A Mechanism of Resistance to Partial Macrolide and Streptogramin B Antibiotics in *Staphylococcus aureus* Clinically Isolated in Hungary

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A plasmid pEP2104 originated from *Staphylococcus aureus* was clinically isolated in Hungary during 1977. The plasmid mediates inducible resistance to PMS-antibiotics; partial macrolide [the 14-membered macrolides, erythromycin (EM) and oleandomycin and the 16-membered macrolides mycinamicin I (MCM I) and mycinamicin II (MCM II)] and type B streptogramin (MKM-B) antibiotics.

The sequence of 31 amino acid residues obtained by N-terminal analysis of the 63 kDa protein (MsrSA) present in the membrane from 8325 (pEP2104) cells, whose PMS-resistance was induced by a concentration of 1.35 μg EM/ml [EM-induced 8325 (pEP2104)], was identical to the corresponding sequence in a membrane protein MsrA related to promoting efflux of [14C]EM [Ross J. I., et al., Mol. Microbiol., 4, 1207 (1990)].

A constitutive PMS-resistant strain 8325 (pMC38) was obtained from the 8325 (pEP2104) strain in the presence of 1 μg MCM/1 ml. No inactivation of EM in EM-induced 8325 (pEP2104) was observed. Moreover, poly(A)-directed polylysine system containing ribosomes from EM-induced 8325 (pEP2104) cells and S100 from *Escherichia coli* was inhibited by not only EM but spiramycin and MKM-B [Matsuoka M., et al., Biol. Pharm. Bull., 16, 1288 (1993)]. In addition, ribosomes from both EM-induced 8325 (pEP2104) and 8325 (pMC38) strains showed about the same affinity as those from the host strain, NCTC8325.

These results suggest that, like MsrA protein, active drug-efflux due to MsrSA protein may be responsible for PMS-resistance. How can the 8325 (pMC38) strain discriminate PMS-antibiotics from most of 16-membered macrolides and lincosamides? A possible explanation is discussed in terms of the pKₐ-value related to the physicochemical nature of the antibiotics.

Key words  *Staphylococcus aureus*; erythromycin; macrolide antibiotics; macrolide resistance; pKₐ

Resistance in most staphylococcal strains to macrolide antibiotics is accompanied by the phenotypically decreased accumulation of the drugs. ³ This phenotype can arise from any of the following three resistance mechanisms: (1) Decreased permeability or impaired uptake responsible for the decreased drug accumulation. This suggestion was initially proposed as a macrolide-resistant mechanism. ² (2) Alteration of ribosomes due to dimethylation of a specific adenine residue of 23S rRNA. The altered ribosome causes the decreased antibiotic binding. ³ After all, staphylococcal cells that contain the altered ribosome exhibit a decreased accumulation of macrolides. ¹ Such staphylococcal strains that specify an inducibly or constitutively resistant phenotype to macrolide-lincosamide-streptogramin B (MLS) antibiotics occur more often in hospitals. (3) Active efflux as a mechanism to explain decreased macrolide-accumulation was suggested later. Such strains show inducibly and constitutively unusual resistance (mnrA and erpA genes coding for macrolide-resistance due to active efflux, respectively) to the 14-membered macrolides such as erythromycin (EM), oleandomycin (OL), the 15-membered macrolides such as azithromycin, but not the 16-membered macrolides, or lincosamide such as clindamycin.⁴

Recently, energy-dependent or active efflux systems (responsible for resistance to not only macrolide but also other antimicrobial agents such as hydrophilic quinones,⁵ ethidium bromides,⁶ antiseptics and disinfectants, ⁷ and a heavy metal⁸ in *Staphylococcus aureus*) are now observed frequently in clinical isolates. The enhanced macrolide-efflux is based on the result that energy inhibitors or uncouplers such as 2,4-dinitrophenol(DNP) and carbonylreanide m-chlorophenylhydrazone cause staphylococcal cells to increase drug accumulation.⁹

A strain of *S. aureus*, not *Staphylococcus epidermidis*, harboring plasmid pEP2104 which mediates inducibly unusual macrolide resistance was isolated in Hungary.⁰ This phenotypic trait was comparable to that of the *S. epidermidis*; *S. aureus* 8325 (pEP2104) showed inducible resistance to EM and OL, streptogramin B antibiotics such as pristinamycin Iₐc and mikamycin B (MKM-B), and the 16-membered macrolides mycinamicin (MCM I) and II, but not to the other 16-membered macrolide or lincomamide antibiotics.⁹,¹⁰ Such a phenotype is referred to as PMS (partial macrolide and streptogramin B antibiotics) resistance. ¹¹ When the PMS-resistance of the 8325(pEP2104) strain was induced by 1.35 μg EM/ml, a 63 kDa-protein (hereafter referred to as MsrSA) present in the cytoplasmic membrane of the strain increased considerably. The protein is closely related to PMS-resistance.¹¹

In this paper, the characteristics of the MsrSA protein responsible for PMS-resistance in *S. aureus* 8325(pEP2104) and its constitutive mutant strain 8325(pMC38) are described.

MATERIALS AND METHODS

**Bacterial Strains**  *S. aureus* strains used in this experiment were inducible PMS-resistant 8325 (pEP2104),¹² constitutive MLS resistant 8325 (pI258), and a susceptible NCTC8325. The 8325 (pMC38) strain was a spontaneous
constitutive-resistant mutant derived from the inducible PMS-resistant strain 8325(pEP2104) by spreading the cells (2.0 × 10^9 cells) on brain heart infusion agar (BHHIA) plates containing MCM I (1 μg/ml) and a susceptible mutant strain 8325(pSP6) was isolated from the same inducible resistant strain by using a mutagen, N-methyl-N'-nitro-N-nitrosoguanidine. 13)

**Chemicals** EM, OL, spiramycin (SPM), tylosin (TS), and tris(hydroxymethyl)aminomethane (tris) were obtained from Sigma Chemical Co.; MCM I and II and rokitamycin (RKM) from Asahi-kasei Co.; lincomycin (LCM) from Japan Upjohn Co.; penicillin G potassium (PC-G) from Meiji Co.; [N-methyl-14C] EM (14C-EM; 54.0 μCi/mmol) from Amersham Japan Co.; MKM-B was purified from a mikamycin mixture (obtained from Banyuu Co.) using liquid chromatography. Other chemicals were purchased from commercial sources.

**Effect of pH on Susceptibility to EM** This test was basically carried out by measuring the minimum inhibitory concentration (MIC). 5,20) Each of the MICs was determined by using overnight cultured cells according to an agar dilution technique. A two-fold dilution and a suitable narrow dilution of EM, from 0.01 to 500 μg/ml, were distributed into culture medium H. 14) The medium is capable of resisting persistently large changes in pH such as 6.6, 7.3, 7.6 and 8.6. The concentrations of cell cultures were adjusted to 10^9 cells/ml. Five μl of the cell suspension (5 × 10^3 cells) was inoculated onto antibiotic-containing plates with a Microplanter (Sakuma Seisakuju, Tokyo). The plates were incubated for 18 to 20 h at 37°C. The inhibitory concentration was the lowest concentration of drug that prevented visible growth.

**Isolation of Membrane Proteins** Staphylococcal membrane proteins were obtained and SDS-polyacrylamide gel (8%) electrophoresis of the proteins (equivalent to 20 μg) was conducted as previously described. 11)

**EM Binding to Ribosomes** Ribosomes were prepared and the binding affinity of EM to ribosomes was measured according to the methods of Nakajima et al. 15) and Rafael et al., 16) respectively. Under our experimental condition that *S. aureus* cells were exponentially grown in medium H, a value of 3.2 × 10^3 ribosomes per cell was estimated by disrupting protoplasts (equivalent to about 2.3 × 10^11 cells, and 99.7% of the intact cells yielded their protoplasts) which were obtained by the slightly modified method of Chopra, et al. 17): the protoplasts were disrupted for 10 min at 0°C in a glass homogenizer (5 ml, No. 24240 Tokyo Rikakikai Co.), and the estimated value of the ribosomes was corrected by addition of known amounts of staphylococcal ribosomes as an internal standard.

**Partial Sequencing of Amino Acids of Membrane Protein** This procedure was basically performed using the methods of Matsudaira. 18) After electrophoresis, the gel that was left unstained was equilibrated in Tris-glycine transfer solution (0.1 M Tris, 0.19 M glycine, 0.1% (w/v) methanol) for 30 min at room temperature. During this time a polyvinylidene difluoride (PVDF) membrane of the same size as the gel was soaked in 100% methanol for 20 s and again in the transfer solution for 30 min. Protein transfer to the membrane was performed using an AE-6670P Horizelot transfer system (ATTO) in the Tris-glycine transfer solution, at 12 V, 90 min. The PVDF membrane was washed in deionized H2O and stained in 50% methanol-10% acetic acid. The PVDF membrane was finally rinsed in deionized H2O, air dried, and stored at -20°C. The transferred proteins were sequenced on an Applied Biosystems Model 477A Protein Sequencer.

### RESULTS AND DISCUSSION

**Constitutive Strain Derived from the Parental Strain 8325(pEP2104)** In order to avoid a time-consuming procedure for induction of PMS-resistance by EM, we tried to obtain a constitutively PMS-resistant mutant from strain 8325(pEP2104). Twenty-eight spontaneous mutants were obtained by spreading 2.0 × 10^9 cells of 8325(pEP2104) on BHHIA plates containing 1 μg MCM I/ml. The effect of EM on the susceptibility of one constitutive mutant strain 8325(pMC38) out of the 28 mutants was compared with that of other strains: NCTC8325 (susceptible), NCTC8325 containing plasmid pEP2104, and NCTC8325 containing plasmid pSP6 (Fig. 1). Strain 8325(pSP6) is a susceptible mutant derived from pEP2104 mediating inducible PMS-resistance and mutation probably takes place on a regulatory gene whose product is a regulator protein which regulates the transcription of at least one structural gene related to PMS-resistance (Fig. 1d and lane 6 in Fig. 2), since any one of revertants from the 8325(pSP6) strain, whose 63 kDa-protein was deficient, gave the same phenotype as the parent strain 8325(pEP2104).

Strain 8325(pMC38) exhibits constitutive resistance to PMS-antibiotics (Fig. 1c), whereas the strain 8325(pEP2104) is an inducible PMS-resistant one, since the latter strain produced a D-shaped zone of inhibition around each disc containing MCM I and MKM-B (Fig. 1b). No zone of inhibition around EM or OL disc could be found using the agar disc diffusion test (Fig. 1b). By using a liquid culture medium, however, strain 8325(pEP2104) revealed inducible resistance to EM and OL, 12) and strain 8325(pMC38) constitutive resistance to the drugs (data not shown). In contrast, *S. aureus* strains NCTC8325 and 8325(pSP6) were susceptible to the macrolide antibiotics used, LCM and MKM-B (Fig. 1a and d), whereas strain 8325(pJ25), a typical MLS resistant one, was resistant to all drugs used (Fig. 1e). The resistant profile of these strains towards PC-G and heavy metals was used as a convenient marker of the presence of plasmid pEP2104 or plasmids, such as pMC38 and pSP6, derived from plasmid pEP2104 (Fig. 1b, c, and d).

**Effect of pH on EM-Susceptibility** The macrolides EM and OL are well known to be most active at alkaline pH, e.g. 8.5. 19) Since the pK of values of EM and OL are, respectively, 8.6 and 8.5, the non-protonated antibiotics are associated with more antibacterial activity, as Mao and Wiegand have observed previously. 19) Hence, we investigated a relationship between the change in the amount of non-protonated EM and the effect of pH on susceptibility to EM, focusing our particular attention on the PMS-resistant strain 8325(pMC38). Except for strain 8325(pJ25), that shows constitutive MLS-resistance due to...
Fig. 1. Effect of Erythromycin on the Susceptibility of \( S. \) \( aureus \) to Macrolide, Lincosamide, and Mikamycin B Antibiotics and Inorganic Compounds

Each disk contains (μg/disc): top row (from left to right), OL, 10, SPM 20, MCM 15, MKM-B 25, RKM 20, and TS 20; second row, EM (each of six discs) 10; bottom row, LCM 5, PC-G5, mercuric chloride (Hg) 10, cadmium sulfate (Cd) 50, sodium arsenate, dibasic (As) 50, and lead acetate (Pb) 1000.

to \( N^6, N^\theta \)-dimethylation of a specific adenine residue in 23S rRNA, the MIC values in the PMS-resistant strain 8325(pMC38) and susceptible strain NCTC8325 tend to increase as the pH goes from 8.6 to 6.6 (Table 1). However, the concentration of non-protonated EM molecules present in the culture medium at different pH values was fairly constant in each strain: for example 7.0 x 10^{-9} \( \text{M} \) (pH 6.6), 2.5 x 10^{-9} \( \text{M} \) (pH 7.3) and 1.2 x 10^{-8} \( \text{M} \) (pH 7.6) for strain NCTC8325, and 1.5 x 10^{-9} \( \text{M} \) (pH 6.6), 3.25 x 10^{-9} \( \text{M} \) (pH 7.3), and 3.68 x 10^{-8} \( \text{M} \) (pH 7.6) for strain 8325(pMC38). In contrast, there was more than a 10-fold difference in the concentration of uncharged EM between 8325(pMC38) and NCTC8325 strains (Table 1). On the other hand, strain 8325(pMC38) was no more than twice as resistant as NCTC8325 strain, when exposed to 16-membered macrolide and lincosamide antibiotics whose \( pK_a \) values are around 7.5 (Table 2). There is no doubt that such a great difference in EM-resistance between the 8325(pMC38) and NCTC8325 strains has been brought about by pMC38 plasmid. However, it is unclear how the NCTC8325 strain containing plasmid pMC38 is able to distinguish between PMS-antibiotics and most of the 16-membered macrolides and lincosamides.

Moreover, neither cells nor SM100 (105000 x g supernatant obtained from them) of 8325(pMC38) or EM-induced 8325(pEP2104) inactivated macrolide antibiotics such as EM or josamycin (JM111)

**Affinity of \( S. \) \( aureus \) Ribosomes for EM** In a previous paper, ribosomes from EM-induced 8325(pEP2104) cells were as sensitive to EM, SPM, and MKM-B as those from either plasmid-free strain NCTC8325 or strain 8325 (pEP2104) without induction of PMS-resistance by EM, when the poly(A)-directed polylysine synthesizing system of cell-free-extracts containing ribosomes from any one of those \( S. \) \( aureus \) strains and SI100 from *Escherichia coli* Q13 was used. In this paper, the degree of EM-binding to staphylococcal ribosomes was tested as an indicator of the drug-sensitivity of ribosomes. The affinity was expressed in terms of an apparent dissociation constant \( (k_{\text{d}}) \) which

![Diagram](image-url)
was determined by Scatchard-plot analysis. Table 3 shows that ribosomes from all *S. aureus* strains NCTC8325, 8325(pEP2104), EM-induced 8325(pEP2104), and 8325(pMC38), but not strain 8325(pIP258) are sensitive to EM, since ribosomes from 8325(pMC38) and EM-induced 8325(pEP2104) cells had about the same affinity as those from NCTC8325 and uninduced 8325(pEP2104) cells: those apparent *K*_A had an order of magnitude of 10^{-8} (M).

**Partial Amino Acid Sequence of the 63 kDa Protein** The MsrSA was isolated from EM-induced 8325(pEP2104) cells and sequenced from the first NH2-terminal to the 31st COOH-terminal amino acid residues using an Applied Biosystems Model ABI-477A Protein Sequencer using a slight modification of the Matsudaira method19 (Fig. 3). The partial sequence of 31 amino acid residues of the MsrSA protein was identical to the corresponding sequence in a membrane protein MrSA that mediates inducible resistance to 14-membered macrolides and type B streptogramins (referred to as MS-resistance), the amino acid sequence of which was deduced from base sequences obtained from MS-resistance gene *msrA*. The resistance gene *msrA* on a 31.5 kb plasmid, pUL5050, was initially detected in *S. epidermidis* which was clinically isolated in the UK during 1989, and subcloned on a SauSA fragment (1.9 kb) into a high copy-number vector pSK265 in the *S. aureus* RN4220 strain.60 The genes MsrA and MsrSA found individually in *S. epidermidis* and *S. aureus*, respectively, seem to originate from a common ancestor. The MsrA protein appears to promote energy-dependent efflux of EM, since energy inhibitors such as arsenate and DNP increase the uptake of EM in resistant cells of *S. aureus* RN4220(pUL5054) strain, and since the two ATP-binding motifs present in the protein are homologous to those of a family of transport-related proteins from gram-negative bacteria and eukaryotic cells, the proteins that are responsible for multidrug resistance. Accordingly, MsrSA protein in EM-induced 8325(pEP2104) and 8325(pMC38) strains may also be related to active efflux of the 14-membered macrolides EM and OL, 16-membered macrolides MCM I and II, and MKM-B. If this is the case, *S. aureus* containing plasmids pMC38 must be able to discriminate PMS antibiotics from antibodies including 16-membered macrolides and LCM (MIC values at pH 7.3 in Tables 1 and 2). It is also noteworthy that, in a culture medium at pH 7.3, the p*K*_A value of EM which is determined from its MIC to the 8325(pMC38) strain is about 130 times as resistant compared with the value from the MIC to the NCTC8325 strain. In contrast the value of JM, one of the low-p*K*_A-antibiotics, in the former resistant strain is only twice as resistant as the latter sensitive strain. Accordingly, as far as p*K*_A is concerned, *S. aureus* containing pMC38, which carries the gene that specifies PMS-resistance, could discriminate high-p*K*_A antibiotics (from 8.5 to 9.0) from low-p*K*_A ones (from 7.0 to 7.7) (Table 4). Further detailed study of the mechanism of resistance to PMS-antibiotics is in progress.

### Table 1. Effect of pH on Susceptibility to Erythromycin in *S. aureus* NCTC8325, 8325(pMC38), and 8325(pIP258)

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (10^{-7} m) at various pH values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.6</td>
</tr>
<tr>
<td>NCTC8325</td>
<td>6.8</td>
</tr>
<tr>
<td>(0.07)^a</td>
<td></td>
</tr>
<tr>
<td>8325(pMC38)</td>
<td>150</td>
</tr>
<tr>
<td>(15)</td>
<td>(32.5)</td>
</tr>
<tr>
<td>8325(pIP258)</td>
<td>&gt;6800</td>
</tr>
<tr>
<td>(&gt;67.4)</td>
<td>(&gt;325)</td>
</tr>
</tbody>
</table>

*a* The values in parenthesis are concentrations of non-protonated erythromycin.

### Table 2. Susceptibility of *S. aureus* NCTC8325 and 8325(pMC38) to Macrolide and Lincomycin Antibiotics at pH 7.3

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (10^{-7} m) at pH 7.3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SPM</td>
</tr>
<tr>
<td>NCTC8325</td>
<td>18.5</td>
</tr>
<tr>
<td>(5.3)^a</td>
<td></td>
</tr>
<tr>
<td>8325(pMC38)</td>
<td>37.1</td>
</tr>
<tr>
<td>(10.5)</td>
<td>(11.2)</td>
</tr>
</tbody>
</table>

*a* The values in parenthesis are concentrations of the non-protonated antibiotics.

### Table 3. Apparent Dissociation Constant (*K*_A) of the Erythromycin-ribosome Complex

<table>
<thead>
<tr>
<th>70S ribosome from</th>
<th><em>K</em>_A (m) of erythromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC8325</td>
<td>1.7 x 10^{-8}</td>
</tr>
<tr>
<td>8325(pEP2104)</td>
<td>3.4 x 10^{-8}</td>
</tr>
<tr>
<td>EM-induced 8325(pEP2104)</td>
<td>2.8 x 10^{-8}</td>
</tr>
<tr>
<td>8325(pMC38)</td>
<td>9.5 x 10^{-9}</td>
</tr>
<tr>
<td>8325(pIP258)</td>
<td>N.D. 49</td>
</tr>
</tbody>
</table>

*a* PMS-resistance was induced by 1.35 μg erythromycin/ml, an optimum inducer concentration.  
*b* N.D.: *K*_A value could not be determined since ribosomes of this strain showed low affinity due to dimethylation of a specific adenine residue in 23S rRNA.

### Fig. 3. Sequence Alignment of 31-NH2 Terminal Amino Acids of MsrSA and MrSA Proteins

### Table 4. p*K*_A Value of Macrolide and Lincosamide Antibiotics

<table>
<thead>
<tr>
<th>Antibiotics of</th>
<th>Low p<em>K</em>_A</th>
<th>High p<em>K</em>_A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TL</td>
<td>JM</td>
</tr>
<tr>
<td>7.0</td>
<td>7.5</td>
<td>7.6</td>
</tr>
</tbody>
</table>

*Abbrevs.: See the legend in Fig. 1.  
a) and b: p*K*_A values of the drugs have been determined in methanol and 40% aqueous methanol, respectively.*

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REFERENCES