An Increase of 63 kDa-Protein Present in the Cell Membranes of *Staphylococcus aureus* That Bears a Plasmid Mediating Inducible Resistance to Partial Macrolide and Streptogramin B Antibiotics

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A plasmid, pEP2104 (23.9 kilobase pairs), from *Staphylococcus aureus* carries a gene that specifies inducible resistance to 14-membered (erythromycin, EM, and oleandomycin, OL) and 16-membered macrolide (mycinamicin I and II), but not to all of the latter, and to streptogramin type B antibiotics (partial macrolide- and streptogramin-B-antibiotic resistance: PMS-resistance) (I. Jánosi, É. Bán, Acta Microbiol. Acad. Sci. Hung., 29, 187 (1982) and Y. Nakajima et al., J. Pharmacobiodyn., 15, 319 (1992)). The induced cells of strain 8325(pEP2104) did not inactivate EM, OL, josamycin, rokitamycin or mikamycin B (MKM-B), and the cell-free extract of the strain did not inactivate EM or MKM-B, either. Ribosomes from the cells whose PMS-resistance was induced by EM were sensitive not only to EM or spiramycin, but also to MKM-B. A 63000-dalton protein increased to a great extent only in the cell membrane fractions of induced 8325(pEP2104), and may be involved in PMS-resistance.

**Keywords** *Staphylococcus aureus*; erythromycin; macrolide antibiotic; inducible resistance; inducibly decreased permeability

Most of the clinically isolated *Staphylococcus aureus* resistant to macrolide antibiotics show inducible resistance to macrolide, linosamide, and streptogramin type B antibiotics (inducible MLS-resistance). The MLS-resistance is based on the dimethylation of a specific adenine residue of 23S rRNA present in the 50S ribosomal subunit, in which the dimethylation renders ribosomes unable to bind MLS antibiotics. The induction of RNA methylase occurs translationally.

Other clinical isolates of *S. aureus* exhibiting an unusual type of resistance to macrolide antibiotics were first observed during an outbreak at day-nurseries in Hungary in 1977 and later at 26 other well-defined epidemiological foci throughout that country. These strains were inducibly resistant to 14-membered macrolide antibiotics such as erythromycin (EM), oleandomycin (OL), and to streptogramin type B antibiotics such as pristinamycin I and II (MKM-B). However, they remained susceptible to most 16-membered macrolide antibiotics, but not to mycinamicin I and II (MCM), or to and lincosamide antibiotics. Such a phenotype was referred to as partial macrolide- and streptogramin-B-antibiotic resistance (PMS-resistance). The present study is an attempt to discover a clue for inspecting the mechanism of PMS-resistance.

**Materials and Methods**

**Bacteria** *S. aureus* strains, PMS-resistant 8325(pEP2104), and inducible and constitutive MLS-resistant ISP447 and 8325(pJ258), respectively, were used. Plasmid pEP2104 carries genes that specify resistance to 14-membered macrolide, MCM, and streptogramin type B antibiotics, as well as to Hp, CCl, and β-lactam antibiotics (due to penicillinase production). *Erretherhia coli* Q13 was used for preparing cell-free extracts. *E. coli* BM694/pAT63, which exhibits a high level of EM-resistance due to inactivation of the drug, was provided by P. Courvalin, Institute Pasteur, France. *Micrococcus luteus* ATCC9341 was used as an indicator organism for the test of antibiotic activity.

**Chemicals** EM was obtained from Japan Upjohn Co.; spiramycin (SPM) and rokitamycin (RKM) from Asahi-kasei (formerly Toyoyodo Co.); OL from Pfizer Taito Co.; josamycin (JM) from Yamanouchi Pharmaceutical Co. MKM-B was purified from a mikamycin mixture

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shown) was inactivated by the cells of \textit{S. aureus} 8325 (pEP2104) during 72-h incubation, whereas \textit{E. coli} BM694/pAT63 cells inactivated EM within 24 h. Nor could strain 8325 (pEP2104) cells inactivate 16-membered macrolides such as JM or RKM, or MKM-B (data not shown). The MICs of EM against \textit{S. aureus} NCTC8325 and 8325 (pEP2104) and \textit{E. coli} BM694/pAT63 were 0.78, and more than 100 and 1600 \textmu g/ml, respectively.

Next, we investigated whether EM or MKM-B is inactivated by S100 from 8325 (pEP2104) cells induced by EM. In several inactivation systems\textsuperscript{14} adopted so far, no EM- or MKM-B-inactivation due to (a) phosphorylation, (b) hydrogenation, (c) acetylation, or (d) hydrolysis using S100 from the EM-induced or uninduced cells of strain 8325 (pEP2104) was found. Consequently, inactivation of the macrolide and MKM-B may be ruled out in the strain as a mechanism of PMS-resistance.

On the contrary, S100 from \textit{E. coli} BM694/pAT63 exhaustively hydrolyzed EM within 2 h (data not shown). This result confirms the report of Barthélémy \textit{et al.}\textsuperscript{10}

\textbf{Sensitivity of Ribosomes \textsuperscript{a}} A known mechanism of MLS-resistance is due to the \textit{N}^\textit{b}-\textit{N}^\textit{c}-dimethylation of a specific adenine residue in 23S ribosomal RNA.\textsuperscript{13} In order to compare the mechanism of PMS-resistance with that of MLS-resistance, a sensitivity of ribosomes from the induced cells of 8325 (pEP2104) against EM, SPM and MKM-B was tested using a poly(A)-directed polylysine synthesizing system. The incorporation activity of \textsuperscript{14}C]lysinine into polylysine by homologous cell-free extracts containing ribosomes and S100 from \textit{S. aureus} was about 1/5 of that (5.64 \times 10^3 dpm/mg of ribosomal protein) of the heterologous extracts containing staphylococcal ribosomes and S100 from \textit{E. coli} Q13. But the ribosomes in the homologous cell-free system showed the same sensitivity profile to the drugs as those in the heterologous system.

With the intention of detecting the fine response of ribosomes to the antibiotics, a system containing the heterologous cell-free extracts was adopted.

From both the dose response curves and the same experiments on the antibiotic-sensitivity of uninduced and induced ISP447 strains' ribosomes, 50\% inhibitory concentrations (ID\textsubscript{50}) of the drugs were determined (Table I). Ribosomes from induced cells of the inducibly MLS-resistant strain ISP447 showed resistance to EM, SPM and MKM-B, whereas ribosomes from induced 8325 (pEP2104) cells were as sensitive to both macrolide and MKM-B as those from either NCTC8325 or uninduced 8325 (pEP2104) cells. Ribosomes from the plasmid-free strain NCTC8325 and the uninduced- and induced-cells of strain 8325 (pEP2104) bound to \textsuperscript{14}C]EM ([N-methyl-\textsuperscript{14}C]EM, 3.68 mCi/mmol) had an approximately similar affinity: apparent dissociation constant, \textit{K\textsubscript{d}}, with values of 1.7 \times 10\textsuperscript{-8}, 3.4 \times 10\textsuperscript{-8} and 2.8 \times 10\textsuperscript{-8} \textmu M, respectively. These \textit{K\textsubscript{d}}s were determined in terms of respective Scatchard plots.\textsuperscript{15,16}

Table I indicates that there is no alteration in ribosomes from EM-induced strain 8325 (pEP2104).

If EM-binding to ribosomes causes inhibition of the poly(A)-directed polylysine synthesis adopted, one should be able to demonstrate the same degree of relative inhibition per concentration of EM in relation to the amount of the drug capable of binding to ribosomes. For instance, the molar concentration of 705 ribosomes (one OD\textsubscript{260} unit equivalent to 31 pmol)\textsuperscript{17} is 7.75 \times 10\textsuperscript{-8} M according to our experiment (Table I). The concentration of EM at ID\textsubscript{50} is about 0.30 \mu g/ml (equivalent to 4.1 \times 10\textsuperscript{-7} M) in the cells of plasmid-free strain NCTC8325 (Table I). Assuming non-protonated molecules alone bind to the ribosomes, the molecules present in 4.1 \times 10\textsuperscript{-7} M of EM (p\textsubscript{K\textsuperscript{a}} = 8.6: p\textsubscript{K\textsuperscript{a}} value determined by Seiler's method,\textsuperscript{18}) which is essentially based on a titrimetric method at pH 7.6 are calculated at 3.7 \times 10\textsuperscript{-8} M. This agrees with about half a concentration of the ribosome molecules (7.75 \times 10\textsuperscript{-8} M) present in the polylysine synthesizing system (Table I). This supports the result demonstrated by Mao and Wiegand that EM inhibits protein synthesis to a greater degree in alkaline solutions.

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Strain} & \textbf{ID\textsubscript{50} (\mu g/ml)} & \textbf{EM} & \textbf{SPM} & \textbf{MKM-B} \\
\hline
NCTC8325 & 0.30 & 0.34 & 0.76 \\
8325 (pEP2104) & 0.26 & 0.40 & 0.68 \\
ind. 8325 (pEP2104) & 0.30 & 0.49 & 0.76 \\
ISP447 & 0.34 & 0.30 & 0.97 \\
ind. ISP447 & 891 & >1600 & >25 \\
\hline
\end{tabular}
\caption{Effect of Antibiotics on Poly(A)-Directed Polylysine Synthesis by Cell-Free Extracts Containing Ribosomes from \textit{S. aureus} and S100 from \textit{E. coli} Q13}
\end{table}
which make EM more non-protonated.\textsuperscript{19) }

These results suggest that neither S100 nor ribosomes from induced cells of strain 8325(pEP2104) determine the resistance to PMS-antibiotics.

**Membrane Proteins** *S. aureus* NCTC8325 containing plasmid pEP2104 was found to increase, to a great extent, a protein (63 kDa) present in cell membranes, when the PMS-resistance in the strain was induced by EM (Fig. 2).

On the other hand, when 1 \( \mu g \) EM/ml ([N-methyl-\textsuperscript{14}C]EM: 11.4 nCi/mm mol) (a favorable concentration required for the induction of PMS-resistance) was present in medium H, the EM-accumulation in pEP2104-carrying NCTC8325 cells (5.6 \( \times 10^{-3} \) \( \mu g \) EM/mg of the dry weight cells) was about a half as much as that found in NCTC8325 cells.\textsuperscript{20) } The 63 kDa-protein might provide PMS-resistance as a result of inducibly decreased PMS-antibiotic accumulation. Plasmid pEP2104 (23.9 k) showed similar, but not identical, macrolide-resistant plasmids (from *S. epidermidis*), pNE24 and pUL5050 (26.5 and 31.5 k) respectively.\textsuperscript{21,22) } The latter two plasmids carry the macrolide-resistant genes that encode proteins present in cell membranes, i.e. Erp (60 kDa) and MsrA (56 kDa), respectively. Goldman and Capobianco, and Ross et al. have suggested that the two proteins, Erp and MsrA, may be related to active efflux, which could be the macrolide resistant mechanism.\textsuperscript{21,22) } However, it is unclear at present whether the half decrease of EM-accumulation in the induced cells of 8325(pEP2104) is due to active efflux or to impaired uptake of the drug. Further study is in progress on the cause of the low EM-accumulation responsible for PMS-resistant *S. aureus*.

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**References**