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# Mechanisms of Heteroresistance in Methicillin-Resistant *Staphylococcus aureus*

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**Characteristic for methicillin-resistant (Mc$^r$) staphylococci is the heterogeneous expression of the intrinsic methicillin resistance. The majority of the cells express resistance to low concentrations of methicillin, and a minority of the cells express resistance to much higher concentrations. We show here (i) that the presence of the *mecA* encoding region on plasmid pBBB79 was sufficient to render a methicillin-susceptible (Mc$^s$) *Staphylococcus aureus* strain heteroresistant and (ii) that this Mc$^r$ strain segregated highly resistant subclones which retained the high-resistance phenotype under nonselective growth conditions. The Mc$^r$ strain with only *mecA* on plasmid pBBB79 thus behaved identically to a Mc$^r$ strain carrying the complete *mec* determinant integrated at its proper chromosomal site. (iii) Curing a such highly resistant subclone from plasmid pBBB79 yielded an Mc$^s$ strain that was as susceptible as the original Mc$^s$ parent strain. (iv) Comparisons were made between the original parent and the cured Mc$^s$ strain by backcrossing pBBB79 into them and looking at their progeny. Transductants derived from the formerly highly resistant cured strain became resistant to high concentrations of methicillin, whereas transductants derived from the original parent strain were resistant to lower concentrations of methicillin and showed the typical heterogeneous resistance. We deduced therefrom that the high-level resistance expressed by the minority of the population of Mc$^r$ *S. aureus* was due to a chromosomal mutation(s) (*chr**) involving neither *mecA* nor the additional 30 kb of *mec*-associated DNA. Moreover, we could show that this postulated mutation *chr** was not linked to the *femAB* operon, which is known to affect methicillin resistance levels.**

Clinical isolates of methicillin-resistant (Mc$^r$) *Staphylococcus aureus* contain the methicillin resistance determinant *mec*, which confers an intrinsic resistance to all β-lactam antibiotics. The *mec* determinant is located on additional DNA of unknown origin and nature and integrates into a specific site in the chromosome of *S. aureus* (14). The *mec* determinant codes for *mecA*, the structural gene for low-affinity penicillin-binding protein (PBP2′ or PBP2a). This PBP2′ adds to the existing PBPs of the cell and is the biochemical correlate of methicillin resistance (13, 18, 28). Some strains contain *mecR1-mecI*, which code for elements regulating *mecA* transcription (23, 26, 27). Only a small part of the remaining 30 kb of DNA associated with *mecA* (3) has been characterized. It carries at least one insertion sequence-like element IS*431* (2) or IS*257* (15), thought to act as an integration site for additional, not related resistance determinants, a direct-repeat (*dru*) element, and a *ugpQ*-like open reading frame (22). It is yet unknown whether this or any further parts of the additional DNA contribute in one or another way to physiological functions involved in β-lactam resistance.

Characteristic for the *mec*-encoded methicillin resistance is its heterogeneous expression. Mc$^r$ strains show a basal resistance to low concentrations of β-lactams and give rise to a few subclones or mutants, which are capable of growing at high concentrations of the antibiotic. The level of methicillin resistance is influenced by osmolarity, temperature, growth medium, other external factors, and growth phase (25; reviewed in reference 19). These phenotypic variations, however, have to be clearly separated from the resistance expression of the highly resistant subclones or mutants formed by Mc$^r$ strains. Most of these highly resistant subclones retain, when grown under nonselective growth conditions, their resistance to high concentrations of methicillin (13a, 24). The genes responsible for the mutation to high-level resistance have not yet been identified. Amplification of *mec*-associated sequences was observed after step selection of Mc$^r$ strains for growth on high concentrations of methicillin (20). However, loss of these duplications was not correlated with loss of high resistance. Amplification seems, therefore, not to be involved in high-level resistance. Alterations in the inducibility of PBP2′ were proposed to be responsible for higher resistance in the highly resistant subpopulation (8). Mc$^r$ strains with a functioning negative regulatory region *mecR1-mecI* upstream of *mecA* (27) express very low resistance when uninduced (23). Mutations or deletions in *mecI* relieve this repression (26), and resistance is increased. An analogous, though not as stringent, control of *mecA* occurs through *blaR1-blaI* (11, 23), the regulatory elements of staphylococcal β-lactamase. However, the amount of PBP2′ produced apparently does not correlate with resistance levels. Mc$^r$ strains, i.e., strains devoid of *mecR1-mecI* and *blaR1-blaI* and therefore capable of high constitutive synthesis of PBP2′, show heterogeneous resistance and segregate into highly resistant subclones. It is unknown which genetic and physiological alterations have occurred in these subclones.

Besides PBP2′, genes termed *fem* (4, 5) or *aux* (21) factors are needed for the expression of the resistance. They are part of the *S. aureus* genome and are involved in peptidoglycan metabolism (9, 16). Inactivation of the *femAB* operon decreases resistance to methicillin without affecting PBP2′ production (5, 12) and might, therefore, be involved in one way or another in high resistance.

In the present study we could show that the mutation(s) leading to high resistance is located on the chromosome but that it must differ from *femAB*.

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TABLE 1. Bacterial strains and plasmids[a]

| Strain or plasmid | Relevant genotype | Phenotype | Source and/or reference |
|---|---|---|---|
| Strains | | | |
| RN4220 | 8325-4 r- | Restriction mutant | R. Novick |
| BB413 | 8325, *mec* Ω8(*chr*::Tn*551*) | Heterogeneously Mc$^r$, Em$^r$ | This laboratory[b] |
| BB291 | 8325, *mec* Ω2000(*chr*::Tn*551*) | Heterogeneously Mc$^r$, Em$^r$ | This laboratory[b] |
| BB892 | 8325, *mec chr** Ω8(*chr*::Tn*551*) | Highly Mc$^r$, Em$^r$ | This study; highly resistant colony derived from BB413 |
| BB894 | 8325, *mec chr** Ω2000(*chr*::Tn*551*) | Highly Mc$^r$, Em$^r$ | This study; highly resistant colony derived from BB291 |
| BB255 | 8325; parent strain | Mc$^s$ | 4 |
| BB270 | 8325, *mec* | Heterogeneously Mc$^r$ | Transduction of *mec* from a clinical isolate into BB255[c] |
| BB749 | 8325, pBBB79 | Mc$^r$ Cm$^r$ | This study; transduction of pBBB79 into BB255 |
| BB749s | 8325 | Mc$^s$ | This study; spontaneous loss of plasmid pBBB79 from strain BB749 |
| BB865 | 8325, *chr**, pBBB79 | Cm$^r$, highly Mc$^r$ | This study; highly resistant colony derived from BB749 growing on 256 mg of methicillin per liter |
| BB866 | 8325, pBBB79 | Cm$^r$, heterogeneously Mc$^r$ | This study; transduction of pBBB79 from heteroresistant BB749 into BB255[d] |
| BB867 | 8325, pBBB79 | Cm$^r$, heterogeneously Mc$^r$ | This study; transduction of pBBB79 from highly Mc$^r$ BB865 into BB255[d] |
| BB868 | 8325, *chr** | Cm$^s$ Mc$^s$ | This study; BB865 cured from pBBB79 |
| BB871 | 8325, *chr**, pBBB79 | Cm$^r$, highly Mc$^r$ | This study; transduction of pBBB79 from heteroresistant BB749 into BB868[d] |
| BB872 | 8325, *chr**, pBBB79 | Cm$^r$, highly Mc$^r$ | This study; transduction of pBBB79 from highly resistant BB865 into BB868[d] |
| BB582 | 8325; genetically uncharacterized mutant | Homogeneously Mc$^r$ | In vitro step-selected Mc$^r$ mutant (7) |
| BB936 | 8325; genetically uncharacterized mutant; pBBB79 | Homogeneously Mc$^r$, Cm$^r$ | This study; transduction of pBBB79 into BB582 |
| Plasmids | | | |
| pGC2 | *E. coli-S. aureus* shuttle vector[e] | Amp$^r$ in *E. coli*, Cm$^r$ in *S. aureus* | Constructed by and obtained from P. Matthews |
| pBBB79 | pGC2 *mecA* insert | Amp$^r$ in *E. coli*, Cm$^r$ Mc$^r$ in *S. aureus* | This study; *Hin*dIII fragment carrying *mecA* subcloned from pBBB21 (28) into the *Hin*dIII site of pGC2 |

[a] Abbreviations: Amp, ampicillin; Cm, chloramphenicol; *chr**, an unknown chromosomal mutation leading to high-level methicillin resistance in the presence of *mecA*; Em, erythromycin; Mc, methicillin.
[b] B. Berger-Bächi.
[c] By phage 80α and selection for Mc$^r$ (4).
[d] By phage 80α and selection for Cm$^r$.
[e] pC194 cleaved at the *Hin*dIII site and inserted into the *Pvu*II site of pGEM-1 (Promega).

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** Plasmids and *S. aureus* strains are listed in Table 1. The strains were grown in Luria-Bertani (LB) broth (tryptone, yeast extract, NaCl [10, 5, and 5 g/liter, respectively]) at 37°C unless mentioned otherwise. Ampicillin, chloramphenicol, and methicillin (50, 20, and 5 mg/liter, respectively) were added when needed.

**DNA manipulations.** DNA preparation, isolation of plasmids and DNA fragments, restriction of DNA, ligation of DNA fragments, separation of DNA fragments by gel electrophoresis, transfer to membranes, random primed labelling of DNA with [α-$^{32}$P]dCTP, and Southern hybridizations were performed according to the protocols of Maniatis et al. (17) and Ausubel et al. (1).

**Construction of plasmid pBBB79, transformation, and transduction.** The 4.2-kb *Hin*dIII fragment from plasmid pBBB21 (28) carrying *mecA* was isolated and ligated into the *Hin*dIII site of shuttle vector pGC2. Transformation was first done in *Escherichia coli* DH5α. The plasmid was subsequently isolated, purified, and introduced into restriction-deficient *S. aureus* RN4220 by electroporation as described earlier (7). Transduction into appropriate *S. aureus* strains was by phage 80α (6).

**Curing of plasmid pBBB79.** Plasmid pBBB79 was lost spontaneously with a rate of approximately 5 to 30% from *S. aureus* strains when grown overnight on LB medium containing no chloramphenicol. By replica plating, single colonies cured from pBBB79 were identified as unable to grow in the presence of chloramphenicol and methicillin. The total loss of any plasmid and *mecA*-related sequences was checked by Southern hybridization using pBBB79 as a probe.

**Population analysis and selection of high-level resistant subclones.** Aliquots of an overnight culture were plated on LB plates containing increasing concentrations of methicillin. The CFU were determined after 48 h of growth at 35°C. High-level resistant mutant subclones were picked from the plate containing 256 mg of methicillin per liter and purified for single colonies on 256 mg of methicillin per liter. Stability of the high resistance was checked over 2 weeks by daily transfer of 0.1 ml of culture into 20 ml of fresh LB medium, containing chloramphenicol for maintenance of the plasmid.

**Screening for high- and low-level methicillin resistance by replica plating.** Highly resistant strains grow confluently on plates containing 100 mg of methicillin per liter. Heterogeneously Mc$^r$ strains, in contrast, produce no or only a few single colonies at this concentration, corresponding to the proportion of cells giving rise to the high-level resistant subpopulation. The strains to be analyzed for heterogeneous or high-level resistance were patched onto an LB master plate. By replica plating a fresh master plate not older than 2 h on plates containing either 5 or 100 mg of methicillin per liter we could

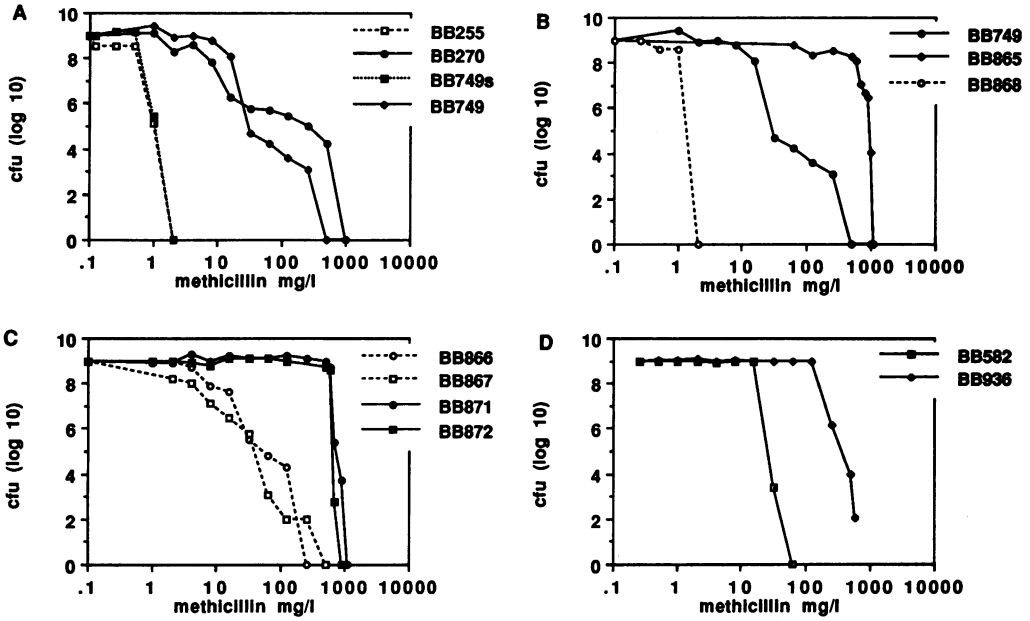


FIG. 1. Population analysis of different strains. The CFU on increasing concentrations of methicillin were determined as described in Materials and Methods after a 48-h incubation at 35°C. (A) Susceptible strain BB255, Mc$^r$ strain BB270, Mc$^r$ strain BB749 containing plasmid pBBB79, and cured Mc$^s$ strain BB749s. (B) Heterogeneous Mc$^r$ strain BB749; BB865, a highly resistant subclone derived from BB749; and BB868, BB865 cured from pBBB79. (C) BB866, parent strain BB255 transduced with pBBB79 of the BB749 origin; BB867, parent strain BB255 transduced with pBBB79 of the BB865 origin; BB871, cured strain BB868 transduced with pBBB79 of the BB749 origin; BB872, cured strain BB868 transduced with pBBB79 of the BB865 origin. (D) BB582, step-selected Mc$^r$ mutant; BB936, Mc$^r$ mutant containing pBBB79.

distinguish between heterogeneously and highly methicillin-resistant clones. Highly resistant strains formed a lawn at both concentrations, whereas heterogeneously Mc$^r$ strains formed a lawn on 5 mg/liter only and did not grow on 100 mg of methicillin per liter.

## RESULTS

A population analysis of the typical heterogeneously Mc$^r$ strain BB270 was compared with the population analysis of the isogenic susceptible parent, into which the *mecA* gene has been introduced by transduction of plasmid pBBB79. The methicillin resistance profile of Mc$^r$ transductant BB749 was essentially identical to the profile of the isogenic Mc$^r$ strain BB270 (Fig. 1A). Curing of strain BB749 from its plasmid yielded a susceptible strain, BB749s, indistinguishable from the parent Mc$^s$ strain BB255.

To see whether the highly resistant subclones which arose in BB749 at a frequency of $10^{-5}$ to $10^{-6}$ retained their high resistance as those derived from Mc$^r$ strain BB270, a highly resistant subclone, BB865, selected from strain BB749 at 256 mg of methicillin per liter was picked and purified. It retained its high resistance upon daily subcultures over 2 weeks on nonselective medium (Fig. 1B). Curing this highly resistant mutant from its plasmid yielded susceptible strain BB868, essentially indistinguishable by population analysis profile from susceptible strains BB255 and BB749s.

Since the cured strains apparently did not differ from each other in terms of behavior towards methicillin, we addressed the question of whether the information for high-level resistance was still present. If yes, it might be located either on the plasmid of the highly resistant mutant or on the genome of the cured strain. Phage 80α-mediated transductions were therefore performed with either susceptible parent strain BB255 or susceptible strain BB868, the cured descendant from high-level resistant subclone BB865, as a recipient. The donor of the *mecA*-containing plasmid pBBB79 was either heterogeneously Mc$^r$ strain BB749 or highly Mc$^r$ strain BB865. Transductants were selected for growth on chloramphenicol and replica plated on 5 or 100 mg methicillin per liter, to discern between heterogeneous and high methicillin resistance. Independently of the origin of the plasmid, it was the genetic background of the recipient's genome which determined the resistance levels of the transductants (Fig. 1C). When strain BB255 was used as a receptor, heterogeneously methicillin-resistant transductants, namely, strains BB866 and BB867, were obtained, independently from the origin of pBBB79. When strain BB868 was the receptor, all transductants, represented here by BB871 and BB872, respectively, were highly resistant.

Methicillin resistance can be acquired in vitro by stepwise selection of a susceptible strain for growth on increasing concentrations of methicillin. This yields homogeneously Mc$^r$ mutants, as shown earlier (7) and in Fig. 1D for the fourth-step Mc$^r$ mutant BB582. We wondered whether introduction of *mecA* on pBBB79 into mutant BB582 would increase the resistance and would lead to heterogeneity. Resistance was increased in the resulting transductant BB936 (Fig. 1D), but there was no pronounced heterogeneity apparent. We verified that the transductant BB936 indeed had obtained pBBB79 and a functioning *mecA* gene by backcrossing pBBB79 from strain BB936 into susceptible strain BB255, selecting for Cm$^r$, and screening for acquisition of Mc$^r$. All transductants became Mc$^r$, showing that the increase in resistance in BB936 was due to PBP2′.

Since *femAB* affects the level of methicillin resistance (5, 12), we wondered whether any mutations in the vicinity of *femAB* were responsible for high-level resistance. For this purpose we chose two Mc$^r$ strains, BB413 and BB291, which carried a silent Tn*551* insertion at a distance of less than 5 kb integrated

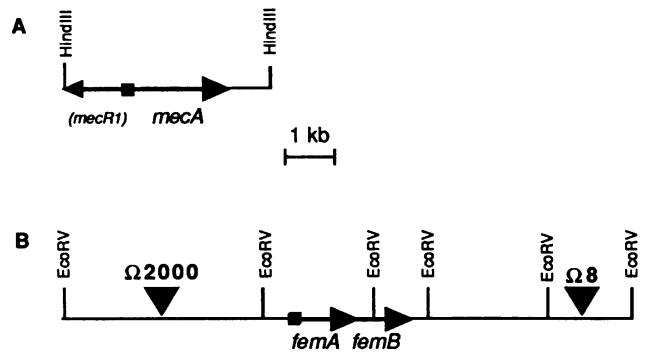


FIG. 2. (A) Insert of plasmid pBBB79. The *Hin*dIII fragment carries *mecA* which codes for PBP2′ and a truncated *mecR1* gene. Open reading frames are indicated by arrows. (B) *Eco*RV restriction map of the chromosome surrounding the *femAB* operon. The *Eco*RV fragments carrying silent insertions of Tn*551*, namely, Ω8 and Ω2000, are indicated (▼). They are cotransducible with *femAB*.

left or right of the *femAB* region (Fig. 2), known to be cotransducible with *femAB* (6). High-level resistant mutants BB892 and BB894, respectively, were isolated therefrom. BB892 and BB894 acted as donors, and heterogeneous Mc$^r$ strain BB270 acted as a recipient in phage 80α-mediated transductions of Tn*551*. All erythromycin-resistant transductants obtained were replica plated on 100 or 5 mg of methicillin per liter to see whether high-level resistance was linked to any of these Tn*551* inserts close to *femAB*. However, from over 100 transductants analyzed all were still heterogeneously Mc$^r$; none had acquired high-level resistance.

## DISCUSSION

The heterogeneous expression of methicillin resistance is typical for Mc$^r$ *S. aureus*. The level of resistance is strain specific and varies from borderline resistance to high basal resistance depending upon the genetic background of the strains (29). Independently from these variations, there appears within each culture a highly resistant subpopulation with high-level resistance. High-level resistant subclones are generally stable and retain their high-level resistance trait upon repeated passages in drug-free broth. We showed that an Mc$^r$ strain with *mecA* on plasmid pBBB79 produced a highly resistant minority as do clinical Mc$^r$ isolates containing the *mec* determinant in the chromosome. Since these high-level resistant mutants arose in the presence of *mecA* alone, in the absence of the additional 30 kb of *mec*-associated DNA, we could rule out that the additional DNA plays any role in high-level resistance. Resistance to high concentrations of methicillin was shown here to be the consequence of alterations encoded by the chromosome. It was the genetic background of the recipient strain which determined the outcome of the resistance level in the transductants which had acquired pBBB79. The previous passages of the pBBB79 plasmid, whether in highly or heterogeneously Mc$^r$ strains, did not affect plasmid-encoded genes. We termed the unknown chromosomal mutation(s) leading to high resistance in the presence of PBP2′ *chr**. The mutation rate of $10^{-6}$ to high resistance in our strain points presumably to one single mutational event.

In susceptible strains selected for growth on increasing concentrations of methicillin, high resistance is acquired in small increments, resulting in changes in affinity to penicillin and the amount of the existing PBPs and in further, not yet characterized alterations (7). Since pBBB79 in strain BB936 increased the resistance of the in vitro Mc$^r$ mutant but apparently did not produce heterogeneity, it might be postulated that the *chr** mutation had already occurred during step selection.

The *chr** mutation is not cotransducible with the two silent Tn*551* insertions surrounding *femAB*. Its chromosomal location is still not known.

The highly resistant subclones are not genetically stable in all strains (24), as shown here. In some strains subclones picked at high concentration revert more readily. Recently (10), resistant subclones selected at 25 mg/liter which reverted readily to their original resistance pattern were described. They were uncharacterized strains, and their behavior is reminiscent of an induction phenomenon. Induction of resistance by β-lactams, especially in strains with the negative regulatory element *mecR1-mecI* can, once induced, be mistaken for the genetically determined high-level resistance discussed above. Expression of methicillin resistance is very complex. The nature of *chr** is intriguing; the phenotype of *chr** is not apparent in the susceptible strain BB868 and appears only after introduction of PBP2′. The mapping and isolation of the putative chromosomal high-resistance gene *chr** will be a step forward in methicillin resistance genetics and will help to elucidate the molecular mechanism of high methicillin resistance.

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