nasal decolonization of MRSA (5, 12, 30).

IRS is a class I tRNA synthetase, a group characterized by an ATP-binding Rossman fold possessing consensus HXGX and GXKMSKS motifs (Fig. 1) (7). In clinical *S. aureus* isolates expressing low-level mupirocin resistance, amino acid substitutions have only been detected at two sites (V₅₈₈F and V₆₃₁F)

in IRS that are located in the ATP-binding domain of the Rossman fold, a region which also overlaps the mupirocin binding site (2, 8, 26, 32). However, *S. aureus* exhibiting incremental increases in resistance to mupirocin can be selected in the laboratory after repeated exposure to increasing concentrations of the antibiotic (28). Such mutants, which probably arise from multiple genetic changes in *ileS*, have not yet appeared in clinical isolates of *S. aureus* (2, 8). Mutations in drug targets that confer resistance to other antibiotic classes can impose fitness costs on the organism (1). The occurrence of the V₅₈₈F and V₆₃₁F mutations in clinical isolates of mupirocin-resistant staphylococci appears to reflect the low-cost of these mutations in terms of bacterial fitness (15). In contrast, other *ileS* mutations may impose fitness costs, precluding their appearance in the clinic.

We provide here an extensive analysis of the in vitro and in vivo fitness costs for mupirocin resistance genotypes that have been identified in the clinic, as well as other novel mutations in the IRS of *S. aureus*. We also demonstrate that reversion, pseudoreversion, and compensatory evolution are all strategies capable of alleviating fitness costs associated with development of mupirocin resistance. Furthermore, we provide structural explanations for mupirocin resistance and accompanying reductions in fitness by molecular modeling of mutant IRS enzymes.

MATERIALS AND METHODS

Bacteria and growth conditions. The mutants described in the present study are derivatives of *S. aureus* 8325-4 (22). Strains were cultured aerobically at 37°C by using Iso-Sensitest broth (ISB) or Iso-Sensitest agar (ISA) (Oxoid, Basingstoke, United Kingdom).

Determination of susceptibility to mupirocin. The lithium salt of mupirocin was a gift from Glaxo SmithKline Pharmaceuticals (Harlow, United Kingdom). MICs were determined by agar dilution on ISA with an inoculum in ISB of 10⁶

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(i)

Q²

S.a	53	HDGPPYANGNL	<u>FMGH</u>	ALNKILKDFIVRYKTMQGFYAPYVPGWDTHGLP	100
E.c	54	HDGPPYANGSI	<u>HIGH</u>	SVNKILKDIIVKSKGLSGYDSPYVPGWDCHGLP	101
M.t	45	LDGPPYCSGRI	<u>HLGT</u>	AWNKIMKDSYLRFKSMRGFNVRQPGWDTHGLP	92
T.t	43	YEGPPTANGLP	<u>HVGH</u>	AQARSYKDLFPRYKTMRGYYAPRRAGWDTHGLP	90

(ii)

L²

S.a	528	WFDSGS----	SHRGVL-----	ETRPELS-----	FPADMYLEGS	<u>DQYRGW</u>	FNSSITTSVATRGVSPYK	580
E.c	534	WFDSGS----	THSSVV-----	DVRPEFA-----	GHADMYLEGS	<u>DQHRGW</u>	FMSLMISTAMKGKAPYR	586
M.t	519	WIDSGV----	AGWAAL-----	HYPREKE--	LFSEWFPYDF	<u>ITEGHDQ</u>	TRGWFYSQLCGGVIALDETPYR	576
T.t	518	WYDSGA----	MPFASLHYPFE	HEEVFRES-----	FPADFIAEGID	<u>QTRGW</u>	FNSLHQLGVMLFGSIAFK	576

F^{1,2} V¹ **F^{1,2}**

S.a	581	FLLSHGFVMDGE	<u>GKKMSKS</u>	LGNVIVPDQVVKQKGADIAR---	LWVSSTDYL---	ADVRIS	634
E.c	588	QVLTHGFTVDGQ	<u>GRKMSKS</u>	IGNTVSPQDVMNKLADILR---	LWVASTDYT---	GEMAVS	641
M.t	577	RVLMHGFTLDEE	<u>GRKMSKS</u>	LGNVVEPEDVIEKYGADVLRFYLLWANKP---	W---	EDLKFFV	631
T.t	577	NVICHGLILDEK	<u>GQKMSKS</u>	LGNVVDPWDIIRKFGADALR---	WYIYVSAPP--	EADRRFG	631

(iii)

C²

S.a	792	QALLDK-WRTFMNLRDDVNRALETARNEKV	820
E.c	802	EAMNDAFWDELLKVRGEVNKVIEQARADKK	831
M.t	793	EDAVDGKLEAMMDIVREIIEACARARDTAR	822
T.t	791	PALADEALVAQMRAVLKVVDLARAARAKSG	820

FIG. 1. Amino acid sequence alignment of three regions in the *S. aureus* (S.a) IRS enzyme involved in mupirocin resistance, with the IRS enzymes of *Escherichia coli* K-12 (E.c; GenBank accession no. SYECIT), *M. thermoautotrophicum* Marburg (M.a; SYEXI), and *Thermus thermophilus* (T.t; P56690). The consensus sequences involved in ATP binding are boxed. Important amino acids involved in isoleucine binding are underlined. First- and second-step mutations (superscripts 1 and 2) in the *S. aureus* IRS are shown in boldface above the wild-type amino acid.

CFU/spot (31). The MIC was defined as the lowest concentration of mupirocin that inhibited visible growth after 18 h of incubation at 37°C.

Determination of the mutation frequencies to resistance and MPC. Mutation frequencies were determined by previously described methods (23). The mutant prevention concentration (MPC) is defined as the lowest concentration of an antibiotic that prevents bacterial colony formation from a culture containing 10¹⁰ CFU (33). Accordingly, late-logarithmic-phase cultures of the *S. aureus* 8325-4 growing in ISB were harvested by centrifugation and resuspended in fresh ISB at a final concentration of 10¹¹ CFU/ml. Aliquots (200 µl) were incorporated into ISA plates containing serial twofold mupirocin dilutions in the range 0.06 to 1.0 µg/ml. The MPC was determined after incubation at 37°C for 48 h.

Determination of bacterial fitness. Maximum doubling time and relative competitive fitness determinations were performed exactly as described previously (15).

PCR amplification and DNA sequencing of the *ileS* gene. PCR amplification and sequencing of *ileS* genes from genomic DNA was performed as described previously (15).

Survival of *S. aureus* in vivo. As a measure of in vivo fitness, the survival of *S. aureus* strains was determined in a murine wound abscess infection model (14). Mice were subcutaneously injected with 10⁸ bacteria suspended in phosphate-buffered saline. After 7 days, the mice were killed, and the abscesses were recovered and homogenized in ISB. Bacteria in the homogenized lesions were quantified by plating on ISA, and the survival of mupirocin-resistant mutants was compared statistically with strain 8325-4 by using one-way analysis of variance

and evaluation of minimum significance difference ($P < 0.05$) between means by the T method (27). The stability of mupirocin resistance genotypes during infection was confirmed by comparing the growth rates and susceptibility to mupirocin of in vivo isolates with their starting strains.

Selection of fitness-restored mutants. Fitness-restored mutants were selected from unfit parent strains after serial transfer in nonselective ISB over a period of 7 to 9 days. Cultures of mupirocin-resistant mutants were grown in ISB at 37°C for 24 h, followed by transfer of aliquots to fresh ISB to initiate cultures containing ca. 10⁶ CFU/ml. Serial transfer was continued until an increase in growth rate was detected, which is indicative of increased fitness in the population. Cultures were then plated onto nonselective ISA, and large, fast-growing colonies were selected for further study.

Molecular modeling. Molecular models of the mutated enzymes were constructed by using the Swiss-PDB viewer v3.7 software (11) in conjunction with the structure files 1FFY.pdb (26) and 1JZQ.pdb (21). The lowest-energy conformations of the resulting mutated enzymes were then subjected to full energy minimization by using the MM2 force field within MacroModel (18) on Silicon Graphics O2 or Indy workstations. In order to simplify the computations, all calculations were performed on a 25-Å spherical portion of the protein centered on the ligand. For the energy minimizations, a 12-Å spherical region within this substructure centered on the ligand was defined in which all atoms, including those from the ligand, were allowed to freely move and atoms outside this region were frozen.

TABLE 1. First-step, second-step, and compensatory mutations associated with mupirocin resistance and their associated effects on resistance, bacterial growth, and fitness

Strain	Parent	Mupirocin MIC ($\mu\text{g/ml}$)	Mean $\pm$ SD		Substitutions ^a	
			Doubling time (min)	Competitive fitness (<i>W</i>)	Nucleotide substitutions in <i>ileS</i>	Amino acid substitutions in IRS
8325-4		0.016	37.5 $\pm$ 0.5	1.00	NA	NA
First-step mupirocin-resistant mutants						
JGH-1	8325-4	1	40.9 $\pm$ 1.6	0.92 $\pm$ 0.02	G₁₇₇₈T	G₅₉₃V
JGH-2	8325-4	2	43.4 $\pm$ 0.8	0.94 $\pm$ 0.05	G₁₇₇₈T	G₅₉₃V
JGH-3	8325-4	2	38.9 $\pm$ 0.3	0.99 $\pm$ 0.08	G₁₈₉₁T	V₆₃₁F
JGH-4	8325-4	2	41.6 $\pm$ 0.7	1.00 $\pm$ 0.03	G₁₈₉₁T	V₆₃₁F
JGH-5	8325-4	8	40.7 $\pm$ 0.7	0.94 $\pm$ 0.04	G₁₇₆₂T	V₅₈₈F
JGH-6	8325-4	8	41.0 $\pm$ 1.0	0.95 $\pm$ 0.01	G₁₇₆₂T	V₅₈₈F
JGH-7	8325-4	8	42.0 $\pm$ 0.7	0.93 $\pm$ 0.01	G₁₇₆₂T	V₅₈₈F
JGH-8	8325-4	8	38.8 $\pm$ 1.8	0.96 $\pm$ 0.05	G₁₇₆₂T	V₅₈₈F
JGH-9	8325-4	16	39.1 $\pm$ 2.0	0.99 $\pm$ 0.03	G₁₇₆₂T	V₅₈₈F
JGH-10	8325-4	16	40.0 $\pm$ 0.5	0.94 $\pm$ 0.04	G₁₇₆₂T	V₅₈₈F
Second-step mupirocin-resistant mutants						
JGH-11	JGH-1	16	66.7 $\pm$ 4.6	0.63 $\pm$ 0.03	G₁₇₇₈T , G₁₈₉₁T	G₅₉₃V , V₆₃₁F
JGH-12	JGH-4	32	58.8 $\pm$ 0.7	0.57 $\pm$ 0.03	G₁₈₉₁T , G₁₇₆₂T	V₆₃₁F , V₅₈₈F
JGH-13	JGH-5	64	102.6 $\pm$ 5.2	0.32 $\pm$ 0.06	G₁₇₆₂T , C₂₄₄₆T	V₅₈₈F , R₈₁₆C
JGH-14	JGH-6	128	120.4 $\pm$ 4.0	0.24 $\pm$ 0.08	G₁₇₆₂T , T₁₆₈₇C	V₅₈₈F , F₅₆₃L
JGH-15	JGH-8	128	141.0 $\pm$ 29.0	0.33 $\pm$ 0.01	G₁₇₆₂T , T₂₀₁A	V₅₈₈F , H₆₇Q
Fitness-restored mutants						
JGH-16	JGH-11	2	40.4 $\pm$ 0.8	0.99 $\pm$ 0.02	G₁₇₇₈T , G₁₈₉₁T , C₅₈₇T	G₅₉₃V , V₆₃₁F , A₁₉₆V
JGH-17	JGH-11	2	40.2 $\pm$ 1.0	0.94 $\pm$ 0.07	G₁₇₇₈T , G₁₈₉₁T , C₅₈₇T	G₅₉₃V , V₆₃₁F , A₁₉₆V
JGH-18	JGH-11	2	38.3 $\pm$ 0.4	1.00 $\pm$ 0.00	G₁₇₇₈T , G₁₈₉₁T , G₅₆₈A	G₅₉₃V , V₆₃₁F , E₁₉₆K
JGH-19	JGH-11	2	40.1 $\pm$ 1.5	1.00 $\pm$ 0.01	G₁₇₇₈T , G₁₈₉₁T , G₅₆₈A	G₅₉₃V , V₆₃₁F , E₁₉₆K
JGH-20	JGH-12	32	39.2 $\pm$ 2.5	1.01 $\pm$ 0.05	G₁₈₉₁T , G₁₇₆₂T , not detected	V₆₃₁F , V₅₈₈F , not detected
JGH-21	JGH-13	0.5	40.0 $\pm$ 0.4	0.83 $\pm$ 0.08	G₁₇₆₂T , C₂₄₄₆T , G₅₈₃A	V₅₈₈F , R₈₁₆C , E₁₉₅K
JGH-22	JGH-13	4	38.6 $\pm$ 2.4	0.95 $\pm$ 0.01	G₁₇₆₂T , C₂₄₄₆T , G₅₈₃A	V₅₈₈F , R₈₁₆C , E₁₉₅K
JGH-23	JGH-13	8	39.3 $\pm$ 0.4	0.93 $\pm$ 0.08	G₁₇₆₂T , C₂₄₄₆T , C₅₈₇T	V₅₈₈F , R₈₁₆C , A₁₉₆V
JGH-24	JGH-14	8	37.8 $\pm$ 1.5	0.98 $\pm$ 0.01	G₁₇₆₂T , T₁₆₈₇C , not detected	V₅₈₈F , F₅₆₃L , not detected
JGH-25	JGH-15	8	39.3 $\pm$ 0.7	0.96 $\pm$ 0.05	G₁₇₆₂T , T₂₀₁A , A₂₀₁T	V₅₈₈F , H₆₇Q , Q₆₇H
JGH-26	JGH-15	8	49.6 $\pm$ 4.5	0.79 $\pm$ 0.07	G₁₇₆₂T , T₂₀₁A , C₁₁₈₈A	V₅₈₈F , H₆₇Q , H₃₉₆Q
JGH-27	JGH-15	16	52.0 $\pm$ 5.0	0.79 $\pm$ 0.07	G₁₇₆₂T , T₂₀₁A , C₁₁₈₈A	V₅₈₈F , H₆₇Q , H₃₉₆Q

^a The mutations in *ileS* and IRS occurring at each step-wise selection are shown in boldface. NA, not applicable.

RESULTS AND DISCUSSION

First-step mupirocin-resistant mutants. Selection of antibiotic-resistant mutants through chromosomal mutations occurs in a mutant selection window in which the drug concentration lies between the MIC and the MPC (33). A mutant selection index of 31 (i.e., MPC divided by MIC) was obtained for *S. aureus* 8325-4 on the basis of an MPC of 0.5 $\mu\text{g/ml}$ and MIC of 0.016 $\mu\text{g/ml}$. This indicates an intermediate vulnerability to the development of mupirocin resistance in *S. aureus*, which is less than that exhibited by rifampin but greater than that displayed by the majority of fluoroquinolones (33). We isolated and characterized mutants of strain 8325-4 that arose spontaneously after selection with 0.064 μg of mupirocin/ml (four times the MIC). The mutants displayed low-level resistance to mupirocin with MICs in the range 1 to 16 $\mu\text{g/ml}$ (Table 1). The mutants arose with an overall frequency of $(7.2 \pm 0.9) \times 10^{-8}$, which is consistent with single mutational events. Several mutants selected for sequencing of *ileS* revealed the **V₅₈₈F** and **V₆₃₁F** IRS amino acid substitutions also found in clinical isolates (Table 1). However, a third mutant class was identified involving a **G₅₉₃V** mutation (Table 1). This genotype has not previously been identified in mupirocin-resistant *S. aureus*,

but mutation at the equivalent locus in *Methanobacterium thermoautotrophicum* Marburg (**G₅₈₉D**) confers mupirocin resistance (17) (Fig. 1).

Second-step mutations in *ileS* conferring elevated mupirocin resistance. Mutants of *S. aureus* exhibiting elevated levels of resistance to mupirocin (MIC $\geq$ 16 $\mu\text{g/ml}$) have been isolated in vitro after stepwise exposure to increasing concentrations of the antibiotic (28). Although these resistant derivatives probably arise by accumulation of separate mutations within *ileS*, there are no DNA sequence data to support this hypothesis. Strains JGH-1, JGH-4, JGH-5, JGH-6, and JGH-8, which contain single mutations in *ileS* (Table 1), were therefore used as starting strains for the selection of higher-level resistant second-step mutants.

Second-step mutants were selected by plating onto ISA containing mupirocin at four times the respective MICs for the starting strain. This resulted in the recovery of mutants for which mupirocin MICs were in the range 16 to 128 $\mu\text{g/ml}$ (Table 1). Short regions of the *ileS* were resequenced and resistance mutations detected. However, where no mutations were identified (JGH-13, JGH-15), the entire *ileS* gene was sequenced to reveal second-step mutations. In many cases the second mutation

occurred at one of the sites ($V_{588}F$ or $V_{631}F$) already identified in low-level mutants, resulting in the generation of double $V_{588}F/V_{631}F$ or $G_{593}V/V_{631}F$ mutants (Table 1). Antonio et al. (2) have suggested that $V_{631}F$ and $V_{588}F$ mutations may be mutually exclusive since considerable distortion of the hydrophobic domain of IRS would occur. Nevertheless, we show here that these mutations can coexist in IRS. Similarly, $G_{593}V$ and $V_{631}F$ mutations can be simultaneously tolerated in IRS. Three second-step mutations occurred at loci not previously implicated in mupirocin resistance (Table 1) involving the following changes: $R_{816}C$ (strain JGH-13), $F_{563}L$ (strain JGH-14) and $H_{67}Q$ (strain JGH-15).

Second step mutants arose with an overall frequency in the range of 4×10^{-10} to 8×10^{-10} , which is less than the frequency for selection of first-step mutants. The reason that second-step mutations occur less frequently is unclear. However, this does not appear to be related to mutational target size since the numbers of sites at which new mutations arose during first- and second-step selections were similar (Fig. 1).

In vitro fitness of first-step mupirocin-resistant mutants. Resistance to antibiotics arising from mutations in target genes can result in fitness costs that affect bacterial growth and survival (1, 20). Fitness costs associated with development of mutational resistance to mupirocin could be a factor that influences the prevalence of certain staphylococcal resistance genotypes in the clinic. In preliminary studies, we recently reported that the $V_{588}F$ mutation in clinical isolates of GISA is associated with low fitness costs (15). The fitness of other genotypes has not been examined, but it is possible that fitness costs may account for the low frequency of detection of the $V_{631}F$ resistance allele and, similarly, the apparent absence of $G_{593}V$ in clinical specimens. We have now expanded our preliminary analysis by determining the in vitro fitness of a number of strains derived from 8325-4 that contain $V_{588}F$, $V_{631}F$, or $G_{593}V$ mutations in IRS. Fitness was assessed by determining the doubling times of mutants and also by comparing their performance with the parental strain 8325-4 in mixed culture competition assays (Table 1 and Fig. 2).

The doubling times of six mutants containing the $V_{588}F$ alteration, two mutants containing the $V_{631}F$ alteration and two mutants containing the $G_{593}V$ alteration were not considerably different from the parental starting strain 8325-4 (Table 1). In mixed culture competition assays five of six strains containing the $V_{588}F$ mutation displayed a small loss of fitness, but the relative competitive fitness (W) never fell to <0.9 . The $G_{593}V$ mutation was also associated with a small loss (6 to 8%) of fitness detectable by the mixed culture competition assay.

Strains carrying the $V_{631}F$ mutation (JGH-3 and JGH-4) were as fit as the parental strain (Table 1). However, although there were no significant differences in doubling times, there was an apparent reduction in W of ~ 0.2 for three other strains containing $V_{631}F$ mutations (data not shown). In other systems, it is well known that compensatory mutations can arise in unfit antibiotic-resistant mutants that restore fitness and yet retain resistance (1, 20). Therefore, the $V_{631}F$ mutation might be initially associated with a small fitness cost that is ameliorated during subculture through intragenic or extragenic suppression. Nevertheless, no evidence for intragenic compensatory adaptation was found in fit strains possessing the $V_{631}F$ mutation.

The relatively modest fitness costs associated with the $V_{588}F$

FIG. 2. Correlation between fitness of mupirocin-resistant mutants evaluated by doubling time and competitive fitness (W).

and $V_{631}F$ mutations are in agreement with our earlier studies on GISA and consistent with the occurrence of these resistance genotypes in clinical isolates (2, 8, 15). Since the $G_{593}V$ resistance mutation does not appear to incur significant fitness costs, at least in vitro, it might arise in clinical isolates. Currently, this mutation has not been identified in clinical strains (2, 8, 15). However, more extensive surveys may reveal this genotype in the future.

In vitro fitness of second-step mupirocin-resistant mutants. In addition to the very low mutation frequencies for the generation of double *ileS* mutants, another reason for the absence of such bacteria in clinical isolates might be poor fitness of strains with two mutations in IRS. Indeed, second-step mupirocin-resistant mutants displayed reduced fitness both in vitro and in vivo (Table 1 and Fig. 3).

The doubling time of mutants containing two *ileS* mutations was longer than that for first-step mutants or for the original mupirocin-sensitive parental strain 8325-4. For example, JGH-15 (carrying mutations $V_{588}F$ and $H_{67}Q$) grew approximately three times more slowly than first-step mupirocin-resistant mutants or 8325-4. The second-step mutants also displayed loss of fitness in the mixed-culture competitive fitness assay with W values from 0.24 to 0.63 (Table 1).

Correlation between fitness costs measured by maximum doubling times and mixed-culture competition. As previously mentioned, the exponential growth of resistant bacteria was affected by mupirocin resistance, and this was most pronounced in second-step mutants. Furthermore, a positive ($R^2 = 0.89$), correlation was observed between the doubling times and W of mupirocin-resistant staphylococci (Fig. 2). Consequently, at least in the case of mupirocin resistance in *S. aureus*, growth rate determinations provide a simple representative measure of fitness trends without the need for laborious mixed-culture competition assays.

In vivo fitness of first- and second-step mupirocin-resistant mutants. Significant costs of resistance observed in vitro usually indicate situations where resistance would impose a fitness burden on the organism in natural populations (1). Nevertheless, the survival of *S. aureus* 8325-4 and selected first- and second-step mupirocin-resistant mutants, which carry mupirocin resistance genotypes previously identified in clinical strains,

FIG. 3. Survival of mupirocin-resistant mutants in a murine wound abscess model.

was also examined in a murine wound abscess infection model (Fig. 3). The number of viable organisms in the infectious dose was established at the start of the experiment, and the number of survivors after 7 days of infection was determined by plating samples of homogenized skin lesions onto ISA. The surviving fraction of strain JGH-12 (double IRS mutant, V₆₃₁F/V₅₈₈F) was significantly lower than the wild-type 8325-4 (Fig. 3). In contrast, survival of the first-step mupirocin-resistant mutants JGH-4 (V₆₃₁F) and JGH-6 (V₅₈₈F) was not significantly different from 8325-4. The mupirocin MICs and doubling times of independent colonies recovered from the mice were similar to the values for the strains used to initiate the infections (data not shown). This implies genetic stability of mupirocin resistance within *ileS* during the murine infections.

Although the experiments reported here did not reveal a statistically significant difference in the *in vivo* fitness of strains 8325-4 and JGH-6, there is, nevertheless, an indication that the V₅₈₈F mutation in strain JGH-6 might be associated with increased fitness compared to the parental starting strain 8325-4 (Fig. 3). However, further experiments involving infection of five mice with mixed cultures of strains 8325-4 and JGH-6 provided no evidence that JGH-6 was fitter than 8325-4 (data not shown).

Overall, the murine data provide a correlation between *in vitro* and *in vivo* fitness and further indicate that the clinically occurring V₅₈₈F and V₆₃₁F mutations are not associated with significant fitness costs, whereas second-step mutants, such as JGH-12 (V₆₃₁F/V₅₈₈F), which have not been reported clinically, possess significant fitness burdens.

Stability of second-step mupirocin-resistant mutants. In the absence of continued antibiotic selection pressure, the fitness of unfit antibiotic-resistant mutants can be restored by several mechanisms, one of which involves reversion of resistant alleles by back mutation (1). Consequently, if mupirocin-resistant mutants with more than one mutation in *ileS* can arise in the clinic, the failure to detect them in clinical isolates could reflect reversion to produce fitter strains containing only one resistance allele. We therefore examined whether resistance was lost and fitness restored when unfit second-step mupirocin-resistant mutants (JGH-11 to JGH-15) were serially subcultured in the absence of mupirocin for up to 9 days by daily transfer into fresh medium.

At the end of each subculture, samples were taken for de-

termination of mupirocin MICs. Serial subculture of mupirocin-resistant strains JGH-11, JGH-13, JGH-14, and JGH-15 led to progressive reduction of organisms expressing higher levels of resistance to mupirocin within the cultures and replacement by derivatives for which mupirocin MICs were lower (Fig. 4). For instance, the MIC of mupirocin against JGH-15 was initially 128 μ g/ml. However, after serial subculture in the absence of mupirocin for 4 days, the culture contained daughter cells with only low-level resistance (mupirocin MIC = 8 μ g/ml), which remained stable for the remainder of the serial subculture. With the exception of JGH-12 (see below), similar results were obtained with the other higher level mupirocin-resistant mutants, which all gave rise to stable, lower-level mupirocin-resistant populations within 4 to 7 days of serial subculture (Fig. 4).

In contrast to the other higher level mupirocin-resistant strains, resistance was stable in strain JGH-12 during the course of 9 days serial subculture since starting and final MICs were 32 μ g/ml. However, organisms recovered at the end of this experiment were fitness compensated (see below).

Fitness of strains recovered from serial subculture and further genetic analysis. During serial subculture the emergence of lower-level mupirocin-resistant subpopulations in cultures of JGH-11, JGH-13, JGH-14, and JGH-15 coincided with pro-

FIG. 4. Changes occurring in mupirocin susceptibility (A) and doubling time (B) during passage of unfit second-step mupirocin-resistant mutants.

TABLE 2. Calculated mupirocin and Ile-AMP binding energies for IRS in relation to mupirocin susceptibility and fitness costs in *S. aureus*

Amino acid substitutions in IRS	Binding energies of mupirocin (ΔE_{mup} [kcal/mol])	MIC ($\mu\text{g/ml}$)	Binding energies of Ile-AMP ($\Delta E_{\text{Ile-AMP}}$ [kcal/mol])	Fitness cost ^a
None	47.31	0.016	48.50	NA
First-step mupirocin-resistant mutants				
G ₅₉₃ V	42.84	1–2	46.04	Low
V ₆₃₁ F	44.24	2	45.66	Low
V ₅₈₈ F	42.31	8–16	46.79	Low
Second-step mupirocin-resistant mutants				
G ₅₉₃ V, V ₆₃₁ F	34.11	16	35.62	High
V ₆₃₁ F, V ₅₈₈ F	34.73	32	36.29	High
V ₅₈₈ F, F ₅₆₃ L	28.75	128	30.09	High
V ₅₈₈ F, H ₆₇ Q	29.52	128	35.59	High

^a Refer to Table 1 for fitness data.

gressive increases in growth rates of the mixed populations (Fig. 4). This is consistent with reversion of one of the mutated alleles in the *ileS* gene of the parent strains to produce derivatives with restored fitness and reduced resistance or accumulation of further mutations that act to suppress both the resistance phenotype and fitness cost of the existing allele (pseudoreversion). Elevated growth rates were also recorded in cultures of JGH-12 after 3 days of subculture even though resistance of JGH-12 to mupirocin was unaltered through 9 days of serial subculture. This probably reflects the appearance of an intragenic or extragenic compensatory mutation that restores fitness but allows resistance to be retained (1).

The more rapidly growing colonies obtained from the mupirocin-resistant strains were therefore presumptive fitness-restored mutants. A number of these colonies were retained for further analysis. They were designated strains JGH-16 to JGH-27 (Table 1). Strains JGH-16 to JGH-27 exhibited doubling times (range, 38 to 52 min) and *W* values (range, 0.79 to 1) that indeed indicated partial or complete restoration of fitness compared to their original unfit parental starting strains (Table 1).

To determine whether compensatory or back mutations were responsible for fitness restoration, the entire *ileS* gene of the fitness-restored mutants was sequenced and mutations were identified. Back mutation of the second-step mutation (Q₆₇H) in strain JGH-15 to create JGH-25 restored fitness by reinstating the HMGH sequence (Fig. 1 and Table 1). However, back mutation of resistance alleles was not detected in other fitness-restored strains. Intragenic and extragenic compensatory mutations that suppressed the effects of the resistance genotypes were inferred from sequencing data. For example, in strains JGH-22 and JGH-23 (both derived from JGH-13) either E₁₉₅K (JGH-22) or A₁₉₆V (JGH-23) mutations were found, in addition to the previously identified resistance mutations V₅₈₈F and R₈₁₆C. One strain, JGH-21 (derived from strain JGH-13) seemed to have acquired an extragenic mutation in addition to the compensatory mutation E₁₉₅K. Acquisition of this uncharacterized extragenic mutation may explain why strain JGH-21 showed greater susceptibility to mupirocin (MIC = 0.5 $\mu\text{g/ml}$) compared to JGH-22 (MIC = 4 $\mu\text{g/ml}$). Compensatory mutations (E₁₉₀K or A₁₉₆V) were detected in strains JGH-16 to JGH-19 (each derived from JGH-11) in regions adjacent to compensatory mutations found in JGH-22 and JGH-23 (each derived from JGH-13). Intragenic mutations were not found in JGH-20 (derived from JGH-12) or JGH-24 (derived from

JGH-14), a finding consistent with a role for extragenic compensatory mutations in restoring the fitness of these strains.

Restoration of fitness by intragenic suppression usually involves compensation by second site mutations that restore fitness and retain resistance (1, 20). Therefore, the situation described here is somewhat unusual since intragenic (and indeed some extragenic) mutations produced derivatives that exhibited lower-level resistance to mupirocin, despite retention of both original mutations that conferred high-level resistance. Fitness costs may be alleviated in these pseudorevertants by introduction of compensatory mutations that alter IRS conformation, restoring catalytic activity and susceptibility to mupirocin, despite the presence of mutations previously conferring high-level mupirocin resistance whose expression is now partially suppressed.

Molecular modeling of the mupirocin binding site in the IRS of resistant mutants. In order to probe the molecular effects of mutations upon mupirocin binding, we constructed molecular models of both the first- and second-step IRS mutants and used these to both quantify the effect of the mutations upon the binding energy for mupirocin and to try to establish the precise interactions responsible for any changes in binding energy. The calculated binding energies of mupirocin within these complexes are summarized below (Table 2). In general, there appears to be a broad correlation between the calculated mupirocin-IRS binding energy and the measured mupirocin MIC.

As can be seen, all of the mutant enzymes contained lower mupirocin-protein binding energies than that calculated for the wild type (Table 2). In addition, the most dramatic decreases were found within the second-site mutants, in keeping with elevated levels of mupirocin resistance. Detailed examination of the molecular models appeared to reveal some of the structural factors affecting the mupirocin binding of these mutants.

G₅₉₃V. Glycine 593 is located in the loop that makes direct contact with the tRNA via a hydrogen bond between the backbone carbonyl and the 2'-OH of G-68 (Fig. 5). Substitution for valine locates the new hydrophobic side chain close to this hydroxyl and appears to result in a net repulsion. This induces a slight movement of the loop toward the side chain of the neighboring methionine 589, which in turn appears to move the backbone carbonyl unit of valine 588 nearer to the hydrophobic region of mupirocin bordering C-8' and C-9'. The re-

FIG. 5. Detail of mupirocin-binding region in *S. aureus* IRS. Residues that have been mutated are shown in green, and the mutation is indicated in italics. Nucleoside residue G68, in Ile tRNA, is also included.

sulting repulsion also appears to lengthen the H-bond between the valine 588 NH and the O = C1 carbonyl of mupirocin by 0.2 Å (Fig. 5). This apparent relaying of the steric interaction experienced within the newly mutated residue also appears to move some of the carbon atoms within the fatty acid side chain of mupirocin. Since this resides in a fairly narrow hydrophobic channel, this movement may also contribute to weaker binding of antibiotic to the enzyme.

V₅₈₈F. In the wild-type enzyme, valine 588 lines the hydrophobic pocket that interacts with the fatty acid side chain of mupirocin. Upon mutation to phenylalanine, the increased bulk of the benzyl side chain results in steric repulsion at C-1 and C-2 of mupirocin and lengthens the valine 588 NH/O = C1 H bond by 0.67 Å (Fig. 5).

V₆₃₁F. As in the case of G₅₉₃V, V₆₃₁F appears to affect mupirocin binding via a "steric relay" mechanism. Valine 631 is stacked above valine 588 and, upon mutation to phenylalanine, the increased bulk of the benzyl side chain repels that of valine 588. The resulting torsional movement pushes the carbonyl oxygen of valine 588 closer to mupirocin and again, distorts the fatty acid side chain of the antibiotic, and weakens the valine 588 derived H bond, which becomes lengthened by 0.22 Å (Fig. 5).

G₅₉₃V/V₆₃₁F. Increasing the size of the side chain at position 631 from isopropyl to benzyl in combination with the effect of the G₅₉₃F mutation, as described previously, increases further

the steric repulsion between the side chains at positions 631 and 588 and, again, leads to further stretching of the V₅₈₈-mupirocin H bond by 0.13 Å and, perhaps more importantly, further increases the distortion along the fatty acid side chain of the antibiotic (Fig. 5).

V₆₃₁F/V₅₈₈F. Since these two amino acids are essentially stacked one above the other, the steric repulsion between the two benzyl side chains in this mutant appears to be considerable such that the movement of F₅₈₈ away from F₆₃₁ appears to abolish the F₅₈₈-mupirocin H bond with considerable distortion of the mupirocin fatty acid moiety by up to 1.18 Å from its position in the wild-type enzyme.

V₅₈₈F/F₅₆₃L. In addition to the effect of the V₅₈₈F mutation via its direct interaction with mupirocin as noted above, the F₅₆₃L mutation appears to be a further example of a "relayed" steric clash with mupirocin. The calculated structure indicates that the leucyl side chain at position 563, which is located within a looped region, will undergo steric repulsion with L₅₈₂, which is directly opposite on the adjacent peptide chain. This appears to result in torsional strain within the loop and in particular moves the side chain of tryptophan 562 close to the C-14 methyl group of mupirocin.

H₆₇Q/V₅₈₈F. As for the previously described double mutant, in addition to the direct effect of the V₅₈₈F mutation on mupirocin binding, the H₆₇Q mutation also appears to directly af-

FIG. 6. Structures of mupirocin (structure 1), the reactive intermediate (structure 2), and the sulfonamide-based mimic (structure 3).

fect the interaction with mupirocin and locates the carbonyl group from the amide side chain of glutamine in close proximity to the hydrophobic portion of the sugar-like ring of mupirocin, centered around C-16 and C-8.

Comparison of computational predictions for mupirocin binding with in vitro IRS binding data. It has been reported (3) that an ethyl ester variant of mupirocin (therefore containing a much-shortened alkyl chain) shows a 10-fold reduction in activity against *S. aureus* IRS compared to mupirocin ($k_i = 0.19$ versus 0.017 nM, respectively). Using the equation $\Delta G = -RT \times \ln k_i$ (wherein ΔG is the change in free energy, R is the gas

constant, T is the temperature, and k_i is the dissociation constant of the enzyme-inhibitor complex), free-energy changes upon dissociation (ΔG) of 13.25 and 14.68 kcal/mol, respectively, are obtained. Clearly, the magnitudes of these measured free energies reflect the total change in the energetics of the system during binding (including changes in solvation, entropy, bond enthalpies, etc.). Therefore, they cannot be directly compared to the calculated gas-phase enthalpies of binding shown in Table 2, which simply reflect the relative changes in bond enthalpy between substrate and enzyme resulting from substrate binding. Nevertheless, the reduced affinity of the short-

FIG. 7. Overlay of the X-ray crystal structure of IRS from *T. thermophilus* (green) containing the reactive intermediate mimic (yellow) on the modeled reactive intermediate (Ile-AMP) bound within the IRS of *S. aureus*.

ened alkyl chain variant for IRS compared to that of mupirocin is consistent with our present findings concerning the effect of mutations on the region of the protein bordering the binding pocket of the alkyl chain of mupirocin.

Molecular modeling of isoleucyl-AMP binding. The variation in fitness costs associated with the various mutants could result from changes in the binding of the enzyme to the reactive isoleucyl-AMP intermediate generated during acylation of the tRNA. We have therefore probed the structural details within a series of models that examine the binding energies of the reactive isoleucyl-AMP intermediate (Fig. 6, structure 2) generated during acylation of the tRNA. The models of this reactive complex were constructed first by docking a model of structure 2 into the enzyme active site by using the automated molecular docking program AutoDock (19). The lowest-energy complex was then used to create the various mutant structures, which were then subjected to energy minimization as described above. To date, there is no available X-ray structural data for the *S. aureus* IRS containing the reactive intermediate, with which direct comparison of IRS/inhibitor models could be made.

However, the X-ray crystal structure of a sulfonamide-based inhibitor (Fig. 6, structure 3), designed to mimic a reactive intermediate (Fig. 6, structure 2) with an IRS, has been reported (21). Comparison of the models with this structure reveals remarkable similarity with essentially all of the observed inhibitor/enzyme contacts present within the active complex/enzyme models (Fig. 7). In particular, hydrogen bonds are predicted between the 2'-OH and backbone N of Q₅₅₄; the 3'-OH and side chain of Q₅₅₄; a phosphate oxygen and backbone NH of Y₅₈, and between the leucyl NH₃ and the carbonyl of P₅₆. Similarly, the purine ring takes part in H bonding involving N5 and the backbone NH of V₅₈₈, and between N7 and H₅₈₅ (Fig. 7). In addition, the reactive intermediate and mupirocin bind in very similar regions within the enzyme (Fig. 8), with the epoxide ring of mupirocin occupying the same space as the phosphate unit in the intermediate. The central sugar core of both systems is also seen to overlap. The close similarity in binding regions for mupirocin and the reactive intermediate may further explain why major conformational changes that result in higher-level resistance also incur significant fitness reductions. However, an important difference occurs in the region of the extended hydrophobic side chain of mupirocin, which is essentially unoccupied in the reactive intermediate-containing models.

Table 2 lists the calculated binding energies of the model of the reactive intermediate within the mutant enzymes. Interestingly, the binding energies within all of the first-step mutants are essentially the same as that calculated for the wild-type system. In particular, as outlined above, the G₅₉₃V mutation occurs in a region several angstroms away from the phosphorylated intermediate binding cavity. Similar observations apply for the V₆₃₁F and V₅₈₈F first-step mutations (Fig. 9). These mutations appear to have little effect upon the binding of the acylated intermediate due to the absence of any part of the ligand in these regions.

For the second-step mutants the calculated binding affinities show a marked decrease relative to the first-step mutants, with that from V₅₈₈F/F₅₆₃L exhibiting the lowest value (Table 2). Inspection of the models reveals the origins of the decreased binding energies. For the G₅₉₃V/V₆₃₁F mutant, the presence of the V₆₃₁F mutation in particular appears to induce a small

FIG. 8. Comparative views of bound structures of mupirocin (top), reactive intermediate (red, bottom), and superposition of structures (middle) in the IRS of *S. aureus*.

movement in the adjacent peptide strand at V₅₈₈ which in turn induces a further movement (by ~ 0.55 Å) in the neighboring peptide loop at position M₆₅. This widens the hydrophobic pocket holding the purine ring of the substrate, and results in movement of the substrate to better fit the wider pocket. This in turn alters the hydrogen bond distances between the substrate and enzyme in the region of the isoleucine fragment, most noticeably involving Y₅₈ NH and one of the phosphate oxygens, which is lengthened by ~ 0.1 Å and also that between the NH₃ of the substrate and the side chain of D₉₅, which increases in length by 0.2 Å. This widening of the pocket binding the purine ring is also seen in the V₆₃₁F/V₅₈₈F mutant and analogous side chain interactions appear to induce the same type of distortion in substrate binding and H-bond lengths.

The V₅₈₈F/F₅₆₃L mutation also appears to disrupt the H bonding and perhaps also the hydrophobic interactions at the isoleucyl end of the substrate. This seems to result from loss of the edge-to-face interaction between the original aromatic ring

FIG. 9. Detail of the acylated intermediate binding region in IRS of *S. aureus*. Residues that have been mutated are shown in green, and the mutation is indicated in italics.

present at F₅₆₃ and the phenolic ring of Y₅₅₉. This appears to widen the hydrophobic cavity carrying the isoleucyl chain of the substrate and also lengthens the H bonds within this region, particularly that between the NH₃ of the substrate and the side chain of D₉₅ (by 0.23 Å).

This same distortion of the isoleucyl binding pocket also appears to be responsible for the decreased affinity observed for the substrate in the V₅₈₈F/H₆₇Q mutant. In this case, however, it originates from loss of the original face-to-face interaction involving the imidazole rings of H₆₇ and H₆₄. In the mutant, the amide side chain at Q₆₇ is forced to lie nearer the substrate resulting in a steric repulsion, which again lengthens the H bond at the NH₃ and phosphate positions of the substrate by 0.1 and 0.2 Å, respectively.

The general trend within the binding energies broadly correlates with the fitness cost measurements and appears to show that the fitness within the various mutant IRS enzymes is a direct result of changes in the efficiency of binding of the key isoleucyl phosphate ester intermediate.

Conclusions. Mutations conferring antibiotic resistance with only low fitness costs may survive for very long periods in natural populations since there will be no counterselection favoring loss of resistance. The fitness studies reported here suggest that first-step V₅₈₈F and V₆₃₁F *ileS* mutations conferring low-level mupirocin resistance are low-cost mutations both in vitro and in vivo, a finding consistent with the occurrence of these mutations in clinical isolates (2, 8).

We observed in the laboratory that higher levels of mupirocin resistance could be generated by selection of second-step mutants from first-step hosts. Such second-step mutants exhibited fitness costs associated with the accumulation of a second mutation in *ileS*. Studies on fitness costs imposed by other antibiotic resistance mutations (1, 20) do not necessarily preclude the clinical appearance of mupirocin-resistant mutants with more than one *ileS* mutation because compensatory mutations to alleviate fitness might occur. Indeed, the studies presented here show that fitness burdens imposed by two mutations in *ileS* are more frequently relieved by independent

compensatory mutations rather than back mutations in the specific allele conferring resistance, at least in vitro. Nevertheless, there is currently no evidence that mupirocin-resistant mutants with more than one mutation in *ileS* arise in the clinic (2, 8, 15). Furthermore, although several mutations of uncertain significance have been located in the *ileS* of clinical isolates (2, 8), none of them corresponds to the intragenic compensatory mutations we report here in laboratory strains. We therefore conclude that clinical selection of mupirocin-resistant strains possessing two mutations in *ileS*, if it occurs, must be a rare event. There are a number of possible explanations for this situation. In the first instance the probability of a strain simultaneously acquiring two mutations in *ileS* appears to be very low since combination of the individual mutation frequencies for generation of first- and second-step mutants provides a theoretical simultaneous two-step mutation frequency of $\sim 10^{-18}$. Nevertheless, stepwise selection of two mutations conferring resistance could occur as a result of poor compliance or suboptimal application of mupirocin. Consequently, lack of fitness is the most likely explanation for the absence of resistant double mutants in clinical isolates.

In addition to the clinical implications of the work reported here, we provide a structural understanding of the mutations conferring mupirocin resistance and their effects upon bacterial fitness. For example, there was a correlation between binding energies for mupirocin-IRS interactions and degree of resistance to the antibiotic. Furthermore, the lack of fitness in some mupirocin-resistant mutants could be explained by decreased binding of the reactive intermediate Ile-AMP in the IRS enzyme. Unfortunately, it was not possible to extend the structural studies to the third-site fitness-compensated mutants since these were generally located at positions outside the 12-Å sphere centered on mupirocin within which the energy calculations could be performed.

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