Novel Determinants of Antibiotic Resistance: Identification of Mutated Loci in Highly Methicillin-Resistant Subpopulations of Methicillin-Resistant Staphylococcus aureus

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ABSTRACT We identified mutated genes in highly resistant subpopulations of methicillin-resistant Staphylococcus aureus (MRSA) that are most likely responsible for the historic failure of the β-lactam family of antibiotics as therapeutic agents against these important pathogens. Such subpopulations are produced during growth of most clinical MRSA strains, including the four historically early MRSA isolates studied here. Chromosomal DNA was prepared from the highly resistant cells along with DNA from the majority of cells (poorly resistant cells) followed by full genome sequencing. In the highly resistant cells, mutations were identified in 3 intergenic sequences and 27 genes representing a wide range of functional categories. A common feature of these mutations appears to be their capacity to induce high-level β-lactam resistance and increased amounts of the resistance protein PBP2A in the bacteria. The observations fit a recently described model in which the ultimate controlling factor of the phenotypic expression of β-lactam resistance in MRSA is a RelA-mediated stringent response.

IMPORTANCE It has been well established that the level of antibiotic resistance (i.e., minimum concentration of a β-lactam antibiotic needed to inhibit growth) of a methicillin-resistant Staphylococcus aureus (MRSA) strain depends on the transcription and translation of the resistance protein PBP2A. Here we describe mutated loci in an additional novel set of genetic determinants that appear to be essential for the unusually high resistance levels typical of subpopulations of staphylococci that are produced with unique low frequency in most MRSA clinical isolates. We propose that mutations in these determinants can trigger induction of the stringent stress response which was recently shown to cause increased transcription/translation of the resistance protein PBP2A in parallel with the increased level of resistance.

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It is generally agreed that the appearance of methicillin-resistant Staphylococcus aureus (MRSA) strains among clinical isolates represents the single most serious blow to the chemotherapy of S. aureus infections, since the unique resistance mechanism carried by all MRSA strains provides protection against the single largest family of antibacterial agents—the β-lactam antibiotics (1). Since their first appearance in clinical specimens in 1960, this resistance mechanism has made its way into a large variety of S. aureus lineages and diverse clones of MRSA have spread throughout the globe to cause serious and often life-threatening infections both in hospitals and in the community (2–5).

Most MRSA strains carry an identical—acquired—genetic determinant mecA (6, 7) which is part of a mobile genetic element (staphylococcal cassette chromosome mec element [SCCmec]) (8) inserted into the S. aureus chromosome at a unique chromosomal site. mecA encodes a protein, PBP2A, a peptidoglycan transpeptidase with extremely low affinity for the entire large family of β-lactam antibiotics (9), and the presence of this protein plays a critical role in allowing MRSA strains to continue synthesis of peptidoglycan and bacterial growth in the presence of high concentrations of β-lactam antibiotics. A model for the mechanism of action of PBP2A on the molecular level has been proposed (10).

In contrast to the common molecular mechanism of resistance, individual MRSA clinical isolates differ widely in their susceptibility to β-lactam antibiotics with individual MRSA strains presenting methicillin MIC values as low as a few µg/ml up to several hundred µg/ml depending on the particular MRSA clone, and this variation in resistance level cannot be explained by transcriptional regulation of mecA through the activity of regulatory elements such as the mecR1 and mecR1 or blal and blalR1 genes (11, 12).

A detailed examination of the β-lactam susceptibility of cultures of MRSA strains presents an even more complex and intriguing picture. MRSA grown from single-cell inocula produce cul-
tures that are highly heterogeneous with respect to their antibiotic susceptibility with most cells (more than 99%) showing only moderate- or low-level resistance often close to the MIC values of methicillin-susceptible isolates. On the other hand, the same cultures also contain bacteria with an extremely high level of resistance—in the MIC range of several hundred µg/ml—and the frequency of such highly resistant cells in a given culture (10⁻⁴ to 10⁻⁵) appears to be specific for the particular MRSA clone. This phenomenon has become known as the “heterogeneous” phenotype. It was first recognized and described in 1960, in the microbiological analysis of the historically first MRSA infection by Jevons who was surprised to recover two MRSA populations with widely different methicillin MIC values from a patient with an MRSA infection (13). The methicillin MIC of the majority of the bacteria was 2 µg/ml, but upon prolonged incubation of the specimen, more bacteria were recovered with a much higher antibiotic MIC value in the range of several hundred µg/ml.

Most contemporary clinical isolates of MRSA express β-lactam resistance in a similar heterogeneous fashion (14). Plotting the number of bacteria capable of forming colonies against the concentration of the antibiotic in the agar plates produce phenotypic profiles called population analysis profiles (PAPs), and the shape of the PAP is characteristic for the particular MRSA strain (15, 16). The PAP was subsequently shown to be a unique phenotypic marker of MRSA clones—highly reproducible in chronologically distinct isolates of the same MRSA lineage (17). The presence of highly methicillin-resistant cells in cultures of MRSA is of obvious relevance both for the detection of MRSA in clinical specimens and also for therapeutic options (18–20).

The stability of PAP for a given MRSA clone indicates that the heterogeneous composition of MRSA cultures is genetically controlled, i.e., the highly resistant subpopulations of bacteria must carry mutations in some genetic determinants that are “wild type” in the majority of less-resistant cells of the same clone.

Full genome sequences of a large number of MRSA strains are now available in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). However, most of the sequenced strains show heterogeneous antibiotic resistance, and therefore, the sequence information available relates only to the genetic makeup of the “cell majorities” in each of these particular MRSA clones and provide no information about unique mutated genes that must be present in highly resistant subpopulations carried by each one of the sequenced MRSA strains.

The purpose of this study was to identify mutated genes associated with the rare highly resistant bacteria that are produced with a low frequency (10⁻⁴ to 10⁻⁵) during growth of four historically early MRSA lineages belonging to the archaic clone of MRSA (17). The four strains selected for the study carry the mecA determinant on SCCmec type I cassettes that do not contain an active form of the mecl or mecR regulatory genes (12). The β-lactam antibiotic (methicillin or oxacillin) MIC values for the majority of bacteria in these four strains were in the range of 2 up to 12 µg/ml of oxacillin. When these four “parental” MRSA strains were plated on oxacillin-containing agar for PAPs, rare colonies (10⁻⁴ to 10⁻⁵) capable of growing on agar containing 100 µg/ml of oxacillin were also detected. Cultures of such colonies—named “H*R”—(for homogeneous and high-level resistance)—pro-

![FIG 1](https://mbio.asm.org/mbio.asm.org) Heterogeneous expression of β-lactam antibiotic resistance in four MRSA strains. (A to D) Population analysis profiles for strains UK13136 (A), UK63/458 (B), E2125 (C), and E4278 (D) are shown by the lines with solid squares. H*R isolates capable of growing in the presence of 100 µg/ml oxacillin were picked from the agar plates as indicated by an asterisk. Population analysis profiles of cultures of H*R isolates are shown by the lines with empty squares.
| Mutation no. | Locus in S. aureus COL | Description | Nucleotide change | Amino acid change | Functional category |
|------------|------------------------|-------------|------------------|------------------|--------------------|
| 1          | SACOL0314              | Transcriptional regulator (rpiR family) | C349448T         | Thr216Ile        | b                  |
| 2          | SACOL0403              | Transcriptional antiterminator (bgG family) | C409253 Del     | Frameshift after Ser461 | b |
| 3          | SACOL0434              | Hypothetical protein 1 | C440981T         | Gln41Stop        | f                  |
| 4          | SACOL0460              | IMP dehydrogenase (guaB) | C463203T         | Arg310Cys        | a                  |
| 5          | SACOL0461              | GMP synthase (guaA) | A463937 Del      | Deletion after Ser27 | a |
| 6          | SACOL0495              | Hypothetical protein 2 | G464191T        | Pro414Leu        | f                  |
| 7          | SACOL0533              | Methionyl-tRNA synthetase (metS) | G497663T         | Asp777Stop       | f                  |
| 8          | SACOL0544              | Ribose-phosphate pyrophosphokinase (prsA) | C52272T        | Pro291Leu        | a                  |
| 9          | SACOL0554              | Hypoxanthine phosphoribosyltransferase (hpt) | G562925A         | Met111Leu        | a                  |
| 10         | SACOL0555              | Cell division protein (ftsH) | C565044A         | Ala249Asp        | e                  |
| 11         | SACOL0562              | Lysyl-tRNA synthetase (lysS) | G570441T         | Arg111Leu        | c                  |
| 12         | SACOL0574              | Glutamyl-tRNA synthetase (gltX) | G570684A         | Arg93His         | c                  |
| 13         | SACOL0576              | Cysteinyl-tRNA synthetase (cysS) | G597283A         | Glu439Lys        | c                  |
| 14         | SACOL0583              | Ribosomal protein L11 (rplK) | C60393T         | Ile140Val        | c                  |
| 15         | SACOL0588              | DNA-directed RNA polymerase, β-subunit (rpoB) | C608417T        | Ala477Val        | b                  |
| 16         | SACOL0589              | DNA-directed RNA polymerase, γ-subunit (rpoC) | G611391A        | Arg239His        | b                  |
| 17         | SACOL0758              | 1-Phosphofructokinase (fruK) | G61672T         | Gly333Cys        | e                  |
| 18         | Intergenic             | Hypothetical protein 3/glucose-6-phosphate isomerase | G61927A         | Leu418Ile        | c                  |
| 19         | SACOL0991              | Oligopeptide ABC transporter, permease (oppB) | G612859A        | Ile479Phe        | c                  |
| 20         | SACOL1689              | GTP pyrophosphokinase (relA2) | G613291A        | Ala749Glu        | c                  |
| 21         | SACOL1710              | Valyl-tRNA synthetase (valS) | G613500T        | Thr942Ile        | b                  |
| 22         | Intergenic             | Valyl-tRNA synthetase/DNA-3-methyladenine glycosylase | G613517T        | Val948Leu        | b                  |
| 23         | SACOL1717              | Porphobilinogen deaminase (hemC) | G613594T        | Thr265Ile        | d                  |
| 24         | SACOL1745              | Ribosome binding site of pyruvate kinase (pyk) | G6178697        | Deletion of RBS  | d                  |
| 25         | SACOL2038              | tRNA N6-adenosine threonylcarbamoyltransferase (gpp) | G779646 Ins     | Frameshift after Ala35 | d |
| 26         | SACOL2072              | DEAD box ATP-dependent RNA helicase (srnB) | C968358T        | Asp265Asn        | d                  |
| 27         | SACOL2108              | Translation factor SUA5 (sua-5) | C1719144 Del    | Frameshift after Met383 | a |
| 28         | SACOL2117              | Fructose-bisphosphate aldolase (fbuA) | G1719336T Del   | Gln255Stop       | f                  |
| 29         | Intergenic             | Ribosomal protein S13/S18 (rpsM) | C1719336T Del   | Asp177Val        | c                  |
| 30         | SACOL2215              | Hypothetical protein 4/3-hydroxymethylglutaryl-CoA reductase | G1719336T Del   | Frameshift after Lys167 | d |

a CoA, coenzyme A.
b C349448T, C at position 349448 changed to T; C409253 Del, deletion of the C at position 409253; G779646 Ins, insertion of G at position 779646.
c Functional categories a through g as defined in Table 2.
produced highly and homogeneously resistant populations of bacteria with MIC values in the range of several hundred μg/ml.

Chromosomal DNA was prepared from the H*R cultures along with DNA prepared from the corresponding heteroresistant “parental” (majority) cells followed by full genome sequencing. Mutated genes unique to the particular H*R culture were identified by comparison to the status of the same gene in the “parental” culture.

Mutations in 27 genes and 3 intergenic sequences were identified in the highly resistant H*R derivatives recovered from the four heteroresistant “families” of MRSA. While the mutated genes represent a range of functional categories, we suggest that a common feature of these mutations may be their capacity to induce a stringent stress response in the bacteria. This proposal is consistent with recent evidence that identified a key role of the relA gene complex in defining the level of β-lactam resistance in laboratory models of MRSA strains (20, 21). As experimental evidence strongly suggested this, each of the four heteroresistant “parental” MRSA strains described in the present communication could be made to change their mode of expression of resistance from heterogeneous to homogeneous by the use of mupirocin, i.e., by experimentally inducing the stringent stress response in the bacteria (22–30).

**RESULTS**

The majority of clinical MRSA isolates express β-lactam resistance in a heterogeneous fashion. As a first attempt to better understand the genetic basis of this phenomenon, we selected four genetically related and historically early MRSA strains which had very similar heteroresistant phenotypes, as indicated by the virtually superimposable population analysis profiles (PAPs) (Fig. 1). The four strains included UK13136, the historically first MRSA, isolated in 1960 in the United Kingdom (13) named in our study as the “parental” strain of family A. This strain has already been characterized by molecular techniques (31). The second MRSA strain, ST63/458, was also isolated in the United Kingdom in 1963 and was the “parental” strain of family B in our study. Both of these strains are of sequence type ST250 and carry an SCCmec type I. The two additional parental strains, E2125 (parental strain of family C) and strain E4278 (parental strain of family D), were both isolated in Denmark in 1964 and 1967, respectively, and both were ST247 carrying SCCmec type I. All four strains belonged to the “archaic” clone of MRSA (17).

Figure 1 shows the rather similar PAPs of the four “parental” MRSA strains; in each strain, the majority of bacteria had relatively low oxacillin MIC values between 2 and 12 μg/ml. However, each culture also contained—with a low frequency of about 10−5—highly resistant (mutant) subpopulations of bacteria with an oxacillin MIC of ≥400 μg/ml.

Overnight cultures of the four parental strains were grown in tryptic soy broth (TSB), and 1-ml portions of the turbid overnight cultures were used to prepare the “parental” DNAs for sequencing. Aliquots of the rest of the overnight parental cultures were plated for population analysis, and 20 of the rare highly resistant colonies (named “H*R”) that appeared on the agar plates supplemented with 100 μg/ml oxacillin (see the asterisks in Fig. 1), were picked from the progeny of each of the four parental cultures. The H*R colonies were resuspended in TSB and restreaked on tryptic soy agar (TSA), and cultures of the 10 H*R colonies were grown in TSB, restested for oxacillin resistance by PAP, and used to prepare DNA for sequencing. Eventually, the DNA sequence of each H*R colony was compared to the sequence of the corresponding “parental” culture in order to identify the genes that were mutated in the H*R cultures.

On the basis of this comparison, mutations in 3 intergenic sequences and 27 genes were identified in the stably resistant H*R strains recovered from the four heteroresistant MRSA strains (Table 1).

The mutated genes, their putative functions, and the nature of the nucleotide and amino acid change are listed in Table 1. The following six genes carried multiple mutations: fbaA (3 mutations), guaA (2 mutations), hpt (2 mutations), oppB (2 mutations), rpoC (9 mutations), and relA2 (2 mutations).

These genes may be involved in (p)ppGpp-mediated stringent stress response: guaA, hpt, and relA2 are directly linked to the synthesis of (p)ppGpp which targets RNA polymerase, the product of rpoB and rpoC (32). The fbaA gene encoding fructose bisphosphate aldolase was reported to be downregulated in (p)ppGpp-mediated stringent stress response induced by serine hydroxamate (33). Of the 30 genetic loci carrying mutations, 27 are expected to alter function either by point mutations or by frameshifts. The remaining 3 determinants were in intergenic sequences with a change in a single nucleotide. Thus, mutations in 27 different genes would appear to be responsible for the increase in the resistance of H*R isolates to oxacillin either singly or through a concerted effect of all mutations.

### Table 2: Functional categories of mutations associated with highly resistant (H*R) isolates

| Functional category | No. of determinants | Mutation(s)* associated with H*R isolates in the following strain: |
|---------------------|---------------------|---------------------------------------------------------------|
| Guanine metabolism (category a) | 5 | guaA, guaB |
| Transcription (category b) | 5 | bglG, rpoB, RNA helicase |
| Translation/ribosomal structure (category c) | 9 | rpsM, lysS |
| Transport/metabolism (category d) | 5 | pyk, hemC |
| Cell division (category e) | 1 | ftsH |
| Unknown function (category f) | 5 | HP2, Intergenic |

| | UK13136 (family A) | ST63/458 (family B) | E2125 (family C) | E4278 (family D) |
|---------------------|---------------------|---------------------|---------------------|---------------------|
| Guanine metabolism | guaA, guaB | guaA, relA2, prsA | relA2, hpt | rpiR, rpoB, rpoC |
| Transcription | bglG, rpoB, RNA helicase | rpoC | gcp, cysS, valS | lysS, gltX, metS, sau-5 |
| Translation/ribosomal structure | rpsM, lysS | rplK | oppB, ftsK |
| Transport/metabolism | pyk, hemC | fbaA | fbaA, hemC |
| Cell division | ftsH | HP2, Intergenic | Intergenic |

*Genes potentially involved with induction of a stringent stress response are indicated in boldface print. The fbaA and oppB genes are downregulated by (p)ppGpp-mediated stringent stress response (33). HP stands for hypothetical protein.
Table 2 lists functional categories of the mutated genes identified in H*R isolates of the four MRSA families identified by capital letters A through D. Of the 27 mutated genes, 21 were in guanine metabolism (a), in transcription (b), in translation/ribosomal structure (c) and/or in transport (d). Interestingly, in most H*R isolates, mutation in a single gene was sufficient to produce the highly resistant phenotype (Table 3). Thirteen out of 17 genes listed in Table 3 are included in 3 functional categories: four (guaA, prsA, hpt, and relA2) in guanine metabolism; two (rpoB and rpoC) in transcription; and seven (metS, lysS, cysS, valS, gcp, sua-5, and rpsM) in translation/ribosome structure. Each of these mutations would be expected to trigger the stringent stress response and produce high and homogeneous resistance.

Twelve H*R isolates each carrying a single mutation (Table 3) were compared to their respective parental strains for the relative amounts of PBP2A. Cells were grown in the presence of 0.5 μg/ml oxacillin to induce the mecA gene, and membrane fractions were prepared for Western blotting. All H*R isolates showed at least 2-fold increase in PBP2A compared to their parental strains (Fig. 2) suggesting that each mutation resulted in the recruitment of increased amounts of PBP2A into the cell membranes.

### TABLE 3  H*R isolates carrying mutations in a single gene

| Mutation no. | Locus in S. aureus COL | Gene | H*R strain | Functional category |
|--------------|------------------------|------|------------|---------------------|
| 5            | SACOL0461              | guaA | A3, BB9    | a                    |
| 7            | SACOL0533              | metS | DD8        | c                    |
| 8            | SACOL0544              | prsA | B5         | a                    |
| 9            | SACOL0554              | hpt  | DD3        | a                    |
| 11           | SACOL0562              | lysS | AA9        | c                    |
| 13           | SACOL0576              | cysS | CC3        | c                    |
| 15           | SACOL0588              | rpoB | DD9        | b                    |
| 16           | SACOL0589              | rpoC | B4, B8, B9, BB2, BB3, DD6, DD7 | b |
| 17           | SACOL0758              | ftaK | DD5        | d                    |
| 20           | SACOL1689              | relA2| BB8, D3    | a                    |
| 21           | SACOL1710              | valS | C8         | c                    |
| 23           | SACOL1717              | hemC | AA2        | d                    |
| 24           | SACOL1745              | pyk  | A5         | d                    |
| 25           | SACOL2038              | gfp  | CC1        | c                    |
| 27           | SACOL2108              | sua-5| DD1        | c                    |
| 28           | SACOL2117              | fbaA | B10, BB5, BB6 | d |
| 29           | SACOL2215              | rpsM | A2         | c                    |

*Mutation numbers as in Table 1.

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**FIG 2** Determination of PBP2A in membranes of H*R derivatives carrying single mutations. A group of H*R isolates carrying single mutations in a variety of genes associated with high-level oxacillin resistance (Table 3) were analyzed by SDS-PAGE and by testing the relative amounts of PBP2A by Western blotting with a monoclonal antibody prepared against PBP2A. Three isolates (A2, A5, and A2A) from family A, four isolates (B5, BB6, BB8, and BB9) from family B, and five isolates from family D (D3, DD1, DD3, DD5, and DD6) were included in the analysis. Lanes A, B, and D show the SDS-PAGE profiles and the Western blot analysis of PBP2A in these parental isolates. Lanes A_m, B_m, and D_m contain parental samples in which the relative amounts of PBP2A were estimated in the presence of mupirocin. The single mutations carried by the H*R derivatives of family A were as follows: rpsM in lane A2, pyk in lane A5, and hemC in lane AA2. The single mutated genes analyzed in family B were prsA in lane B5, fbaA in lane BB6, relA2 in lane BB8, and guaA in lane BB9. The mutations analyzed in members of family D were relA2 in lane D3, suaA in lane DD1, hpt in lane DD3, ftaK in lane DD5, and rpoC in lane DD6. The M lanes contain molecular size markers (100, 70, 55, and 45 kDa).
DISCUSSION

Inspection of Table 1 through 3 and the figures indicates that a large number and different kinds of mutations can profoundly influence the phenotypic expression of oxacillin resistance in the four heteroresistant MRSA strains. Determinants include genes in cell division as well as genes associated with various aspects of intermediary metabolism. Such a diversity of genetic determinants is reminiscent of the large number of "auxiliary genes" (or fem genes) identified earlier as determinants essential for the optimal expression of high and homogeneous resistance in MRSA strains (34, 35). As a hypothesis to account for the polygenic nature of this phenomenon, it was proposed that the expression of antibiotic resistance involves a bacterial stress response (36).

This proposition, originally suggested to explain the multi-phenotypic expression of oxacillin resistance, seems to also fit the mechanism of heterogeneous resistance analyzed in this communication. In a recent study, we described identification of the critical role that the S. aureus relA gene plays in the phenotypic expression of oxacillin resistance (20). RelA protein plays a central role in the control of biosynthetic activities through its catalytic product—ppGpp and pppGpp—that can interact with and regulate the ribosomal protein synthesis machinery.

The large number and functional diversity of the determinants that influence heterogeneous expression of resistance as described in this communication would fit the model in which a stress response—specifically, the stringent stress—is the ultimate controlling factor of the phenotypic expression of oxacillin resistance in MRSA (20, 21).

Figure 3 shows a modification of the relA model to indicate how diverse genetic determinants identified in the present communication could impact on the level of antibiotic resistance through specific interactions with the relA-controlled RNA polymerase system. In a recent communication (21), we began to test whether the physiological level of antibiotic resistance (i.e., the oxacillin MIC value) is paralleled by the cellular amounts of the mecA gene product PBP2A. In the model systems described in reference 21, increase in the MIC value was accompanied by a parallel increase in the cellular amounts of PBP2A, and increased amounts of PBP2A were also detected in the H*R derivatives described in this communication (Fig. 2).

If the stringent stress response is the central controlling element of the level of oxacillin resistance in MRSA, then one would expect that artificial triggering of the stress response would convert heterogeneously resistant MRSA to highly and homogeneously resistant cultures. In an effort to test this, we repeated the population analysis of the four heteroresistant "parental" strains in the presence of sub-MICs of mupirocin, an agent capable of inducing stringent stress. Figure 4 demonstrates that each one of the four heteroresistant parental MRSA strains described in this communication would exhibit high and homogeneous resistance...
if the phenotype was assayed in the presence of sub-MICs of mupirocin added to the oxacillin-containing agar plates. Identical results were obtained when serine hydroxamate, an inhibitor of seryl-tRNA synthetase, was used instead of mupirocin. These observations may open up so-far untested avenues for the design of antibacterial agents that could influence resistance level of MRSA through a novel type of intervention.

MATERIALS AND METHODS

Aliquots (1 ml) of the four heteroresistant “parental” cultures of UK13136 (parental strain of family A), ST63/458 (parental strain of family B), E2125 (parental strain of family C), and E4278 (parental strain of family D) were inoculated into 5 ml of tryptic soy broth (TSB) and incubated at 37°C overnight with agitation. Portions (1 ml) of the overnight cultures were removed to prepare chromosomal DNAs representing the majority of cells (poorly resistant cells) of these cultures. The overnight cultures were diluted with TSB, and population analysis profiles (PAPs) were done on tryptic soy agar (TSA) plates containing increasing concentrations of oxacillin (Fig. 1). CFU were counted after 48-h incubation of the plates at 37°C. Twenty medium-size colonies capable of growing on TSA plates containing 100 μg/ml oxacillin were picked from the PAP plates of each of the four “parental” MRSA. These colonies were named “H*R” for homogeneous and high-level oxacillin resistance. H*R colonies were recovered from the plates with 1-ml loops and dispersed into Eppendorf tubes containing 200 μl of TSB. Portions (1 ml) from each Eppendorf tube were streaked onto a TSA plate which was incubated at 37°C for 48 h. The H*R isolates were next passed three times onto fresh TSA plates, after which the isolates were restested for resistance level by Etest and population analysis. A total of 42 H*R isolates with high-level and homogeneous oxacillin resistance (oxacillin MIC of ≥400 μg/ml) were inoculated into 5 ml of TSB, incubated at 37°C with agitation overnight, and used to prepare H*R DNAs. The 42 H*R isolates included 10 colonies of UK13136, 12 of UK63/458, 10 of E2125, and 10 of E4278.

The antibiotic resistance profiles of the four heteroresistant strains were also determined by including sub-MICs of mupirocin in the antibiotic-containing plates used for population analysis (37, 38). Mupirocin is a known inducer of the stringent stress response in bacteria.

Genome sequencing. Sequencing libraries were prepared according to previously published methods (39–41). Samples were run on an Illumina HiSeq 2000 sequencer operated according to the manufacturer’s instructions with 100 cycle paired-end runs. Data for the samples have been deposited in the European Nucleotide Archive (see below).

Detection of variations between H*R isolates and the “parental” strains. The sequence of chromosomal DNAs isolated from the H*R colonies was compared to the DNA sequence of the corresponding “parental” strain in order to identify in the H*R isolates mutated loci that may be associated with the high-level oxacillin-resistant phenotype of these clones. This was done using three different approaches to call only high-confidence variants.

The first two approaches are based on de novo assemblers, which are capable of detecting variants (single nucleotide polymorphisms [SNPs]), insertions, and deletions while building the contigs. Both methods completely ignore reference genomes while calling variants between “parental” and H*R isolates. However, to make comparison between the methods easier, the results were mapped back to reference strain COL (GenBank accession number CP000046).

SGA v0.9.19 (42) commands “preprocess” and “index” were run using default settings. Variants were called using “graph-diff” with k-mer (“−k")
Preparation of staphylococcal membrane proteins. Membrane fractions were prepared from isolates belonging to families A, B, and D following the method described previously (21, 38) with a few modifications (21, 38). The membranes were collected by centrifugation at 100,000 g for families A and B and 100,000 g for family D. The relative amounts of PBP2A were determined in each of the isolates using Western blotting. The SDS-PAGE profiles and relative amounts of PBP2A were also compared for each of the parental strains with and without mupirocin (0.03 µg/ml) added to the growth medium. All cultures were harvested at an optical density at 620 nm (OD620) of 0.5, washed, and resuspended in 3 ml of 20 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, 100 µg/ml DNase I, and 50 µg/ml RNase A. The cells were incubated at 37°C for 30 min and disrupted by sonication with pulse of 40% output for 5 min. The supernatants were transferred to fresh ultracentrifuge tubes after centrifugation at 7,000 × g for 20 min. Membrane fractions were collected by centrifugation at 100,000 × g for 1 h. The collected membranes were resuspended in 20 mM Tris-HCl (pH 7.6) and stored at −70°C. The concentration of total membrane proteins was determined by the bicinchoninic acid (BCA) assay.

Western blotting. Western blotting with a monoclonal antibody prepared against PBP2A was used to determine PBP2A in membrane preparations as described previously with a few modifications (21, 38). The membrane proteins (50 µg for families A and B and 100 µg for family D) were loaded on the polyacrylamide gel (8% or 10% resolving gel; 4% stacking gel) for SDS-PAGE. The rabbit anti-PBP2A antibody was used as the primary antibody with dilution of 1:15,000, and the secondary horse- radish peroxidase (HRP)-conjugated anti-rabbit antibody (0.5 mg/ml; PerkinElmer) was diluted to 1:10,000. ChromPure human IgG Fc fragment (Millipore) was added to the blocking solution at a final concentration of 3 µg/ml in order to prevent the antibodies from nonspecific binding. Pierce enhanced chemiluminescence (ECL) 2 (Thermo Fisher Scientific, Inc.) substrate was used for visualization of PBP2A bands with X-ray film exposure.

Nucleotide sequence accession numbers. Data for the genome sequencing samples have been deposited in the National Nucleotide Sequence Database under the sample numbers ERS157365, ERS157381, ERS157396, ERS157409, and ERS157425 to ERS157449.

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