Methicillin-resistant Staphylococcus aureus (MRSA) has become one of the most important nosocomial pathogens worldwide and poses serious infection control problems. As a rule, MRSA are resistant to many antibiotics and increasingly vancomycin-type glycopeptides remain the only rational treatment available. Transfer of vancomycin resistance genes from enterococci to S. aureus has been observed under laboratory conditions and very recently, S. aureus strains with drastically diminished vancomycin susceptibility (MIC $8 \text{ mg/L}$) have been isolated by Hiramatsu and colleagues from patients in Japan followed by first reports about similar isolates from the USA and Slovakia. Spread of such strains or isolates with an enhanced resistance phenotype has been anticipated by many experts in this field, possibly leading to a public health disaster if new therapeutic alternatives do not become available.

New antistaphylococcal approaches might become available as a result of research on new targets for antimicrobials for which no inhibitors are presently in use, preventing cross-resistance to already existing compound classes. A subgroup of these targets currently under research in academia and industry includes proposals to target resistance mechanism(s), thereby allowing the continued use of old, proven compound classes. Within this framework, this article attempts to put into perspective the currently available information about fem factors (factor essential for methicillin resistance) as antistaphylococcal targets.

The concept of fem factors was first developed in 1983 by Brigitte Berger-Bächi, who defined fem genes via a reduction of methicillin resistance obtained by transposon-mediated inactivation of a gene even though the expression of mecA, the primary resistance gene encoding the extra penicillin-binding protein 2a (PBP2a) found in all MRSA, was not influenced. Exploitation of this experimental approach, mainly by the groups of Berger-Bächi as well as that of de Lencastre and Tomasz led to the detection of a large number of fem factors, which have also sometimes been called aux (auxiliary) genes. Today at least six fem genes have been described and it has been estimated that there are at least ten equivalent but distinctly named (e.g. aux) genes.

One of the advantages of this operational definition of the fem factor concept was the relative ease and speed by which new candidate genes and their DNA sequences became available. However, because transposon integration takes place randomly, leading sometimes to only partially functioning proteins or reduced expression levels, it became important to understand the physiological role of the various fem factors in more detail to judge their role in resistance and/or other physiological functions for the staphylococci. Work in this direction led to the important conclusion that, contrary to the postulated recruitment of ‘auxiliary’ genes by MRSA, these genes are ‘housekeeping’ genes and are found in all S. aureus strains, irrespective of their resistance phenotype. In addition, almost all fem factors characterized so far have been shown to be involved in specific steps of cell wall biosynthesis, some of them being common to other bacteria as well; for example, FemF has been shown to catalyse the addition of the characteristic di-amino acid common to all peptidoglycan stem peptides. Since this reaction is an essential step in cell wall biosynthesis, detection of this gene as a fem factor must have been dependent on only partial knockout by transposon mutagenesis. From the foregoing it should be clear that the numerous fem factors include several previously well known genes partial or complete knockout of whose function led to the loss of the MRSA-resistance pheno-
The femAB operon is an S. aureus housekeeping gene and is found in all S. aureus strains, both sensitive and resistant ones. The operon probably arose by gene duplication and codes for two similar cytoplasmic proteins of $\sim 50$ kDa mass, which are produced preferentially during exponential growth. One of the first transposon mutants available for characterizing the function of femA and femB lacked any functional FemB and another, in which the transposon had integrated into the operon control region, had drastically diminished production of both FemA and FemB. Both mutants showed reduced sensitivity to lysozyme, a cell wall lytic enzyme acting within the so-called intermediate bridge of staphylococcal peptidoglycan, which consists of five glycine residues. This long and flexible intermediate bridge allows the extremely high crosslinking between the individual peptide moieties typical for S. aureus cell walls. The pentaglycine intermediate bridge is synthesized in an unusual manner, using glycine residues activated by tRNA and glycine-adding enzyme(s) that can add one glycine at a time at the level of lipid-linked peptidoglycan precursors. A analysis of the cell wall, which is the final product of peptidoglycan biosynthesis, revealed that the femB mutant had a shortened intermediate bridge, with three instead of five glycine residues. The femAB leaky mutant had a mixture of glycine chain lengths, of one, three or five residues. By analogy to FemB function, we speculated that FemA might be responsible for the addition of the second and third glycine residues; this was experimentally proven very recently, when mutants devoid of any functional FemA became available from chemical mutagenesis and allele replacement experiments. The same experiments supported our previous hypothesis that an additional fem factor, tentatively called femX, might exist and be responsible for the addition of the first glycine residue. Unfortunately, mutants defective in the putative FemX protein are not yet available, although a partial, leaky mutant will probably be available. In summary, the genetic and biochemical experiments described so far show that pentaglycine side-chain formation in S. aureus proceeds in distinct steps (cell wall peptides with no, one, three and five glycine units). Although addition of each glycine residue requires an identical chemical reaction, three genes are necessary to proceed from one step to the next, namely femX, femA and femB; despite the significant similarity of their gene products, they cannot complement each other.

In the following, we will now discuss the possible role of these three fem factors as potential anti-staphylococcal targets. First of all, we have to differentiate between stand-alone (i.e. essential) targets and targets suitable for the resistance-breaker concept discussed above. For FemA and FemB, it is now clear from the experiments described that mutants completely devoid of FemA and/or FemB are viable. Their growth is considerably slower and they tend to grow in extremely large clusters as a result of their strongly retarded cell separation. Therefore, neither can be seen as stand-alone targets. However, these mutants are much more susceptible to $\beta$-lactam antibiotics (Table); if one compares isogenic strains such as BB 255 (NCTC 8325, wild-type), its mec-containing MRS A counterpart BB 270, the femAB null mutant AS 145 and the femA mutant UK 17, both constructed from BB 270, it becomes apparent that the femAB and femA mutants are both even more susceptible to $\beta$-lactam antibiotics than the wild-type strain BB 255 even though they are typical of MRS A strains in terms of the production of PBP2a. Correspondingly, femA mutants in a non-MRS A background are hypersusceptible to $\beta$-lactams. Thus $\beta$-lactam hypersusceptibility should occur in all staphylococcal strains, whether methicillin-resistant or-sensitive, after inhibition of the function of either FemA, FemB or both. A further interesting, but not yet fully understood, consequence of FemAB inhibition is an additional sensitization of S. aureus to non-$\beta$-lactam antibiotics (Table). This is not only restricted to other cell wall active antibiotics like vancocin-type glycopeptides (where the effect is relatively small) but has been found for all types of antibiotics tested so far, including quinolones and various protein synthesis inhibitors (Table). With respect to FemX, its role as a possible stand-alone target has not been resolved yet, although data presented by Kopp et al. might be taken as evidence for its existence.

### Table. Comparison of susceptibilities (mg/L) of isogenic wild-type strains and of femA null mutants against various antibiotics

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>BB 255</th>
<th>BB 270</th>
<th>AS 145</th>
<th>UK 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>0.047</td>
<td>4</td>
<td>&lt;0.016</td>
<td>&lt;0.016</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>0.75</td>
<td>&lt;256</td>
<td>&lt;0.016</td>
<td>&lt;0.094</td>
</tr>
<tr>
<td>Imipenem</td>
<td>0.047</td>
<td>0.25</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>Methicillin</td>
<td>0.075</td>
<td>6</td>
<td>0.094</td>
<td>0.25</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>3</td>
<td>3</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>3</td>
<td>1.5</td>
<td>0.125</td>
<td>0.25</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>0.125</td>
<td>0.094</td>
<td>&lt;0.016</td>
<td>0.064</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>0.023</td>
<td>0.016</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.38</td>
<td>0.19</td>
<td>0.064</td>
<td>0.064</td>
</tr>
</tbody>
</table>

*The following isogenic strains were tested using the Etest on Isosensitest agar (37°C, 24 h): BB 255 (wild-type), BB 270 (expressing PBP2a, MRS A), AS 145 (expressing PBP2a, femAB null mutant), UK 17 (expressing PBP2a, femA mutant).*
an indication that femX is an essential gene, since many cells of the putative leaky femX mutant UK 31 already showed spontaneous lysis during growth. In any case, the phenotype observed for femAB mutants, namely hypersusceptibility to β-lactams and grossly enhanced susceptibility to other antibiotics, should be even more exaggerated.

An important aspect for the validity of an antibacterial target is its spread among clinically important organisms. FemA and FemB (and, by analogy, also FemX) occur in all S. aureus strains and, as might be expected from the quite similar (or identical) peptidoglycan interpeptide bridge, also occur in all coagulase-negative staphylococci examined so far. The recent detection of femAB-like genes responsible for incorporation of serine instead of glycine into the interpeptide bridge of staphylococci shows that further femAB-like genes do exist. Thus, for all staphylococci, FemA BX-like proteins involved in cell wall interpeptide bridge formation appear as an attractive, multitarget protein family, which, because of their structural and functional similarity, might be accessible to inhibition by one and the same type of inhibitors (similar to the situation found for PBP and β-lactams).

Staphylococci account for a very large proportion of nosocomial infections. Therefore, a target family specific for both coagulase-positive and -negative staphylococci might be interesting, both medically and economically. Furthermore, more specifically tailored antibiotics have been discussed as one possible approach to avoid or to slow down resistance development. On the other hand, realization of such a concept requires sophisticated, rapid diagnostic tools and, even more importantly, the preparedness of the medical community to cope with the challenges of extremely narrow-spectrum antibiotics. In this respect it is interesting to consider the possibility that femABX-like genes, although primarily considered as antistaphylococcal targets, might occur outside of the staphylococcal world. Interpeptide bridges of chemically distinct, but principally similar type are known to occur in many other clinically important Gram-positive bacteria (while absent in all Gram-negatives), and their biosynthesis seems to follow a very similar scheme to that found in staphylococci. Thus, the femABX target family might be much more widespread than originally thought. In view of the need for new antibiotic classes, directed against novel antibacterial targets, with no cross-resistance to existing compound classes and low propensity for resistance development, we believe that interpeptide bridge synthesis in staphylococci and possibly other Gram-positive bacteria is not only a scientifically interesting but also an important biomedical approach to combat the challenges of bacterial resistance, either by resistance-breaker concepts (FemA BX-like targets) or by stand-alone targets (femX-like genes).

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Acknowledgement

This article is based upon a presentation given at a conference entitled New Targets for New Antibiotics, organized by the Institute Pasteur and held in Paris on June 4–6, 1997.

References

Among the mechanisms identified are over-expression of the drug target. Antibiotics in bacteria have been extensively studied in recent years. An additional resistance mechanism, identified nearly 40 years ago, involves over-expression of the drug target. Elevated levels of drug target confer resistance because higher antibiotic or inhibitor concentrations are required to bind to the excess target to decrease its biological activity to a level that prevents bacterial growth.

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The biochemical mechanisms of acquired resistance to antibiotics in bacteria have been extensively studied in recent years. Among the mechanisms identified are enzymic destruction or modification of the antibiotic, mutation at the target site preventing drug interaction and removal of antibiotic from the cell by efflux.

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Target over-production in bacteria as a mechanism of resistance to antibiotics and inhibitors has received relatively little attention, probably because the first examples were laboratory phenomena with no clinical relevance. However, it has been reported more recently that target over-production probably accounts for acquired resistance to isoniazid and ethambutol in clinical isolates of mycobacteria. This raises the issue of whether resistance due to target over-production could become more prevalent in the clinical setting. This paper reviews examples of antibiotic and inhibitor resistance that arise from target over-expression and considers factors that may limit the emergence of this resistance mechanism in the clinical setting to specific bacterial species.

The Table provides examples of resistance to antibiotics or inhibitors that can be ascribed to over-production of protein targets. Although molecular analysis has not been conducted in all cases, over-expression can result from enhanced transcription of the target gene as a consequence of mutations that affect promoter structure. This results in elevated mRNA production and consequently increased levels of target proteins following translation.

Over-expression of targets through promoter mutations appears to be responsible for resistance in both laboratory-selected mutants and resistant clinical isolates (Table). An alternative mechanism for over-expression of target gene products and, hence, resistance is gene duplication, leading to a gene dosage effect. This does not appear to occur under clinical conditions, but can arise in the laboratory when a target gene is cloned into a multi-copy plasmid vector (Table).

The examples described in the Table indicate that resistance to several antibiotics and inhibitors can arise in a variety of bacterial species through target gene over-expression. However, in some cases it may be impossible to express resistance by this mechanism, e.g. when over-expression of the target gene is lethal to the organism or when the antibiotic- or inhibitor-binding site comprises part of an organelle, when the products of several genes would each have to be independently over-expressed to confer resistance. The limitations for emergence of resistance by coordinated over-expression of several gene products can be illustrated by considering the mechanisms that would be required to confer resistance to drugs inhibiting protein synthesis by interaction with the ribosome. The binding sites in the ribosome for several of these agents probably comprise rRNA, which suggests that over-expression of rRNA structural genes might be a mechanism by which organisms could become resistant to these drugs. However, the synthesis of rRNA and ribosomal proteins occurs with a precise stoichiometry and the production of excess rRNA alone leads, in the absence of elevated ribosomal protein synthesis, to its degradation since insufficient quantities of ribosomal proteins are available for packaging the RNA into ribosomes. Hence, resistance to drugs inhibiting protein synthesis is unlikely to arise by over-expression of rRNA targets.

Regarding the clinical emergence of resistance arising from target site over-expression, it is notable that the only clinical isolates reported to date where this mechanism has occurred are the mycobacteria (Table). Why is this and what is its significance?

It is now well recognized that acquisition of antibiotic resistance determinants on plasmids and transposons is particularly important in the evolution of antibiotic-resistant bacteria. In evolutionary terms, acquisition of plasmid- or transposon-located resistance genes offers advantages to the cell over resistance based on chromosomal mutations (e.g. promoter mutations leading to target gene over-expression) because acquisition of a resistance determinant provides the recipient with pre-evolved genes refined to express resistance at a high level. Furthermore, resistance determinants contained in transposons can become established in diverse bacterial species in which the original plasmid vectors themselves may be unable to replicate. If transposons and plasmids were absent from pathogenic mycobacteria, or the organisms were unable to participate in genetic exchange under natural conditions, then mycobacteria might have to rely upon chromosomal mutations, including those leading to target over-expression, for development of resistance. Is there any evidence to support this conclusion?

Although transposable genetic elements have been described in mycobacteria, the majority are species-specific or present in only a narrow host range. With the exception of transposon Tn610, which encodes resistance to sulphonamides and was isolated from the saprophytic organism Mycobacterium fortuitum, there is no evidence that other transposable elements in mycobacteria naturally encode resistance to anti-mycobacterial agents. Plasmids have been isolated from saprophytic and opportunistic mycobacteria (e.g. Mycobacterium avium complex), but there is no convincing evidence for their presence in pathogenic species such as Mycobacterium tuberculosis, Mycobacterium bovis or Mycobacterium leprae. The functions of many of these plasmids are unknown (i.e. they are cryptic) and none encode resistance to antibiotics. Furthermore, although transposable elements and plasmids do occur in mycobacteria, evidence for genetic exchange between mycobacteria or other organisms under natural conditions has not been reported. This may relate both to the refractory nature of the mycobacterial cell envelope, which probably limits the entry of DNA into the cell, and to the site of the pathogenic mycobacteria within closed lesions. Therefore, unlike other organisms, which can acquire plasmids and transposons encoding pre-evolved genes tailored to express antibiotic resistance, mycobacteria rely upon chromosomal mutations, including those leading to target over-expression, to develop resistance.
Table. Examples of target gene over-expression leading to an antibiotic- or inhibitor-resistant phenotype

<table>
<thead>
<tr>
<th>Antibiotic/inhibitor</th>
<th>Target gene/protein over-expressed</th>
<th>Organism</th>
<th>laboratory derived</th>
<th>clinical isolate</th>
<th>Mechanism of gene over-expression</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-Lactams</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canavanine</td>
<td>arginine-biosynthetic enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-Carbamyl-d-serine</td>
<td>alr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Cycloserine</td>
<td>alr, ddl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diazoborine</td>
<td>envM</td>
<td>E. coli</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethambutol</td>
<td>emb</td>
<td>M. smegmatis</td>
<td></td>
<td></td>
<td>gene dosage, recombinant vector</td>
<td>12</td>
</tr>
<tr>
<td>Ethionamide</td>
<td>inhA</td>
<td>M. smegmatis</td>
<td></td>
<td></td>
<td>gene dosage, recombinant vector</td>
<td>29</td>
</tr>
<tr>
<td>Folate biosynthesis</td>
<td>thymidylate synthetase</td>
<td>Lactobacillus casei</td>
<td></td>
<td></td>
<td>unknown</td>
<td></td>
</tr>
<tr>
<td>inhibitors</td>
<td>dihydrofolate reductase</td>
<td>Enterococcus faecium</td>
<td></td>
<td></td>
<td>unknown</td>
<td>8</td>
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<tr>
<td>Gliobomycin</td>
<td>lsp</td>
<td>E. coli</td>
<td></td>
<td></td>
<td>gene dosage, recombinant vector</td>
<td>32, 33</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>inhA</td>
<td>M. tuberculosis</td>
<td></td>
<td></td>
<td>promoter mutation?</td>
<td>11</td>
</tr>
<tr>
<td>5-Methyltryptophan</td>
<td>tryptophan-biosynthetic enzymes</td>
<td>E. coli</td>
<td></td>
<td></td>
<td>gene dosage, recombinant vector</td>
<td>31</td>
</tr>
</tbody>
</table>

Reference numbers correspond to the literature cited elsewhere in the text.
Thus, there is the possibility that resistance due to target over-expression is unlikely to arise to these agents. However, a number of other agents which do not target protein synthesis are used for chlamydial chemotherapy. Although resistance to antibiotics has been reported in Chlamydia trachomatis, it may emerge as a more widespread problem in the clinic isolates of Mycobacterium tuberculosis. In conclusion, although resistance to these agents might arise in the clinical setting from target gene over-expression, at present, however, there is no evidence that this arises from target over-expression.

In summary, although resistance to antibiotics from target gene over-expression in clinical isolates is currently rare, it may emerge as a more widespread problem in the future, particularly in those organisms which are unable to acquire, or stably maintain, resistance determinants from other sources.

References


