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# Susceptibility of Methicillin-resistant *Staphylococcus aureus* Clinical Isolates to Various Antimicrobial Agents. III. Novel, Inducible Resistance to Macrolide-lincosamide-streptogramin B (MLS) Antibiotics.

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## ABSTRACT

Resistance patterns against 25 antimicrobial agents consisting of β-lactams, aminoglycosides, tetracyclines, fluoroquinolones, macrolides and etc. were examined for 69 strains of methicillin-resistant *Staphylococcus aureus* (MRSA) isolated at Hiroshima University Hospital from July 1991 to April 1992. Regarding overall resistance (the percentage of highly and moderately resistant strains), the following antimicrobial agents were no more effective chemotherapeutics for MRSA infections (%resistance): methicillin (100), flomoxef (100), kanamycin (100), tobramycin (100), amikacin (100), isepamicin (100), gentamicin (78), dibekacin (100), ofloxacin (99), levofloxacin (99), temafloxacin (99), erythromycin (100), clarithromycin (100), tetracycline (93), minocycline (93) and fosfomycin (100). Further spread of arbekacin-resistant strain, which was isolated in April 1991, into a clinical environment could not be recognized during the period covered in the present study.

All the MRSA strains were resistant either constitutively (26 strains) or inducibly (43 strains) to macrolide-lincosamide-streptogramin B (MLS) antibiotics. When expression is constitutive, the strains are resistant to all MLS antibiotics. In contrast, 16-membered macrolide (i.e., jasamycin), lincomycin and mikamycin B escape resistance in the strains with a typical inducible resistance overcome in the presence of 14-membered macrolides by a translational attenuation mechanism. Three of 4 β-lactamase-positive strains, however, can not be classified in these two resistance groups, being exclusively resistant to mikamycin B. The strains grown in the presence of any inducing MLS antibiotic became susceptible to mikamycin B even in the inducer-free culture.

***Key words***: *Methicillin-resistant Staphylococcus aureus (MRSA), Antibiotic resistance, Macrolide-lincosamide-streptogramin B (MLS) antibiotics, Transcriptional regulation*

The low-affinity penicillin binding protein (PBP), designated PBP 2'[22)], PBP 2a[5)] or MRSA PBP[18)] encoded by methicillin (DMPPC)-resistance determinant *mec*A, is responsible for the intrinsic resistance of DMPPC-resistant *Staphylococcus aureus* (MRSA) to β-lactams.

Furthermore, it is troublesome that in most cases MRSA strains are cross-resistant to a variety of antimicrobial agents such as aminoglycosides, tetracyclines, macrolides and fluoroquinolones[12)].

We have been studying the incidence of multidrug resistance in MRSA isolated at Hiroshima University Hospital since 1984[7,19)]. In a previous paper[7)], we reported the isolation of a single arbekacin (ABK)-resistant strain in April 1991: the first time since 1990 when the antibiotic was clinically introduced as a first-choice therapeutic for MRSA. One of the aims of the present study was, therefore, surveillance of the further spread of ABK-resistant isolates in clinical samples.

Translational attenuation is accepted as a mechanism for the inducible resistance to macrolide-lincosamide-streptogramin B (MLS) antibiotics[6)]. However, three newly isolated strains exhibited inducible MLS resistance which could not be explained by the attenuation mechanism. The characterization of the novel, inducible MLS resistance is also a concern of this paper.

## MATERIALS AND METHODS

Sixty-nine MRSA strains were isolated at Hiroshima University Hospital between July 1991 and April 1992. MRSA strain 849, which was isolated from a clinical sample in 1984, and DMPPC-susceptible *S. aureus* FDA 209P were used as references.

The antibiotics and their manufacturers or distributors were as follows: DMPPC and lincomycin (LCM) (SIGMA Chemical Co.); flomoxef (FMOX) and vancomycin (VCM) (Shionogi & Co., Ltd.); isepamicin (ISP) (Toyo Jozo Co., Ltd.); erythromycin (EM) and clarithromycin (CAM) (Taisho Pharmaceutical Co., Ltd.); tetracycline (TC) and minocycline (MINO) (Lederle Japan, Ltd.); fosfomycin (FOM) (Meiji Seika Kaisha, Ltd.); nosiheptide (NH) (Mitsubishi Kasei Corporation); josamycin (JM), ofloxacin (OFLX) and levofloxacin (LVFX) (Daiichi Pharmaceutical Co., Ltd.); temafloxacin (TMFX) (Tanabe Seiyaku Co., Ltd.); mikamycin B (MKM-B) (Kanegafuchi Chemical Ind. Co., Ltd.); kanamycin (KM), tobramycin (TOB), dibekacin (DKB), amikacin (AMK), gentamicin (GM) and ABK (Inst. Microb. Chem.); chloramphenicol (CP) (Wako Pure Chemical Ind., Ltd.); rifampicin (RFP) (Kanto Chemical Co., Inc.); streptomycin (SM) (Irvin Scientific).

The minimum inhibitory concentration (MIC) was measured, unless otherwise specified, by two-fold agar dilution method with Mueller-Hinton agar (DIFCO Laboratories). Test strains grown overnight at 37°C in 5 ml of Meuller-Hinton broth (MHB) (DIFCO Laboratories) were $10^2$-fold diluted with fresh MHB, and about $5 \times 10^3$CFU was applied with multipoint plating apparatus on a surface of agar plate. The plates were incubated at 37°C for 20 hr. The effects of verapamil on the MICs of TC and OFLX were evaluated by 2-fold broth microdilution method[13,14] because the compound was inactive on agar plates, presumably due to the binding to the agar matrix[15].

The production of $\beta$-lactamase by individual MRSA strains was monitored by using BBL cefinase (Becton Dickinson Microbiology Systems).

**Table 1.** Incidence of antibiotic resistance in the MRSA strains isolated at Hiroshima University Hospital from July 1991 to April 1992 (69 strains)

| Antimicrobial agent | Resistant strains | | Moderately resistant strains | | Sensitive strains | | *S. aureus* FDA 209P |
|---|---|---|---|---|---|---|---|
| | No. of strains (MIC, μg/ml) | % | No. of strains (MIC, μg/ml) | % | No. of strains (MIC, μg/ml) | % | |
| Methicillin (DMPPC) | 69 (≧25) | 100 | 0 | 0 | 0 | 0 | (0.10) |
| Flomoxef (FMOX) | 69 (≧6.25) | 100 | 0 | 0 | 0 | 0 | (0.20) |
| Kanamycin (KM) | 69 (≧50) | 100 | 0 | 0 | 0 | 0 | (0.39) |
| Tobramycin (TOB) | 69 (≧12.5) | 100 | 0 | 0 | 0 | 0 | (0.10) |
| Dibekacin (DKB) | 53 (12.5-100) | 77 | 16 (1.56-6.25) | 23 | 0 | 0 | (0.39) |
| Gentamicin (GM) | 54 (12.5-100) | 78 | 0 | 0 | 15 (0.10-0.20) | 22 | (0.10) |
| Amikacin (AMK) | 48 (6.25-12.5) | 70 | 21 (1.56-3.13) | 30 | 0 | 0 | (0.39) |
| Isepamicin (ISP) | 8 (25-50) | 12 | 61 (6.25-12.5) | 88 | 0 | 0 | (1.56) |
| Arbekacin (ABK) | 0 | 0 | 0 | 0 | 69 (0.10-1.56) | 100 | (0.20) |
| Streptomycin (SM) | 4 (>100) | 6 | 0 | 0 | 65 (1.56-3.13) | 94 | (3.13) |
| Erythromycin (EM) | 67 (≧100) | 97 | 2 (6.25-12.5) | 3 | 0 | 0 | (0.20) |
| Clarithromycin (CAM) | 53 (≧25) | 77 | 16 (3.13-12.5) | 23 | 0 | 0 | (0.10) |
| Josamycin (JM) | 28 (≧25) | 41 | 0 | 0 | 41 (0.78-1.56) | 59 | (0.39) |
| Lincomycin (LCM) | 29 (≧100) | 42 | 0 | 0 | 40 (0.39-0.78) | 58 | (0.78) |
| Tetracycline (TC) | 64 (50-100) | 93 | 0 | 0 | 5 (0.20-1.56) | 7 | (0.20) |
| Minocycline (MINO) | 64 (12.5-25) | 93 | 0 | 0 | 5 (0.10-0.39) | 7 | (0.10) |
| Fosfomycin (FOM) | 69 (≧25) | 100 | 0 | 0 | 0 | 0 | (3.13) |
| Vancomycin (VCM) | 0 | 0 | 0 | 0 | 69 (0.39-1.56) | 100 | (0.78) |
| Ofloxacin (OFLX) | 68 (12.5-50) | 99 | 0 | 0 | 1 (0.78) | 1 | (0.20) |
| Levofloxacin (LVFX) | 68 (6.25-25) | 99 | 0 | 0 | 1 (0.39) | 1 | (0.20) |
| Temafloxacin (TMFX) | 68 (3.13-25) | 99 | 0 | 0 | 1 (0.39) | 1 | (0.10) |
| Mikamycin B (MKM-B) | 26 (≧100) | 38 | 0 | 0 | 43 (3.13-12.5) | 62 | (3.13) |
| Nosiheptide (NH) | 0 | 0 | 0 | 0 | 69 (0.003-0.025) | 100 | (0.006) |
| Rifampicin (RFP) | 1 (>6.25) | 1 | 0 | 0 | 68 (0.012-0.10) | 99 | (0.05) |
| Chloramphenicol (CP) | 4 (50) | 6 | 0 | 0 | 65 (6.25-12.5) | 94 | (6.25) |

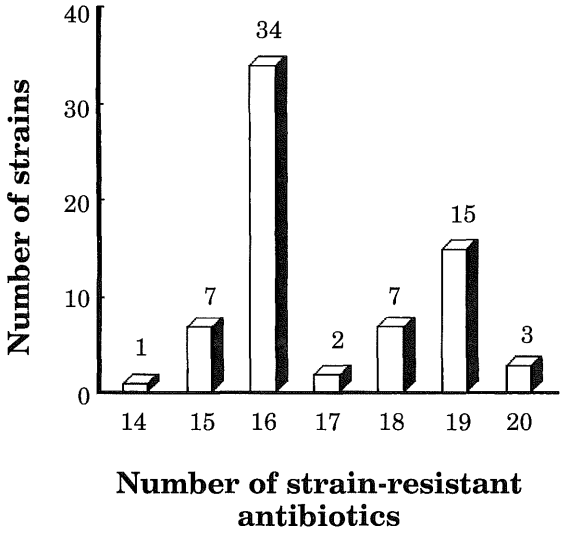


**Fig. 1.** Degree of multiresistance in MRSA strains

## RESULTS

Sixty-nine MRSA strains were classified as: resistant, moderately resistant or susceptible to each antimicrobial agent depending on their MICs according to the definitions of Maple et al[12] and the British Society for Antimicrobial Chemotherapy[2]. The resistance patterns of all the MRSA strains against 25 antimicrobial agents as well as the MIC distribution of individual compounds are shown in Table 1. All the MRSA strains showed resistance to more than 14 antibiotics (Fig. 1). The antimicrobial agents, to which more than 90% strains were resistant, included: DMPPC, FMOX, KM, TOB, DKB, AMK, ISP, EM, CAM, TC, MINO, FOM, OFLX, LVFX and TMFX. GM (resistance, 78%) was slightly less effective than these antibiotics and ca. 40% strains were resistant to JM, LCM and MKM-B. The efficacies of RFP, CP and SM remained satisfactory (resistances, 1%, 6% and 6% respectively) and no resistance was observed against ABK, VCM and NH.

The coagulases produced by MRSA strains were unexceptionally classified into type II. The hydrolysis of nitrocefin was observed with 4 strains.

The resistance phenotype of MRSA to aminocyclitol antibiotics is determined by three inactivating enzymes, in particular bifunctional 6'-acetyltransferase/2"-phosphotransferase AAC(6')/APH(2") and 4'-adenyltransferase AAD(4')[21]. As seen in Table 2, all the strains expressed AAD(4') alone [TOB-resistant ($TOB^r$) type, 15 strains] or in combination with AAC(6')/APH(2") [Mix-resistant ($Mix^r$) type, 54 strains]. However, there was no strain expressing AAC(6')/APH(2") exclusively [GM-resistant ($GM^r$) type].

As for the resistance to typical tetracyclines such as TC and MINO, there are two well-characterized mechanisms. One of these involves the active efflux of antibiotics from bacteria (e.g., plasmid-encoded Tet K determinant) and the other involves ribosomal protection (e.g., chromosomal Tet M determinant)[11,16]. The Tet K determinant mediates high-level resistance to TC but not to MINO. In contrast, the Tet M determinant is related to the resistance to TC and MINO. In 64 MRSA strains with a $TC^rMINO^r$ phenotype (Fig. 2), the resistance to tetracyclines seemed accounted for by the Tet M determinant. The remaining 5 strains were susceptible to tetracyclines, having a $TC^sMINO^s$ phenotype.

**Table 2.** Distribution of aminocyclitol aminoglycoside inactivating enzymes in the MRSA strains

| Phenotype | Aminocyclitol aminoglycoside-modifying enzyme | Number of strains |
|---|---|---|
| $KM^sTOB^sGM^sAMK^sABK^sSM^s$ | — | 0 |
| $KM^rTOB^sGM^sAMK^sABK^sSM^s$ | APH(3') | 0 |
| $KM^rTOB^rGM^sAMK^rABK^sSM^s$ | AAD(4') | 15 |
| $KM^rTOB^rGM^rAMK^sABK^sSM^s$ | AAC(6')/APH(2") or AAC(6')/APH(2")+APH(3') | 0 |
| $KM^rTOB^rGM^rAMK^rABK^sSM^s$ | AAC(6')/APH(2")+AAD(4') or AAC(6')/APH(2")+AAD(4')+APH(3') | 54 |
| | Total ---------- | 69 |

All the 64 strains with a $TC^rMINO^r$ phenotype showed cross-resistance to fluoroquinolones (Fig. 3). Among the remaining 5 strains with a $TC^sMINO^s$ phenotype, only one strain was found to be susceptible to fluoroquinolones and the other strains with a dissociated phenotype ($TC^sLVFX^r$ in Fig. 3) were all aforementioned $\beta$-lactamase-positive strains.

Simultaneous resistance to TC, MINO and LVFX observed in a majority of MRSA strains

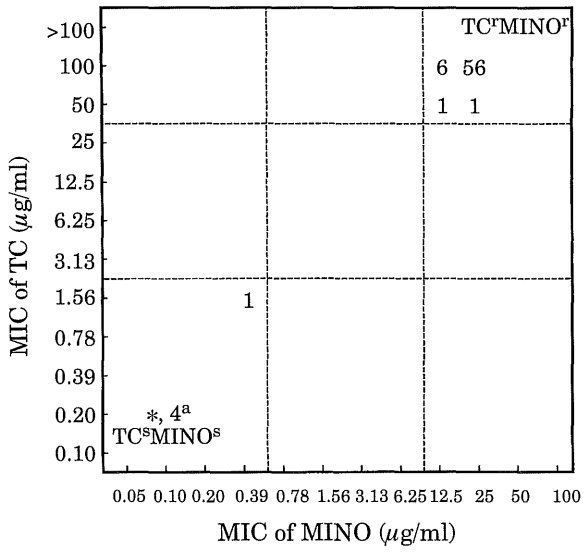


**Fig. 2.** Relationship between resistance to TC and MINO of the MRSA strains isolated at Hiroshima University Hospital
Figures represent the number of MRSA strains with the corresponding MICs.
* represents the MICs of TC and MINO for *S.aureus* FDA 209P.
[a], β-Lactamase-positive strains.

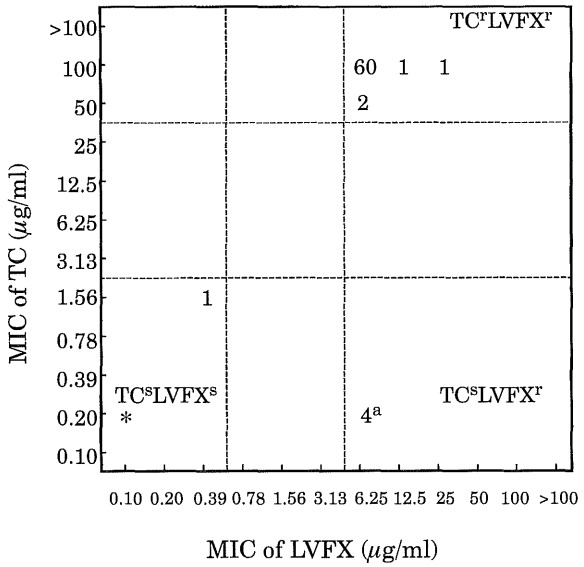


**Fig. 3.** Relationship between resistance to TC and LVFX of the MRSA strains isolated at Hiroshima University Hospital
Figures represent the number of MRSA strains with the corresponding MICS.
* represents the MICs of TC and LVFX for *S. aureus* FDA 209P.
[a], β-Lactamase-positive strains.

employed in the present study may derive from a common efflux mechanism. To work out this hypothesis, the MICs of these antimicrobial agents were measured in the presence of verapamil, which is a well-known inhibitor of various membrane transporters. Strain 849, having a TC$^r$MINO$^s$ phenotype, is supposed to express Tet K protein and the dose-dependent decrease in the MIC of TC is evident from Table 3. In contrast, the resistance of strain 4764 to TC or OFLX was not affected by verapamil even at its highest non-toxic concentration (Table 3).

**Table 3.** Effect of verapamil on TC resistance in the MRSA strains

| Strain | Phenotype | Conc. of verapamil (μg/ml) | MIC of TC (μg/ml) | MIC of OFLX (μg/ml) |
|---|---|---|---|---|
| 4764 | TC$^r$MINO$^r$OFLX$^r$ | 0 | 50 | 25 |
| | | 400 | 50 | 12.5 |
| 4921 | TC$^s$MINO$^s$OFLX$^r$ | 0 | 0.2 | 25 |
| | | 400 | NT | 12.5 |
| 849 | TC$^r$MINO$^s$OFLX$^s$ | 0 | 200 | 0.2 |
| | | 25 | 100 | NT |
| | | 50 | 100 | NT |
| | | 100 | 50 | NT |
| | | 200 | 25 | NT |
| | | 400 | 12.5 | NT |

The MICs were measured by the broth microdilution method in MHB supplemented with 0 - 400 μg/ml verapamil at 37°C for 20 hours.
NT, Not tested.

The MLS resistance was first described in *S. aureus*[3] and is now common in this and other bacteria[1,10]. The MLS resistance is conferred by the function of methylase which converts an

adenosine residue of 23S ribosomal RNA to 6-*N*-dimethyladenosine, thereby reducing the affinity of ribosome for all the MLS-group antibiotics[4,9]. When expression is inducible, MRSA is resistant to 14- and 15-membered macrolides only with a $EM^rCAM^rJM^sLCM^sMKM\text{-}B^s$ phenotype. Structurally related to streptogramin B, MKM-B is included in this group of antibiotics. In contrast, constitutive MLS resistance affords a $EM^rCAM^rJM^rLCM^rMKM\text{-}B^r$ phenotype. The MLS resistance was inducible in 43 strains and constitutive in 26 strains. The number of MRSA strains is plotted as a function of the number of antibiotics to which strains are resistant in Fig. 1. There are two peaks at 16 and 19 antibiotics, representing inducible and constitutive MLS resistances, respectively. Among the 43 strains with inducible resistance, however, strains 4918, 4920 and 4921 are phenotypically differentiated from the others: strains 4918 and 4920, $EM^rCAM^rJM^r LCM^rMKM\text{-}B^s$; strain 4921, $EM^rCAM^rJM^s LCM^rMKM\text{-}B^s$ (Table 4). The typical inducible resistance, which was later explained by a translational attenuation mechanism[6], requires the coexistence of inducers to be expressed against noninducer MLS antibiotics (Table 5). However, strains 4918, 4920 and 4921, which were resistant to EM, JM and LCM but not MKM-B, became resistant to MKM-B when the strains were grown in the presence of EM, JM or LCM. Furthermore, MKM-B resistance did not require the coexistence of inducers (Table 6).

**Table 4.** Type distribution of MLS resistance in the MRSA strains

| Phenotype | Type of resistance | Number of strains |
|---|---|---|
| $EM^rCAM^rJM^rLCM^rMKM\text{-}B^r$ | Constitutive | 26 |
| $EM^rCAM^rJM^sLCM^sMKM\text{-}B^s$ | Inducible (typical)[a] | 40 |
| $EM^rCAM^rJM^rLCM^rMKM\text{-}B^s$ | JM-Inducible (newly discovered)[b] | 3 |
| | Total ---------- | 69 |

[a] When inducible MLS resistance is a typical one, the MRSA strains are resistant to 14-membered macrolides (e.g., EM, CAM), but susceptible to 16-membered macrolides (e.g., JM), lincosamides (e.g., LCM) and streptogramin B-type antibiotics (e.g., MKM-B).
[b] JM-inducible-type MRSA strains are resistant to 16-membered macrolides and lyncosamides as well as 14-membered macrolides.

**Table 5.** Characterization of typical inducible-type MLS resistance

| Strain | Concentration of EM ($\mu$g/ml) | | MIC of JM ($\mu$g/ml) |
|---|---|---|---|
| | seed culture[a] | assay culture[b] | |
| 4764[c] | 0 | 0 | 3.13 |
| | 5 | 0 | 3.13 |
| | 0 | 10 | 200 |

[a] Strain 4764 was grown in MHB or MHB supplemented with 5 $\mu$g/ml EM at 37°C for 20 hours.
[b] The MIC of JM was measured by the broth microdilution method in MHA or MHA supplemented with 10 $\mu$g/ml EM at 37°C for 20 hours.
[c] The MIC of EM is 100 $\mu$g/ml.

**Table 6.** Characterization of newly discovered JM-inducible-type MLS resistance

| Strain | | MIC ($\mu$g/ml) | | | |
|---|---|---|---|---|---|
| | | EM | CAM | JM | MKM-B |
| 4918 | uninduced | >100 | >100 | 25 | 12.5 |
| | JM-incuded[a] | >100 | >100 | >100 | >100 |
| 4920 | uninduced | >100 | >100 | 50 | 12.5 |
| | JM-induced[a] | >100 | >100 | >100 | 100 |
| 4921 | uninduced | >100 | 100 | 1.56 | 6.25 |
| | JM-induced[a] | >100 | >100 | >100 | >100 |
| *S. aureus* 209P | | 0.20 | 0.10 | 0.39 | 3.13 |

[a] The MRSA strains were grown in MHB supplemented with 3 $\mu$g/ml JM at 37°C for 20 hours.

## DISCUSSION

MRSA strains harbor *mec*A gene coding for low-affinity PBP (PBP 2') responsible for their intrinsic resistance to β-lactams[5,18,22], whereas the β-lactamase is thought to contribute to borderline resistance to β-lactams. On the other hand, the regulatory function of β-lactamase plasmid in the induction of PBP 2' was reported[17,24]. The PBP 2' was found to be inducible in the presence of β-lactam antibiotics in strains that carry β-lactamase plasmid, and constitutive in β-lactamase-negative strains. These findings could be explained by the hypothesis that the repressor of β-lactamase gene might also act on *mec*A gene. In fact, low level resistance to DMPPC (MICs, 6.25 - 12.5 μg/ml) was exclusively shown by 4 β-lactamase-positive strains. When induced by DMPPC, these 4 strains became resistant to the antibiotic to the same extent as the other strains (MICs, ≧100 μg/ml).

The incidence of tetracycline and fluoroquinolone resistances increased simultaneously since April 1990[19]. 64 strains were resistant to both groups of antibiotics and one strain was susceptible to both. The remaining 4 strains resistant to fluoroquinolones but not tetracyclines were all β-lactamase-positive strains. It is to be expected that the gene coding for β-lactamase plays a crucial role in the selective expression of fluoroquinolone resistance as in the case of DMPPC resistance.

The resistance to a number of structurally unrelated drugs are reported to be conferred by active-efflux transporter proteins such as *Bacillus subtilis* Bmr[15], Tet K in *S. aureus*[16], Tet A, B and C in gram-negative bacteria[11] and NorA responsible for fluoroquinolone resistance in *S. aureus*[8,23]. In the reference strain with a $TC^rMINO^s$ phenotype (probably expressing Tet K determinant), TC resistance was reversed by verapamil dose-dependently. However, strain 4764 with a $TC^rMINO^r$ phenotype (Tet M-like determinant) remains resistant to TC even at the highest nontoxic concentration of verapamil. Concerning OFLX resistance, it was not affected by verapamil as in the case of TC resistance irrespective of a tetracycline-resistance phenotype, $TC^rMINO^r$ (64 strains) or $TC^sMINO^s$ (4 strains). Based on these findings, the resistance to tetracyclines or fluoroquinolones in the MRSA strains isolated during the period covered by the present study is not attributable to membrane-transport mechanism.

In the MRSA strains with a typical inducible MLS resistance, cells induced by growth at a low dose of EM will not grow when transferred to medium supplemented with JM at the concentrations not less than MIC. They will grow, however, in the same JM concentrations when the medium is also supplemented with high dose of EM[20], since the continuous translation of mRNA for the ribosomal RNA methylase requires the interaction of EM and ribosome (translational attenuation). Strains 4918, 4920 and 4921 were resistant to EM, CAM, JM and LCM but not to MKM-B. The resistance to MKM-B is induced by either EM, JM or LCM and the strains remain resistant to MKM-B after removal of inducers, not requiring the interaction of inducer and ribosome. Although the detailed mechanism for the newly discovered inducible MLS resistance has not yet been established, it might be regulated transcriptionally instead of translational attenuation mechanism for the typical one.

In the previous study[7], a single strain with a significant resistance to all aminoglycoside antibiotics including ABK (therefore referred to as the $ABK^r$ strain) was isolated in April 1991. In the present study, however, the further spread of this strain into a clinical environment could not be recognized.

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