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MECHANISMS OF RESISTANCE

Genetic Determinants of High-Level Oxacillin Resistance in Methicillin-Resistant *Staphylococcus aureus*

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ABSTRACT Methicillin-resistant *Staphylococcus aureus* (MRSA) strains carry either a *mecA*- or a *mecC*-mediated mechanism of resistance to beta-lactam antibiotics, and the phenotypic expression of resistance shows extensive strain-to-strain variation. In recent communications, we identified the genetic determinants associated with the stringent stress response that play a major role in the antibiotic resistant phenotype of the historically earliest "archaic" clone of MRSA and in the *mecC*-carrying MRSA strain LGA251. Here, we sought to test whether or not the same genetic determinants also contribute to the resistant phenotype of highly and homogeneously resistant (H*R) derivatives of a major contemporary MRSA clone, USA300. We found that the resistance phenotype was linked to six genes (*fruB*, *gmk*, *hpt*, *purB*, *prsA*, and *relA*), which were most frequently targeted among the analyzed 20 H*R strains (one mutation per clone in 19 of the 20 H*R strains). Besides the strong parallels with our previous findings (five of the six genes matched), all but one of the repeatedly targeted genes were found to be linked to guanine metabolism, pointing to the key role that this pathway plays in defining the level of antibiotic resistance independent of the clonal type of MRSA.

KEYWORDS oxacillin resistance determinants, MRSA, guanine metabolism

In 2011, four strains belonging to the historically first methicillin-resistant *Staphylococcus aureus* (MRSA) clone (ST250/ST247-SCC*mec*I)—recovered in the United Kingdom and Denmark in the 1960s—were selected for whole-genome sequencing and detailed analysis of the antibiotic resistant phenotype (1). Each strain showed "heterogeneous resistance:" the majority of cells had oxacillin MIC values barely above that of susceptible bacteria, but cells with high-level and homogeneous resistance to oxacillin (H*R) were also present. Whole-genome sequencing performed on both the heterogeneous parental strain and its H*R derivatives identified point mutations in several genes in the H*R strains. A more recent study showed that homogeneous and high-level resistance to oxacillin can also be observed in *S. aureus* strain LGA251, which carries not *mecA* but the *mecC* determinant, and that homogeneous and high-level resistance requires additional genetic determinants (2).

Interestingly, several of these determinants were identified in the H*R strains as part of the (p)ppGpp-dependent stringent stress-response pathway (Fig. 1) (1). Upon activation of the stringent stress response, the protein RelA is activated, leading to the synthesis of the alarmone (p)ppGpp. The accumulation of (p)ppGpp inhibits the activity of several enzymes involved in GTP biosynthesis (e.g., Hpt and Gmk), which causes a drastic reduction in the cellular GTP pool (3). Activation of the stringent stress response was shown to increase the transcription of the *mecA* determinant and the production

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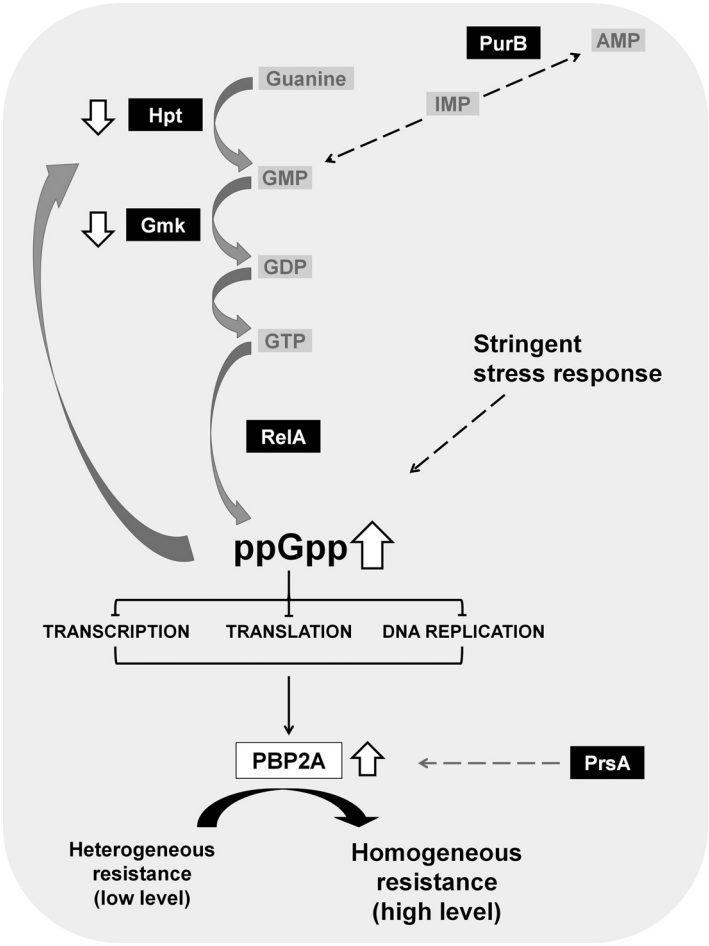


FIG 1 Molecular pathways mediating H*R phenotype with emphasis on guanine metabolism. The activation of the stringent stress response leads to the production of the resistance protein PBP2A, leading to the expression of high and homogeneous antibiotic resistance. This phenotype may be achieved through changes affecting distinct steps of the complex guanine metabolism. Proteins targeted by mutations in H*R strains are highlighted in black boxes.

of the resistance protein PBP2A, accompanied by the expression of high and homogeneous antibiotic resistance (Fig. 1) (4).

In the current work, we aimed to test if these additional genetic determinants are dependent on the clonal type of the MRSA strain. For this, we selected highly oxacillin-resistant isolates from the MRSA clone USA300, which is currently the predominant clonal type of MRSA in the United States (5–7).

A population analysis was used to characterize the resistance phenotypes, and whole-genome sequencing was used to identify mutations in the H*R derivatives of the parental MRSA strains.

RESULTS

Obtaining homogenous highly resistant subpopulations of MRSA strain USA300. Following similar procedures as in previous studies (1), we selected as parental strains four clinical isolates obtained in 2012 from patients with skin and soft tissue infections described in a recent study in the community in New York city (6).

A population analysis was performed on the four parental strains by growing the bacteria on tryptic soy agar (TSA) supplemented with increasing concentrations of oxacillin. Five colonies were selected from plates with 100 μg/ml of oxacillin for each of the four parental isolates. The colonies were stabilized by four passages on TSA in the absence of oxacillin, and a new set of population analyses was performed to confirm their homogeneous and high-level resistance.

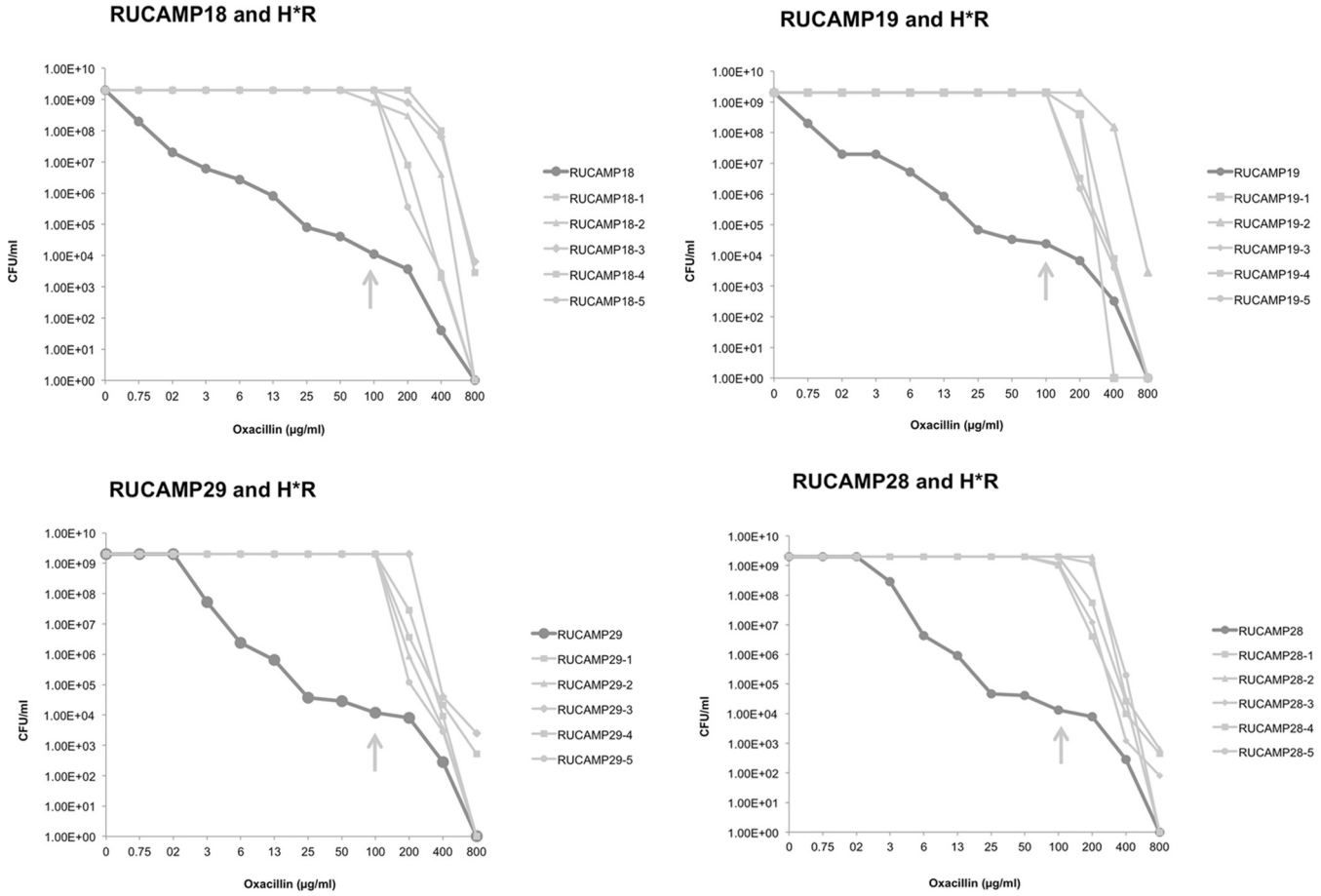


FIG 2 Population analysis profiles of the USA300 parental and H*R strains. RUCAMP18 and RUCAMP19 are the wound and the nare strains recovered from one patient infected and colonized by the same USA300 strain (t008/ST8/SCC*mec*IVa/PVL/ACME); RUCAMP29 and RUCAMP28 are the wound and nare strains of another patient, also infected and colonized by a variant of USA300 (t052/ST8/SCC*mec*IVa/PVL/ACME). Five homogenously resistant colonies were selected at 100 μg/ml of oxacillin from each parental PAP plate, as indicated by the arrows. The graphs show the different profiles between parental and H*R strains.

Population analysis profiles (PAPs) for the USA300 strains and their H*R derivatives demonstrate the heterogeneous phenotype of the parental strains and the homogeneous profile of the H*R strains (Fig. 2).

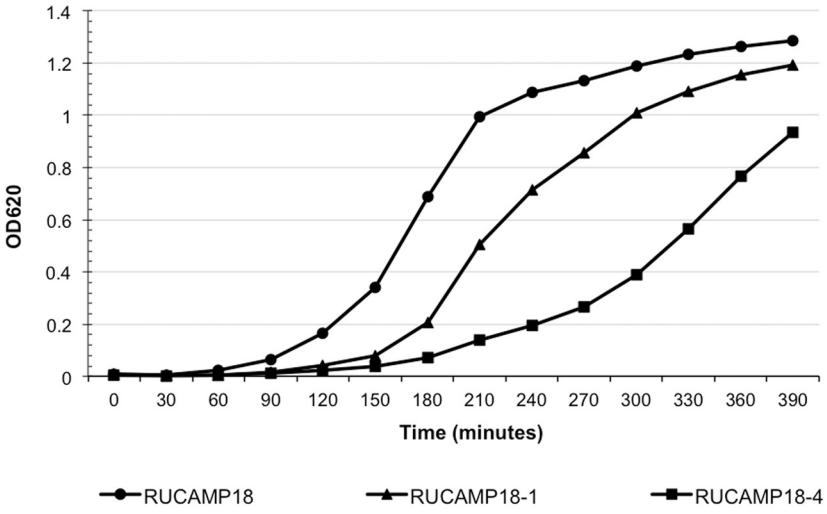
Whole-genome sequencing of parental strains and their subpopulations. The four parental strains, together with the 20 H*R strains, were selected for whole-genome sequencing. Each H*R strain showed a single mutation compared to the parental strain (with the exception of RUCAMP29-1 that carried two mutations). There was a great redundancy in the targeted genes, with only six genes (*fruB*, *gmk*, *hpt*, *purB*, *prsA*, and *relA*) being affected among the 20 H*R strains. All mutations were nonsynonymous or inactivating, with the exception of two mutations that targeted putative regulatory regions: the putative regulatory region of *gmk* (its coding DNA sequence [CDS] was also targeted by a nonsynonymous single nucleotide polymorphism [SNP] in another clone) and a gene encoding a putative DNA-binding transcriptional regulator that is contiguous with *purB* (also targeted by nonsynonymous or inactivating SNPs in other H*R strains). As these likely belong to the same polycistronic operon, the SNP may affect the expression of both genes. Table 1 shows the complete list of mutations observed in the H*R strains in genes that were intact in the parental strains.

Guanine pathway is impacted by most of the mutations affecting resistance level. Except for *fruB*, all genes mutated in the H*R strains (*gmk*, *hpt*, *purB*, *prsA*, and *relA*) participate in various stages of guanine metabolism (Fig. 1). Mutations in four of these genes (*gmk*, *hpt*, *prsA*, and *relA*) have already been identified in the historically

TABLE 1 Mutations found in the H*R strains

H*R clone	Effect	Nucleotide change (5′→3′)	Amino acid change	Affected gene(s)	Locus tag(s) in references[a]	Product
RUCAMP18-1	Stop gained	383T→A	Leu128[a]	*hpt*	SAUSA300_0488/*hpt*; SACOL0554/*hpt*	Hypoxanthine-guanine phosphoribosyltransferase
RUCAMP18-2	Missense variant	500C→A	Ala167Asp	*fruB/lacC_1*	SAUSA300_0684/*fruB*; SACOL0758/*fruK*	Fructose 1-phosphate kinase
RUCAMP18-3	Missense variant	317C→T	Thr106Ile	*hpt*	SAUSA300_0488/*hpt*; SACOL0554/*hpt*	Hypoxanthine-guanine phosphoribosyltransferase
RUCAMP18-4	Stop gained	847G→T	Glu283[a]	*fruB/lacC_1*	SAUSA300_0684/*fruB*; SACOL0758/*fruK*	Fructose 1-phosphate kinase
RUCAMP18-5	Frameshift variant	106delG	Gly39fs	*fruB/lacC_1*	SAUSA300_0684/*fruB*; SACOL0758/*fruK*	Fructose 1-phosphate kinase
RUCAMP19-1	Stop gained	730C→T	Gln244fs	*purB*	SAUSA300_1889/*purB*; SACOL1969/*purB*	Adenylosuccinate lyase
RUCAMP19-2	Missense variant	419C→A	Ala140Asp	*hpt*	SAUSA300_0488/*hpt*; SACOL0554/*hpt*	Hypoxanthine-guanine phosphoribosyltransferase
RUCAMP19-3	Missense variant	523C→T	Pro175Ser	*fruB/lacC_1*	SAUSA300_0684/*fruB*; SACOL0758/*fruK*	Fructose 1-phosphate kinase
RUCAMP19-4	Missense variant	416G→A	Arg139His	*purB*	SAUSA300_1889/*purB*; SACOL1969/*purB*	Adenylosuccinate lyase
RUCAMP19-5	Missense variant	523C→T	Pro175Ser	*fruB/lacC_1*	SAUSA300_0684/*fruB*; SACOL0758/*fruK*	Fructose 1-phosphate kinase
RUCAMP28-1	Stop gained	1860T→A	Tyr620[a]	*relA*	SAUSA300_1590; SACOL1689/*relA2*	GTP pyrophosphokinase
RUCAMP28-2	Missense variant	461C→A	Ala154Glu	*gmk*	SAUSA300_1102/*gmk*; SACOL1221/*gmk*	Guanylate kinase
RUCAMP28-3	Missense variant	911C→T	Ala304Val	*prsA*	SAUSA300_0478/*prs*; SACOL0544/*prsA*	Ribose-phosphate pyrophosphokinase
RUCAMP28-4	Missense variant	394C→T	Arg132Cys	*relA*	SAUSA300_1590; SACOL1689/*relA2*	GTP pyrophosphokinase
RUCAMP28-5	Missense variant	132T→A	Asp44Glu	*prsA*	SAUSA300_0478/*prs*; SACOL0544/*prsA*	Ribose-phosphate pyrophosphokinase
RUCAMP29-1	SNP in IGR, putative regulatory region of gmk/SAUSA_1102 or SAUSA300_1101[a]	C→A (65 bp upstream *gmk*)		*gmk*		Guanylate kinase
RUCAMP29-1	SNP in IGR, putative regulatory region of SAUSA300_1888 or the polycistronic transcript enrolling SAUSA300_1889/purB[a]	G→T (3 bp upstream ATG)		SAUSA300_1888 and/or *purB*		Conserved hypothetical protein/putative DNA-binding transcriptional regulator
RUCAMP29-2	Missense variant	265G→A	Ala89Thr	*prsA*	SAUSA300_0478/*prs*; SACOL0544/*prsA*	Ribose-phosphate pyrophosphokinase
RUCAMP29-3	Missense variant	307A→G	Thr103Ala	*hpt*	SAUSA300_0488/*hpt*; SACOL0554/*hpt*	Hypoxanthine-guanine phosphoribosyltransferase
RUCAMP29-4	Stop gained	2161A→T	Lys721[a]	*relA*	SAUSA300_1590; SACOL1689/*relA2*	GTP pyrophosphokinase
RUCAMP29-5	Missense variant	650C→T	Ser217Leu	*fruB/lacC_1*	SAUSA300_0684/*fruB*; SACOL0758/*fruK*	Fructose 1-phosphate kinase

[a]According to the genome annotation of the reference strains USA300_FPR3757 (accession number CP000255) and COL (accession number CP000046).



Strain	Mutation	Doubling time (minutes)	Growth rate κ (min^{-1})
RUCAMP18		31	0.022
RUCAMP18-1		33	0.021
RUCAMP18-4	*fruB*	47	0.015

FIG 3 Growth curves of mutant in *fruB*. Growth curves of RUCAMP18-4 (mutated in *fruB*), the parental strain RUCAMP18, and RUCAMP18-1, an H*R derivative of RUCAMP18 lacking the mutation in *fruB*. The experiment was performed twice.

earliest MRSA isolates described in the study by Dordel et al. and in *S. aureus* strain LGA251 (1, 2). A mutation in the adenylosuccinate lyase (*purB*) gene was seen for the first time in an H*R strain in this study.

Growth rate alteration associated with point mutations in *fruB*. The gene *fruB* (*lacC_1*) represents one of the most frequently mutated genes in the H*R strains (in six of twenty) identified in this study. Mutations in fructose phosphate kinase are expected to affect the growth rate of the strain (8). To test this, growth curves were generated comparing one H*R strain carrying a mutated form of the gene (strain RUCAMP18-4) with its parental strain (RUCAMP18) and another H*R strain obtained from the same parental strain, but without the particular point mutation (RUCAMP18-1). The growth rate of the mutated H*R strain (RUCAMP18-4) was much lower (0.015 min^{-1}) than the control isolates (0.021 and 0.022 min^{-1}). For RUCAMP18-4, it took 180 min to start the exponential phase compared to 90 min for the parental strain (Fig. 3).

DISCUSSION

In the H*R derivatives of strain USA300, the *mecA* gene is harbored in an SCC*mec*IV cassette (7). The mutated genes observed in these derivatives were virtually identical to the ones previously identified in H*R subpopulations of the "archaic clone" (ST250/ST247) which harbor a different SCC*mec*I cassette (1). Thus, the genetic background defining homogeneous resistance to beta-lactam antibiotics seems to be independent of the cassette harboring *mecA*.

In previous studies, we hypothesized that the stringent response pathway would be associated with the homogeneous high resistance to oxacillin exhibited by H*R derivatives of MRSA (1, 9, 10). In the present study, we further narrowed our suspicion to the pathway of guanine metabolism, as all but one of the repeatedly mutated genes in H*R strains (obtained from either USA300 or the "archaic clone" or even the *mecC*-harboring strain LGA251) were shown to affect one step or another of this particular metabolic pathway (Fig. 1).

Among the genes mutated in H*R derivatives, *fruB* has been associated with vancomycin resistance (11). However, none of our mutants with or without mutations in this gene showed decreased susceptibility to vancomycin (disc diffusion values in the range of 12 to 14 mm) (results not shown).

Under stress, as in the presence of high concentrations of antibiotics in the growth medium, (p)ppGpp is overexpressed, which in turn, will inhibit Gmk in *S. aureus*. Gmk is the enzyme responsible for the conversion of GMP to GDP during *de novo* synthesis of GTP (3). We hypothesize that mutations in RUCAMP28-2 or RUCAMP29-1 could lead to a constitutive inhibition of Gmk; thus, the cellular levels of GTP in these strains are constitutively low, which could help the bacteria to resist the high concentrations of antibiotic.

Mutations found in both *hpt* and *relA* genes were associated with the highly resistant phenotype of the bacteria linked to a constitutive production of (p)ppGpp (1, 12).

The chaperone PrsA was recently identified as a new auxiliary factor of oxacillin resistance in MRSA, affecting presumably the posttranscriptional maturation of penicillin-binding protein 2A (PBP2A), possibly at the stage of export and/or folding of newly synthesized PBP2A (13). More studies are needed to determine the role of mutations in *psrA* in high and homogeneous levels of oxacillin resistance in the mutants RUCAMP28-3, RUCAMP28-5, and RUCAMP29-2, as the mutations identified affect different domains of the protein.

Finally, in this study, *purB*, which is also involved in purine metabolism, has been identified as a novel gene potentially mediating the H*R phenotype. At the molecular level, this phenotype was triggered not only by inactivating nonsynonymous SNPs, but also by a SNP most likely affecting the *purB* mRNA.

In conclusion, of the 20 H*R strains analyzed in this study, a total of only six genes were found mutated. Five of these genes—*prsA*, *hpt*, *fruB*, *gmk*, and *relA*—had been identified in our previous studies associated with the homogeneous high-level resistance to oxacillin (1). The gene *gmk* was shown to be mutated in H*R derivatives of strain LGA251 (2). Four of the other genes (*prsA*, *hpt*, *gmk*, and *relA*), as well as *purB* (the additional gene mutated in USA300 H*R derivatives and identified in the present work), are involved in guanine metabolism, pointing to the key role of this metabolic pathway in defining the level of antibiotic resistance, regardless of clonal type of the MRSA isolate.

MATERIALS AND METHODS

Bacterial strains. The parental strains were RUCAMP18, RUCAMP19, RUCAMP28, and RUCAMP29. RUCAMP18 was obtained from the active wound of one patient, and RUCAMP19 was isolated from a surveillance nasal swabbing performed on the same patient. Similarly, RUCAMP29 was obtained from the wound of a second patient, for which RUCAMP28 comes from the anterior nares. The two pairs of nasal and wound specimens from two different patients were obtained in the course of a study in which only a few patients were found to be both infected and colonized by the same strain. The goal was to study H*R strains in different isolates of the same USA300 clone but from different clinical sources recovered from different patients and to include both harmless colonization as well as active infection samples.

Population analysis profiles. Parental strains were recovered from −80°C stocks by growing in tryptic soy broth ([TSB] Difco Laboratories, BBL, Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C overnight with aeration. Plates of TSA (Difco Laboratories, BBL, Becton Dickinson, Franklin Lakes, NJ, USA) were prepared with increasing concentrations (2-fold) of oxacillin. These plates were inoculated with new dilutions of overnight cultures on TSB. CFU were counted after 48-h incubations of the plates at 37°C (14).

Selection of H*R strains: homogeneously and highly resistant subpopulations. Five medium-size colonies capable of growing on TSA plates containing 100 μg/ml of oxacillin were picked from the PAP plates of each of the four "parental" MRSA isolates. These colonies were named "H*R" for homogeneous and high-level oxacillin resistance. The colonies were resuspended in 200 μl of TSB and used to inoculate plates of TSA and TSA with 100 μg/ml of oxacillin to verify their stability (through four passages in total). TSB cultures were also used for preparations of genomic DNA.

Whole-genome sequencing. Genomic DNA was extracted from both the parental strains and the H*R strains using the Qiagen DNeasy blood and tissue kit (Qiagen, Ambion Inc., Austin, TX, USA). Paired-ended (2 × 250 bp) sequencing was performed at the Instituto Gulbenkian de Ciência ([IGC] Oeiras, Portugal) using the Illumina MiSeq platform. The quality control of reads, *de novo* assembly, contigs quality assessment, and possible contamination search were carried out using the multisoftware pipeline INNUca version 2.6 (https://github.com/B-UMMI/INNUca). Selected assemblies for parental isolates were inspected and subsequently annotated using Prokka (https://github.com/tseemann/prokka) (version 1.12). The mean depth of coverage (after quality control) ranged from 68- to 101-fold. The quality processed reads of each H*R strain were mapped against the corrected assembly of the

respective parental strain using Snippy v3.1 (https://github.com/tseemann/snippy) with the following criteria: (i) a minimum mapping quality of ≥20; (ii) a minimum number of 10 quality processed reads covering the variant position; and (iii) a minimum proportion of 90% of quality processed reads at the variant position differing from the reference. All mutations were inspected and confirmed using Integrative Genomics Viewer (http://software.broadinstitute.org/software/igv/).

Growth properties of the *fruB* mutant. To analyze whether a mutation in *fruB* caused alterations in the growth rate, strain RUCAMP18-4, its parent RUCAMP18, and RUCAMP18-1 (another H*R derivative from the same parental strain but lacking the *fruB* mutation) were tested for their growth rates in TSB, starting with an optical density (OD) of 0.01. The culture densities were tested every 30 min for 8 h. The growth rates were calculated by the formula $k = \log(X_t) - \log(X_0)/0.301t$.

Accession number(s). The sequences for the *S. aureus* isolates are in the Sequence Read Archive (SRA) under BioProject no. PRJEB24730.

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The authors declare no conflict of interest.

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