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Characteristic expression of three genes, *msr*(A), *mph*(C) and *erm*(Y), that confer resistance to macrolide antibiotics on *Staphylococcus aureus*

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Abstract

We have reported that the gene *mph*(C) (formally referred to as '*mphBM*') is located on plasmid pMS97 342 bp downstream of the *msr*(A) gene. *msr*(A) specifies resistance to macrolides by ABC-transporter-mediated efflux, and *mph*(C) has 49% identity to the amino acid sequence of MPH(2′)II, which encodes a phosphotransferase that inactivates some macrolide antibiotics. A strain of *Staphylococcus aureus* NCTC8325 containing plasmid pMS97 inactivated unlabeled and ^{14}C-labeled erythromycin when tested by bioautographic and radioautographic techniques. In addition to erythromycin, other 14-membered ring macrolides (except for ketolides), 15-membered ring macrolides and 16-membered ring macrolides, mycinamicin, rosamicin and YM133, were inactivated by the strain. Erythromycin inactivation products produced by the strain carrying pMS97 were completely different from those produced by *Escherichia coli* BM694 bearing plasmid pAT63, which contains the *ereA* gene encoding an esterase that hydrolyzes macrolide lactones. Constructs formed with the *msr*(A) and *mph*(C) genes, and with the *msr*(A), *mph*(C) and *erm*(Y) genes, showed erythromycin-inactivating activity, but another construct built with the *mph*(C) gene alone failed to show such activity. This result suggests that any region of the *msr*(A) gene is needed for the expression of *mph*(C).
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Keywords: *Staphylococcus aureus*; Macrolide antibiotics; Resistance; MLS antibiotics; Erythromycin; *msr*; *mph*; *erm*; Inactivation

1. Introduction

Macrolide antibiotics have been widely used for treatment of bacterial infections and many clinical isolates of *Staphylococcus aureus* strains resistant to macrolides have been reported [1]. It is well known that there are three resistant mechanisms to macrolide antibiotics in staphylococci: modification of the ribosomal target by a methylase mediated by *erm* genes [2,3], macrolide-specific efflux mechanism by the *msr*(A) gene [4,5], and macrolide inactivation by a gene concerned in antibiotics inertness [6–9].

We have reported that three genes encoding resistance to macrolide–lincosamide–streptogramin B antibiotics (the so-called MLS antibiotics) are located on a single plasmid, pMS97, present in *S. aureus* [10]. They encode ABC-transporter-mediated efflux, inactivation and methylation of 23S rRNA, and have been determined to be arranged in this order. The gene encoding inactivation on plasmid pMS97, *mph*(C), has 49% identity with the amino acid sequence of MPH(2′)II, which is encoded by *mphB* from *Escherichia coli* [11], but no identity with the amino acid sequence of the macrolide esterase encoded by *ereA* or *ereB* from the same strain. The present investigation was conducted to examine the mechanism of regulatory expression of the *mph*(C) gene on plasmid pMS97 by use of a number of constructs built with the *mph*(C) gene alone and together with other genes.

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2. Materials and methods

2.1. Bacteria, plasmids and media

The bacterial strains and plasmids used in this study are described in Table 1. Base agar medium for the assay of inactivation consists of brain heart infusion (BHI; Becton Dickinson, USA) containing 50 mM HEPES (*N*-(2-hydroxyethyl) piperazine-*N'*-3-propanesulfonic acid), 1% of yeast extract, 0.04% of glucose and 1.5% of agar. Soft agar medium had the same composition as the base agar except for substitution of 0.7% of agar for 1.5%. NBY-M medium consists of nutrient broth (NB; Becton Dickinson, USA) containing 0.2% yeast extract and 50 mM MOPS (3-morpholinopropanesulfonic acid; Dojindo Lab., Japan), pH 7.0.

2.2. Antibiotics

Erythromycin (EM), oleandomycin (OL), spiramycin, clindamycin hydrochloride (CLDM) and lincomycin were obtained from Japan Upjohn Co.; azithromycin was from Pfizer Taito Co.; mycinamicin I (MCM) and rokitamycin (RKM) were from Asahi Kasei Co.; roxithromycin, clarithromycin and telithromycin were from Aventis Pharm Ltd.; tylosin and CP were from Sigma; rosamicin was from Yamanouchi Pharmaceutical Co. Ltd; and YM133 was from Mercian Co. Mikamycin B (MKM-B), a streptogramin type B antibiotic, was prepared as described in a previous paper [12]. [*N*-methyl-^{14}C]EM ([^{14}C]EM; 54.0 mCi mmol^{-1}) was obtained from E.I. du Pont de Nemours and Co. Other chemicals were purchased from commercial sources.

2.3. Antibiotic susceptibility

Minimum inhibitory concentrations (MICs) of MLS antibiotics were determined by the serial two-fold dilution method on Mueller–Hinton agar plates according to the standard method recommended by the Japan Society of Chemotherapy [13].

2.4. Assay for resistance pattern

The disk diffusion test was applied to the inducibility of resistance by EM and OL to the other MLS drugs as described previously [14]. The amount of drug on each disk was as follows: EM and OL, 10 μg; RKM and MCM, 20 μg; CLDM, 5 μg; and MKM-B, 30 μg.

2.5. Inactivation of EM

Assay for EM inactivation: after overnight culture in NBY-M, cells were harvested by centrifugation at 10 000 × *g* for 10 min at 4°C. The precipitated cells were resuspended in the same fresh medium containing 10 μg ml^{-1} EM and adjusted to a density of 0.1 at OD_{620}. Following incubation for 24 h at 37°C, the incubation mixture was passed through a 0.45-μm nitrocellulose membrane filter. Eighty microliters of filtrate, corresponding to 800 ng of EM before inactivation, was impregnated onto a paper disk of 8-mm diameter, and the disk was dried.

Table 1
Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics[a]	Source or reference
S. aureus		
NCTC8325	Susceptible to MLS	[19]
RN4220	Restriction-deficient mutant of *S. aureus* NCTC8325	[20]
8325(pMS97)	MLS-resistant strain NCTC8325 transformed with plasmid pMS97	[10]
M. luteus		
ATCC9341	Indicator strain for a drug-susceptibility test	ATCC[b]
E. coli		
BM694(pAT63)	EM-resistant strain due to degradation of the drug	[7]
JM109	Host strains of the shuttle vector pND50	[21]
XL-10-Gold	Host strain of pT7Blue T vector	Stratagene, USA
Plasmid		
pT7Blue T	Cloning vector of PCR, Ap^r	Novagen, USA
pUC118	Vector for cloning of the fragment of plasmid pMS97 by *Pst*I. Ap^r	[10]
pMS97	Plasmid carrying *msr*(A), *mph*(C) and *erm*(Y) genes	[12]
pND50	Cm^r: shuttle vector *S. aureus*/*E. coli*	[22]
pND501	pND50+3096-bp insert, (*msr*(A))	this study
pND502	pND50+2963-bp insert, (*mph*(C))	this study
pND5012	pND50+3778-bp insert, (*msr*(A), *mph*(C))	this study
pND5013	pND50+4548-bp insert, (*msr*(A), *mph*(C) and *erm*(Y))	this study
pND50A	pND50+12563-bp insert, (*msr*(A), *mph*(C), *erm*(Y) and other ORF regions)	this study

[a]MLS, macrolide–lincosamide–streptogramin B antibiotics; Cm^r, chloramphenicol resistance; Ap^r, ampicillin resistance.
[b]ATCC, American Type Culture Collection.

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Table 2
Sequences of construct-specific primers

Constructs[a]	Primers	Oligonucleotide sequence (5′ to 3′)	Position[b]	Product (bp)
pND501	Forward	GAAATAAATATGTGGAAACAGG	5117–5138	3096
pND501	Reverse	ATTTCACCTCCAGCTTTACC	8193–8212	
pND502	Forward	ACAACCGACAGTATGAGTGG	5932–5951	2963
pND502	Reverse	ATCTTTTTTGTTCATTATAA	8875–8894	
pND5012	Forward	GAAATAAATATGTGGAAACAGG	5117–5138	3778
pND5012	Reverse	ATCTTTTTTGTTCATTATAA	8875–8894	
pND5013	Forward	GAAATAAATATGTGGAAACAGG	5117–5138	4548
pND5013	Reverse	GGACTGCCTAGATGGCAATC	9645–9664	

[a]Constructs containing the genes of macrolide resistance are illustrated in Fig. 1.
[b]The positions of primers are based on their location in the sequence deposited with accession number AB092817.

Ten milliliters of soft agar containing *Micrococcus luteus* ATCC9341 (OD_{620} of 0.03), as an indicator microorganism, was poured onto a plate in which 40 ml of base agar had previously been allowed to harden. The impregnated disks were laid on top of the plate and incubated at 37°C for 18–20 h.

Radioautograms and bioautograms of EM: a fresh culture of test strain in BHI (2×10^{10} cfu) was washed with TMK buffer (100 mM Tris–HCl, 10 mM $MgCl_2$, 60 mM KCl and 6 mM 2-mercaptoethanol, pH 7.8) [15]. The precipitated cells were resuspended in 100 μl of the same buffer containing [^{14}C]EM, which was diluted to give 10 μg ml^{-1} (corresponding to 1 585 dpm μl^{-1} at a counting efficiency of 94%) with TMK buffer, and incubated at 37°C for 63 h. The culture was centrifuged after incubation, and 10 μl of supernatant was spotted onto chromatography paper (200 mm × 6 mm; 3MM CHR, Whatman), and chromatographed with a solvent system consisting of chloroform–methanol–aqueous ammonia (9:1:0.1). After development, each strip was cut vertically into two equal parts. One of the strips was tested as a bioautogram with *M. luteus* ATCC9431 as described above. The other was divided into 20 parts from the bottom and radioactivity was counted in a liquid scintillation counter.

2.6. Plasmid construction of resistance gene(s)

All DNA manipulations, including digestion with the indicated restriction enzymes, ligation and agarose gel electrophoresis, were carried out by standard procedures [16]. All the gene fragments used for cloning, except for fragment A (12563 bp), were obtained by polymerase chain reaction (PCR) using the primers listed in Table 2. All primers were synthesized on the basis of the sequence deposited with accession number AB092817.

The PCR reaction mixture consisted of 2.5 U of *Taq* polymerase, 500 nM of each primer, all four dNTPs at 200 mM each, 1.5 mM $MgCl_2$, 20 mM Tris–HCl, pH 8.4, and 50 mM KCl. Plasmid pMS97 as template DNA was added to the PCR reaction mixture and then it was adjusted to a total volume of 100 μl with distilled water. PCR reaction was performed in a 9600 thermal cycler (Perkin Elmer, Japan) with 165 s of denaturation at 94°C, 30 cycles of 45 s at 94°C, 30 s at 55°C and 45 s at 72°C, and 10 min of extension at 72°C. The amplified DNA fragment was cloned into the pT7Blue T vector. These recombinant plasmids were digested with *Xba*I and *Bam*HI, and were ligated into the same site of pND50. The resultant plasmid was transformed into *S. aureus* RN4220 by electroporation [17], and transformants were selected with EM and CP. The constructed plasmid with fragment A was prepared by ligation of the largest fragment of plasmid pMS97 digested with *Pst*I with pND50.

2.7. Sequencing and DNA analysis

The largest fragment A of plasmid pMS97 digested with *Pst*I was sequenced on an automatic sequencer 377 (Applied Biosystems, Foster City, CA, USA) using the dye termination method. The sequence data were analyzed with the BLAST program. The nucleotide sequence data reported in this paper have been deposited in DDBJ under accession number AB092817.

3. Results and discussion

3.1. DNA sequencing of largest fragment of plasmid pMS97 after digestion with PstI

We determined the sequence of 12 563 nucleotides representing the largest DNA fragment of plasmid pMS97 after digestion by *Pst*I, which includes the previously described macrolide resistance genes *msr*(A), *mph*(C) and *erm*(Y) [18]. We found eight open reading frames (ORFs; Fig. 1) other than the three macrolide resistance genes.

3.2. Expression of macrolide resistance genes residing in the constructs

The plasmids constructed by inserting PCR products (3096, 2963, 3778 and 4548 bp) and the *Pst*I-digested fragment (12 563 bp) from pMS97 into vector pND50 were referred to as pND501, pND502, pND5012, pND5013 and pND50A, respectively (Fig. 1). They were transformed

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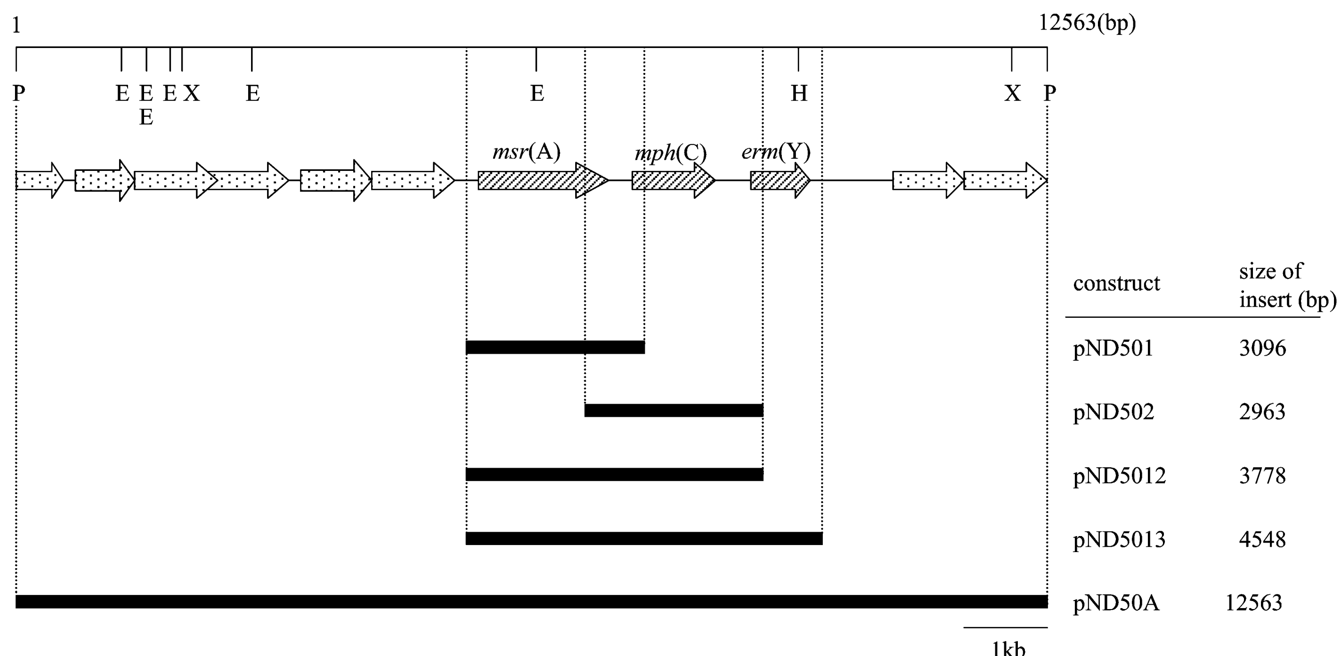

Fig. 1. Schematic representation of constructs containing fragments of genes encoding resistance to macrolides. The constructs pND501, pND502, pND5012, pND5013 and pND50A contain, respectively, *msr*(A), *mph*(C), *msr*(A)–*mph*(C), *msr*(A)–*mph*(C)–*erm*(Y), and *msr*(A)–*mph*(C)–*erm*(Y) including the extra region. The amino acid sequence deduced from the DNA sequence of fragment A (12563 bp) gives eight putative proteins (dotted arrows) in addition to three macrolide resistance regions. The cloned fragment is shown (black bars). Arrows show orientation of cloning (5′ to 3′) and approximate size of the genes of constructs. Slashed arrows show the genes of macrolide resistance: *msr*(A), *mph*(C), and *erm*(Y). Restriction sites: E, *Eco*RI; H, *Hpa*I; P, *Pst*I; X, *Xba*I.

into *S. aureus* RN4220 by electroporation. The transformants were tested for susceptibility to MLS antibiotics in terms of disk diffusion and determination of MIC. Fig. 2 shows susceptibility patterns to MLS antibiotics in the representative transformants (*S. aureus* RN4220 containing pND501, pND502 and pND50A). Strain *S. aureus* 4220(pND501) formed 'D-shaped' inhibition zones around both MCM and MKM-B disks because EM and OL induced resistance to MCM and MKM-B as a result of the presence of the *msr*(A) gene (formally referred to as '*msrSA′*') [10] (Fig. 2A). A similar phenotypic profile was obtained in *S. aureus* 4220(pND5012) despite the presence of *mph*(C) in the construct plasmid. *S. aureus* 4220(pND502) produced a circular inhibition zone around all disks (Fig. 2B) similar to those of the host strain RN4220. If inactivation of any macrolide antibiotics by the product encoded by *mph*(C) residing on plasmid pND502 were to occur, no inhibition zone would appear around the corresponding disks. We could find no differences in the size and shape of inhibition zone between these two strains (*S. aureus* RN4220 and *S. aureus* 4220(pND502)). *S. aureus* 4220(pND50A) shows inducible resistance to RKM, MCM, CLDM, and MKM-B by EM, and its resistance to those antibiotics except for RKM and CLDM is also induced by OL. This suggests that, in the presence of a low concentration of EM, the *erm*(Y) gene could be expressed at the highest efficiency of the three genes (Fig. 2C and Table 3). In addition, *S. aureus* 4220(pMS97) and *S. aureus* 4220(pND5013) also exhibited the same disk pattern as *S. aureus* 4220(pND50A). In our previous paper [18], we reported that *S. aureus* RN4220 containing the constructed plasmid pND503 with *erm*(Y) alone showed exemplary EM-inducible resistance to MLS antibiotics.

The MICs of MLS antibiotics were determined in all strains containing the constructs used in these studies (Table 3). All of these strains, containing any of the constructed plasmids, showed approximately the same susceptibility (MIC determined after 18–20 h incubation) to MCM, RKM, CLDM and MKM-B as the host strain *S. aureus* RN4220. The strains containing the constructed plasmids, except for *S. aureus* 4220(pND502), displayed high MICs for EM and OL approximately similar to those of *S. aureus* 4220(pMS97). However, strains *S. aureus* 4220(pND50A) and *S. aureus* 4220(pND5013), which bear the three genes *msr*(A)-*mph*(C)-*erm*(Y), exhibited MICs for EM (400 μg ml^{-1}) and OL (50 μg ml^{-1}) particularly close to those of *S. aureus* 4220(pMS97) (800 μg ml^{-1} for EM and 50 μg ml^{-1} for OL). This appears to indicate a phenotypic precedence of the *erm*(Y) gene over the other genes, *msr*(A) and *mph*(C), as illustrated by the results in panels A and C in Fig. 2. *S. aureus* 4220(pND501), which has only a single resistance gene, *msr*(A), showed resistance to EM and OL of 100 μg ml^{-1}. On the contrary, *S. aureus* 4220(pND502), which has the single *mph*(C) gene, showed the same susceptibility to EM (0.39 μg ml^{-1}) and OL (1.56 μg ml^{-1}) as the host strain *S. aureus* RN4220. This suggests that *mph*(C) alone

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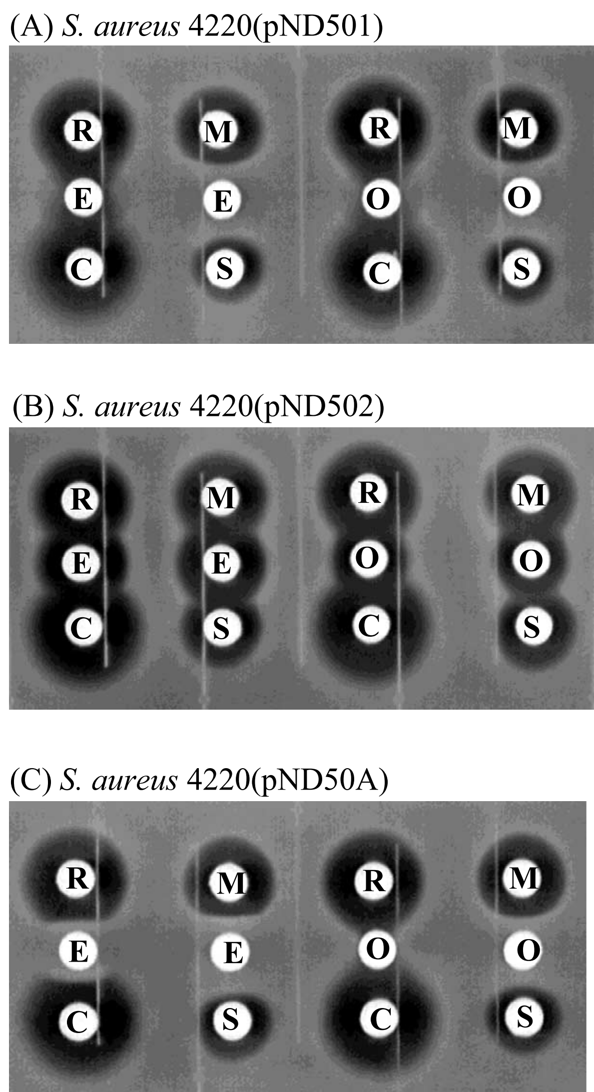

Fig. 2. Susceptibility patterns to MLS antibiotics in the indicated transformants harboring the constructs. A: *S. aureus* 4220(pND501) showed inducible phenotypic resistance. B: *S. aureus* 4220(pND502) showed susceptibility to all drugs. C: *S. aureus* 4220(pND50A) exhibited inducible phenotypic resistance (see the text for details). Disks impregnated with the indicated antibiotics were laid on plates seeded with the indicated strains. The amount of drug on each paper disk was as follows (μg per disk): E, erythromycin, 10; O, oleandomycin, 10; R, rokitamycin, 20; M, mycinamicin, 20; C, clindamycin, 5; S, mikamycin B, 30.

appears insufficient to express resistance to the drugs. This observation of the null-like expression of the *mph*(C) gene was further supported by the fact that *S. aureus* 4220(pND501), bearing only *msr*(A) and *S. aureus* 4220(pND5012), bearing *msr*(A) and *mph*(C), attained approximately the same MICs for EM (100 μg ml^{-1}) and OL (100 μg ml^{-1}).

3.3. Inactivation assay

Inactivation of [^{14}C]EM by *S. aureus* 8325(pMS97) was examined by radioautogram and bioautogram assays. ^{14}C-labeled EM was chromatographed with the indicated solvent on Whatman 3MM chromatography paper, and the developed chromatogram was split into half (each part 3 mm in width). Radioautography of one half of the paper showed a single peak of radioactivity at 15 cm from the origin (illustrated graphically in Fig. 3). When the other half of the paper was assayed by bioautography using *M. luteus* as the indicator microorganism, we detected antibiotic activity at the same position as the radioactivity (panel A in Fig. 3). In contrast, a supernatant sample from a mixture of *E. coli* BM694(pAT63) and [^{14}C]EM incubated for 63 h gave one peak at a position of about 13 cm, but antibacterial activity in the corresponding position had been lost (panel B in Fig. 3). Similarly, a supernatant sample from a mixture of *S. aureus* 8325(pMS97) and [^{14}C]EM incubated for 63 h gave at least two peaks at 0 and 5 cm from the origin. In fact, the 24-h supernatant from the same mixture gave three peaks (data not shown). The transient peak was seen at about 3 cm from the origin, and was gradually lost with time with a corresponding increase in the more mobile peak at 5 cm. These results suggest that the inactivation products produced by the *mph*(C) gene are greatly different from those produced by the *ere* gene from *E. coli* BM694(pAT63).

We further examined *mph*(C)-dependent inactivation of a range of MLS antibiotics, including 14-, 15- and 16-membered macrolides, to determine the spectrum of drugs capable of being inactivated by Mph(C) (Table 4). Inactivation (%) was calculated from the activity of residual drug after incubation with *S. aureus* 8325(pMS97) or

Table 3
MICs of MLS antibiotics against *S. aureus* carrying constructed plasmids

S. aureus	Antibiotics (μg ml^{-1})					
	EM	OL	MCM	RKM	CLDM	MKM-B
RN4220	0.20	1.56	0.20	0.39	0.05	6.25
4220(pMS97)	800	50	0.39	0.39	0.05	6.25
4220(pND50A)	400	50	0.78	0.39	0.05	12.5
4220(pND501)	100	100	0.39	0.39	0.05	6.25
4220(pND502)	0.39	1.56	0.39	0.39	0.05	6.25
4220(pND5012)	100	100	0.39	0.39	0.05	6.25
4220(pND5013)	400	50	0.78	0.39	0.05	12.5

Abbreviations: EM, erythromycin; OL, oleandomycin; MCM, mycinamicin; RKM, rokitamycin; CLDM, clindamycin; MKM-B, mikamycin B.

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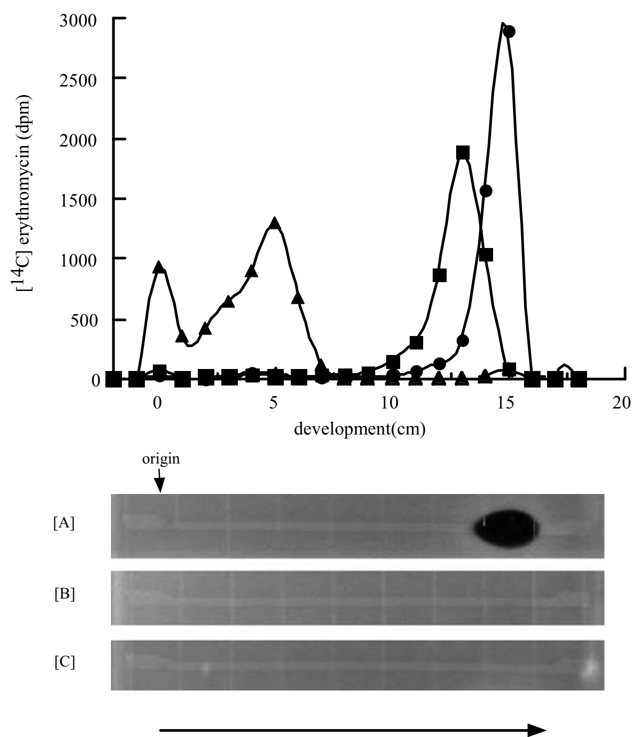

Fig. 3. Radioautogram and bioautogram of [^{14}C]EM (closed circles and panel A) and of [^{14}C]EM incubated for 63 h at 37°C with *E. coli* BM694(pAT63) (closed squares and panel B) and *S. aureus* 8325-(pMS97) (closed triangles and panel C). The direction of development in a solvent system of chloroform–methanol–aqueous ammonia (34.2%) (9:1:0.1) is indicated by arrows.

E. coli BM694(pAT63), as represented by the decrease in the area of the inhibition zone. *S. aureus* 4220(pMS97), coding *msr*(A), *mph*(C) and *erm*(Y), inactivated 14-membered, 15-membered and some 16-membered (MCM, rosamicin and YM133) macrolides. On the other hand, *E. coli* BM694(pAT63), coding the *ereA* gene whose product hydrolyzes the lactone ring of macrolides, inactivated 14-membered macrolides, except for telithromycin which is a type of ketolide, and 15-membered macrolides. Neither strain inactivated lincosamide or MKM-B (belonging to the streptogramin B).

In order to more clearly understand the conditions under which the *mph*(C) gene functions, EM was incubated with the indicated strains carrying the constructed plasmids for 24 h at 37°C and the residual activity of EM present in the filtrate was determined by disk diffusion assay (data not shown). Host strain *S. aureus* RN4220 and strain *S. aureus* 4220(pND502) did not inactivate EM. The other strains, including strain *E. coli* BM694(pAT63), inactivated EM. This observation suggests that any region of the *msr*(A) gene is needed for the expression of the *mph*(C). Although a promoter-like sequence of *mph*(C) is present in the immediate vicinity upstream of the coding sequence, the gene alone was not expressed even in the presence of EM. There are possible explanations for our observation of the phenotypic absence of activity of the Mph(C) protein: the promoter just upstream of *mph*(C) is not recognized by staphylococcal DNA-dependent RNA polymerase; or Mph(C) protein is particularly protease-labile.

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Table 4
Comparison of the spectrum of MLS-antibiotic inactivation after about 60 h of incubation with *S. aureus* NCTC8325 containing *mph*(C) located in plasmid pMS97 and *E. coli* BM694 containing *ereA* located in plasmid pAT63

Antibiotics	Inactivation (%)[a]		
	Group[b]	*S. aureus* 8325(pMS97)	*E. coli* BM694(pAT63)
Erythromycin	A	100	100
Oleandomycin	A	100	100
Roxithromycin	A	32 (100)[c]	100
Clarithromycin	A	100	100
Telithromycin	A	100	4
Azithromycin	B	54 (66)[c]	100
Rokitamycin	C	6	13
Mycinamicin	C	53	6
Spiramycin	C	8 (12)[c]	17
Tylosin	C	12	4
Rosamicin	C	100	0
YM133	C	63	8
Clindamycin	D	6	3
Lincomycin	D	5	4
Mikamycin B	E	0	0

[a]Extent of inactivation (%) is expressed as the ratio of decrease in amount of drug activity (i.e. difference between initial drug activity and residual drug activity after incubation) to the initial amount of drug before incubation with bacteria.
[b]Groups A–E show 14-, 15- and 16-membered macrolides, lincosamide and streptogramin B antibiotics, respectively.
[c]Inactivation rates in parentheses were determined after resistance of *S. aureus* 8325(pMS97) was induced by a small amount of EM (0.05 μg ml^{-1}).

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