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Correlation between Regulation of *mecA* Transcription and Expression of Methicillin Resistance in Staphylococci

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Total RNA was used to study the effect of penicillinase plasmid pI524 and of *mecR*, the regulatory region located on the methicillin resistance determinant (*mec*), on the expression of *mecA*, the gene coding for the low-affinity penicillin-binding protein PBP2′, in methicillin-resistant staphylococci. In the present report, we show that the regulation of methicillin resistance occurs primarily at the level of *mecA* transcription and that in the presence of intact plasmid pI524 or *mecR*, the gene undergoes negative control. The relative amount of *mecA* mRNA present during exponential growth in uninduced cultures matches the type of *mecA* regulation and decreases in the following order: constitutive > pI524 > *mecR*-dependent *mecA* expression. Induction of *mecA* by methicillin is faster in pI524- than in *mecR*-controlled strains. The overall mRNA half-life is similar for all strains analyzed. Our results indicate that methicillin resistance under *mecR* control in certain staphylococcal strains could escape detection by the standard disk diffusion test and broth microdilution test because of the very slow derepression of the *mecA* gene. This finding is of importance for the clinical detection of this type of methicillin resistance.

Methicillin resistance is essentially mediated by a novel penicillin-binding protein, PBP2′, which is unique to methicillin-resistant (Mc^r) staphylococci. Gaisford and Reynolds (17) have provided evidence that, under nongrowing conditions, this PBP2′ could function as a transpeptidase in cell wall synthesis. PBP2′ is characterized by its low affinity for β-lactam antibiotics. This characteristic enables Mc^r staphylococci to support cell wall synthesis at antibiotic concentrations which inhibit the growth of methicillin-susceptible (Mc^s) strains. The *mecA* gene, encoding PBP2′, resides on the methicillin resistance determinant (*mec*), which is located on the *Sma*I-G fragment of the *Staphylococcus aureus* NCTC 8325 chromosome (31).

Several methicillin resistance determinants from different *S. aureus* strains and from one *S. epidermidis* strain have been cloned and partially sequenced (3–5, 24–26, 34, 35, 37, 41, 42). A comparison of three staphylococcal *mecA* sequences revealed a high degree of conservation among different staphylococcal strains and species (37, 44). A striking feature of methicillin resistance expression in staphylococci is its complex regulation. It is known that in Mc^r staphylococci there are at least two modes of phenotypic expression of PBP2′, resulting in strains with constitutive or with inducible PBP2′ synthesis (45, 46).

The nucleotide sequence of the *mecA* promoter region shows similarities to palindrome sequences of a classical penicillinase gene. Therefore, the system seems to have the same induction system as penicillinases. On the basis of these assumptions, the *mecA* gene has been claimed to have evolved by fusion of the regulatory region of a β-lactamase with the structural gene of a penicillin-binding protein of unknown origin (38, 39). However, any conclusive information about the postulated recombinational events and the origin of *mec* is still lacking. The inducibility of PBP2′ by β-lactam antibiotics was demonstrated in certain strains (19, 27, 32, 47), and it was shown that transformation of an intact penicillinase plasmid into constitutive PBP2′ producers resulted in cells inducible by β-lactam antibiotics (41, 43; this report). These plasmids carry, among other genes, the *bla* operon, which is negatively regulated by the repressor *Bla*I. However, the inducibility of PBP2′ was also demonstrated in penicillinase-negative strains (13, 30, 32), indicating that there must be factors other than those encoded by penicillinase plasmids that are able to control the expression of PBP2′. Such a regulatory element, termed *mecR*, which is encoded by some, but not by all, methicillin resistance determinants, has been identified (20, 41). The *mecR* locus was located upstream of *mecA* in the *S. epidermidis* WT55 methicillin resistance determinant and failed to hybridize to sequences of penicillinase plasmid pI524 and to *mec* or chromosomal DNA of Mc^r *S. aureus* BB270. The *mecR* locus was shown to be responsible for the strong repression of PBP2′ synthesis and of methicillin resistance (35, 41). In the present paper, we show for the first time that the expression of PBP2′ is indeed negatively regulated at the transcriptional level and that there are at least two types of *mecA* control. Furthermore, the findings that certain Mc^r staphylococci carrying *mecR* have a strongly repressed methicillin resistance and that in these strains the induction of *mecA* is clearly delayed have repercussions for the standard clinical procedures used to detect methicillin resistance in these particular strains.

MATERIALS AND METHODS

Bacterial strains and plasmid. The *S. aureus* and *S. epidermidis* strains used in this study are listed in Table 1. Plasmid pI524 was electrotransformed into BB270.

Culture conditions and media. For induction experiments with *S. aureus* and *S. epidermidis*, a 1/100 volume of an overnight culture was used to inoculate prewarmed LB medium (23), and the culture was shaken at 37°C until an optical density at 578 nm of 0.5 was reached. The culture was subsequently split into equal volumes, and 0.5 μg of methicillin per ml was added to one portion of the culture.

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TABLE 1. Properties of *S. aureus* and *S. epidermidis* strains and the plasmid used in this study

Strain or plasmid	Relevant genotype[a]	*mecR* locus[b]	Relevant phenotype	Reference or origin
Strains				
S. aureus				
BB270	NCTC 8325 mec_{BB270}	−	Mc^r	8
BB255	NCTC 8325	−	Mc^s	8
BB565	NCTC 8325 mec_{BB565} (pI524)	−	Inducible Mc^{rc}	This study
EK876	mec_{EK876} *bla*	+	Inducible Mc^{rd}	Clinical isolate, 1985 (20)
EK710	mec_{EK710} *bla*	+	Inducible $Mc^{rd,e}$	Clinical isolate, 1978 (20)
S. epidermidis WT55	mec_{WT55} *bla*	+	Inducible $Mc^{rc,e}$	42
Plasmid pI524	*blaI-blaR*		Pc^r Cd^r	28

[a] Subscripts indicate host strains for *mec*.
[b] The presence of the regulatory locus *mecR* on the methicillin resistance determinant is indicated by +, and its absence is indicated by −.
[c] Determined on the basis of transcription analysis.
[d] Determined on the basis of phenotypic expression.
[e] Delayed.

Rifampin assay. To measure the half-life of *mecA* mRNA, we added 0.4 μg of rifampin (Boehringer Mannheim Biochemicals, Mannheim, Germany) to exponentially growing cultures, which had been grown for 1 h in the presence of 0.5 μg of methicillin per ml at 37°C. Aliquots of the rifampin-treated cells were removed at various times and chilled immediately in a dry ice-ethanol bath before further manipulations.

Nucleic acid manipulations. DNA manipulations were done as described by Maniatis et al. (23) or Ausubel et al. (2). For the extraction of total staphylococcal RNA, 8-ml aliquots of exponentially growing cell cultures were pelleted at 10,000 rpm for 3 min in a Sorvall SS34 rotor at 4°C. All of the following manipulations were done rapidly and at 0°C to prevent RNA degradation. The cell pellet was suspended by being vortexed in 700 μl of RNA extraction buffer (7.2 M urea, 1.2% sodium dodecyl sulfate, 35 mM NaCl, 15 mM EDTA, 10 mM Tris-HCl [pH 7.5]), transferred to a microcentrifuge tube containing 300 μl of 25:24:1 phenol-chloroform-isoamyl alcohol (2), and quickly frozen in a dry ice-ethanol bath. The aliquots were thawed at 65°C for 5 min, vortexed, and centrifuged at 4°C for 5 min in an SS34 rotor at 10,000 rpm. DNase I digestion and further purification steps were done as described by Ausubel et al. (2).

The relative amounts of *mecA* mRNA present in the RNA samples were determined by the slot blot method (Bio-Rad Laboratories AG, Glattbrugg, Switzerland); 1 μg of total RNA was applied to each slot, marked by the addition of blue dye to the final blotting wash. The filter was probed with the 1-kb *Xba*I-*Pst*I *mecA*-specific probe (see Fig. 4). The incorporated radioactivity was determined by Cherenkov counting. To investigate the correlation between the expression of *mecA* and the growth of *S. aureus* BB270, we inoculated prewarmed LB medium with an overnight culture (diluted 1/100) and shook the culture at 37°C. The growth curve was determined by monitoring the optical density at 578 nm. Aliquots of the culture were sampled over a 7-h period and used to purify total RNA. Samples were removed at intervals of 30 min for the first 2.5 h and then at intervals of 1 h for the next 4 h. We standardized the RNA yields by decreasing the volume of the samples collected with increasing values of the optical density measured at 578 nm.

Northern (RNA) blotting. Gel electrophoresis and Northern blotting were done as described by Staeheli et al. (40) and Gilman and Chamberlin (18) with GeneScreen Plus (NEF-976; Du Pont de Nemours S.A., Regensdorf, Switzerland) or Biodyne A (Pall, Basel, Switzerland) membranes for transfer. Hybridization was carried out as described by Church and Gilbert (14).

Biochemicals. The enzymes used for DNA and RNA manipulations were purchased from Boehringer or Pharmacia (Uppsala, Sweden), and the basic protocols recommended by the suppliers for their use were followed. Radiolabeled nucleotides were a product of Amersham Corp. (Arlington Heights, Ill.). Methicillin was purchased from Beecham Pharmaceuticals, and rifampin was purchased from Boehringer.

RESULTS

Northern blot analysis of *mecA* expression in constitutive Mc^r *S. aureus* BB270. We examined the expression of *mecA* in Mc^r *S. aureus* BB270 (referred to as $mecA_{BB270}$), which exhibits constitutive expression of PBP2′. We monitored the expression of $mecA_{BB270}$ during the growth cycle of BB270 (Fig. 1A). The level of transcription of the $mecA_{BB270}$ gene was very high throughout the exponential growth phase of the culture. mRNA synthesis reached a maximum just before the onset of the stationary growth phase, when it then sharply fell to background levels. To measure the influence of methicillin on $mecA_{BB270}$ transcription, we prepared total RNA in exponentially growing cultures, in uninduced cells, and in cells induced with subinhibitory concentrations of methicillin (0.5 μg/ml). $mecA_{BB270}$ was expressed at a high level and independent of the presence or absence of methicillin (Fig. 2).

Influence of pI524 on the expression of *mecA* in inducible Mc^r *S. aureus* BB565. *S. aureus* BB565 is essentially BB270 transformed with penicillinase plasmid pI524. By monitoring $mecA_{BB565}$ transcripts during the growth of inducible BB565 cells in the absence of methicillin, we measured a weak $mecA_{BB565}$-specific signal which decreased by the onset of the stationary growth phase (Fig. 1B). The total amount of $mecA_{BB565}$ present before induction by methicillin was remarkably lower than the corresponding amount of $mecA_{BB270}$ mRNA.

The first four lanes and the sixth lane of Fig. 3A show *mecA* transcripts detected during the course of induction by

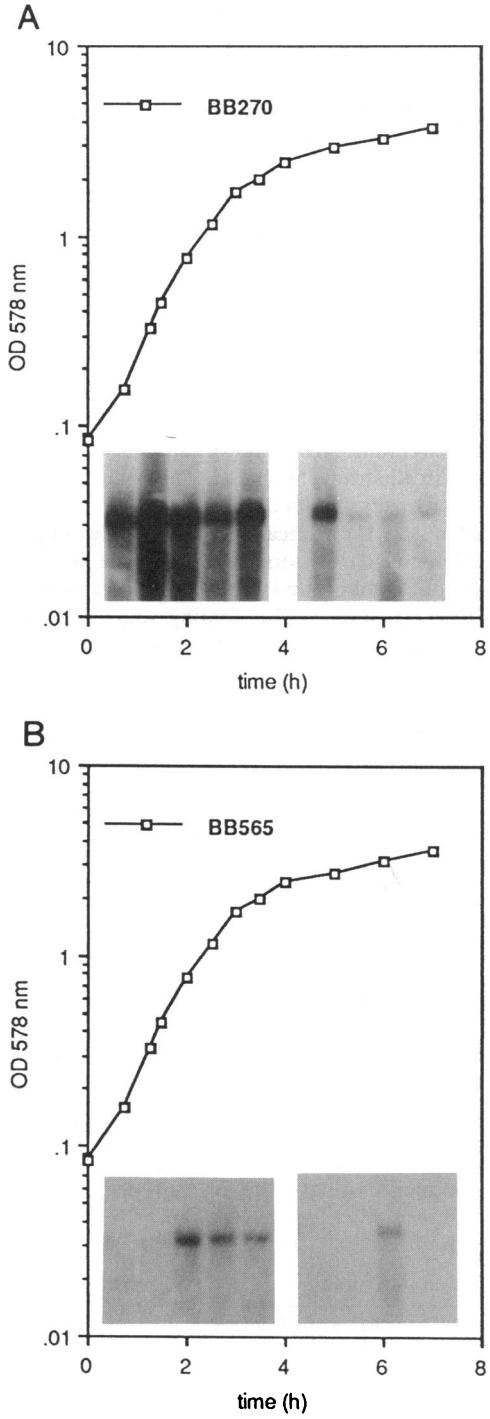

FIG. 1. Synthesis of *mecA* transcripts during the growth of *S. aureus* BB270 (A) and *S. aureus* BB565 (B). Cells were grown in LB medium at 37°C. Growth was monitored by measuring the optical density at 578 nm (OD 578 nm). Aliquots sampled at intervals of 30 min for the first five samples and then at intervals of 1 h were used to prepare total RNA, which was analyzed by Northern blotting. The samples (5 μg per slot) were hybridized to the internal 1-kb *Xba*I-*Pst*I *mecA*-specific DNA probe.

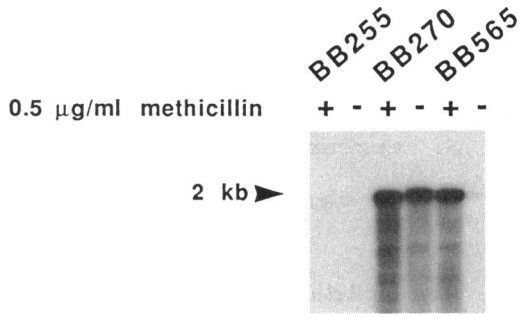

FIG. 2. Induction of *mecA* expression analyzed by Northern blotting. Total RNA (5 μg per slot) isolated from Mc^s *S. aureus* BB255 (control), Mc^r *S. aureus* BB270, and Mc^r *S. aureus* BB565 was analyzed by Northern blotting. The samples were removed from exponentially growing cultures 2 h after induction by 0.5 μg of methicillin per ml (+). As an internal control, no inducer was added to some cultures (−). The samples were hybridized to the internal 1-kb *Xba*I-*Pst*I *mecA*-specific DNA probe.

a subinhibitory concentration of methicillin. At only 10 min after induction, the levels of mRNA expression were clearly above the level of mRNA expression in the uninduced control (Fig. 3A, fifth lane). The signal reached a maximum 2 h after induction, attaining the same high level as that seen in uninduced constitutive Mc^r strain BB270 (Fig. 2).

Regulation of *mecA* transcription in inducible Mc^r *S. epi-*

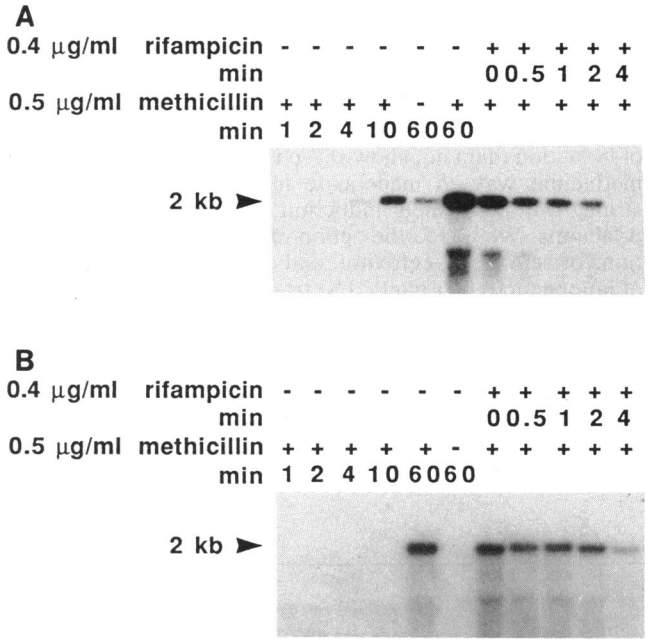

FIG. 3. Time course of *mecA* expression analyzed by Northern blotting. The appearance of *mecA* mRNA after induction by 0.5 μg of methicillin per ml was monitored. Total RNA (5 μg per slot) was analyzed by Northern blotting. From left to right, the first four lanes show the detection of *mecA* transcripts 1, 2, 4, and 10 min after induction; the fifth and sixth lanes show *mecA* mRNA in induced cells (+) and, as an internal control, in uninduced cells (−) 1 h after the addition of methicillin to the cultures; and the last five lanes show the decay of *mecA* transcripts in the presence of 0.4 μg of rifampin per ml 0, 0.5, 1, 2, and 4 min after the addition of the drug. The samples were probed with the internal 1-kb *Xba*I-*Pst*I *mecA*-specific DNA probe. (A) *S. aureus* BB565. (B) *S. epidermidis* WT55.

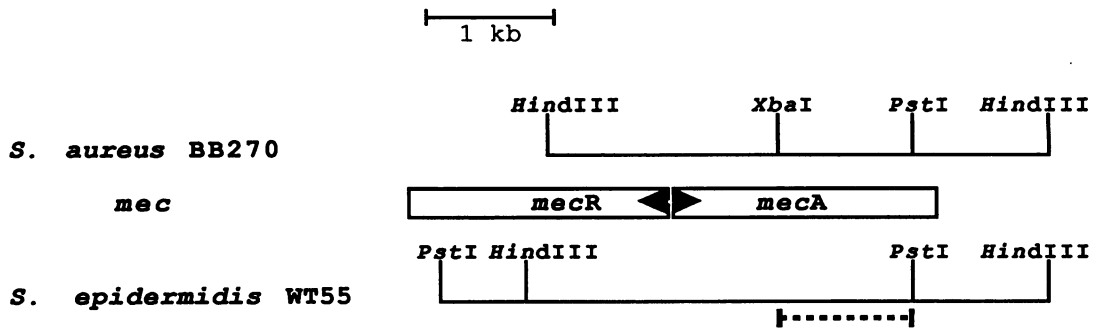

FIG. 4. Comparison of partial restriction maps of *mec* of Mc^r *S. aureus* BB270 and Mc^r *S. epidermidis* WT55. The locations of the *mecA* and *mecR* loci are indicated by boxes. The 1-kb *Xba*I-*Pst*I *mecA*-specific DNA probe used in this study is indicated by a dotted line.

***dermidis* WT55.** *S. epidermidis* WT55 is a clinical isolate carrying the methicillin resistance determinant, which bears $mecA_{WT55}$ closely linked to its own set of regulatory genes, which are encoded by the $mecR_{WT55}$ locus (Fig. 4). By Southern blot hybridization and a DNA sequence comparison, we have established that the sequence of the $mecR_{WT55}$ locus is homologous neither to penicillinase plasmid sequences nor to sequences upstream of $mecA_{BB270}$ (36, 41). This result implies that strain WT55 has a *mecA* control mechanism different from that seen in strain BB565. The time course of $mecA_{WT55}$ mRNA induction in inducible Mc^r *S. epidermidis* WT55 is shown in Fig. 3B. We could not detect any $mecA_{WT55}$ transcripts in strain WT55 during the entire growth cycle of uninduced cells (data not shown). Even after the first 10 min of induction, no signal could be measured, suggesting complete $mecA_{WT55}$ repression and delayed induction of the gene, compared with the results for pI524-controlled $mecA_{BB565}$. A weak but specific signal above the uninduced signal was detectable only after 15 min of induction (data not shown). To rule out the possibility that methicillin was an inadequate inducer for this particular strain, we performed induction experiments with other β-lactams. We tested the action of subinhibitory concentrations of cefotaxim, cefoxitin, and cloxacillin (0.5 μg/ml) and of imipenem (0.05 μg/ml). The transcripts were analyzed 2 h after induction. The results obtained reflected the same low activity that was measured with methicillin as an inducer (data not shown).

Turnover of *mecA* mRNA. The turnover of *mecA* mRNA was analyzed in *S. aureus* BB270 and BB565 and *S. epidermidis* WT55. To ensure that no additional transcription took place, we added rifampin to the cultures. The decay of the *mecA* transcripts is shown in Fig. 3A and B (last five lanes). The mRNA stemming from *mecA* had an average half-life of about 4 min in all strains analyzed. This value was confirmed by slot blot measurements as well.

Implications for the clinical detection of Mc^r staphylococci. The *mecR* locus can strongly repress the *mecA* gene in specific genetic backgrounds and thus affect the expression of methicillin resistance. This finding suggested that methicillin resistance in strains with those particular genetic backgrounds could escape standard clinical detection because of the very slow derepression of the gene. Examples supporting this hypothesis are presented in Table 2. We performed disk diffusion tests and determined the MIC of methicillin by following the guidelines of the National Committee for Clinical Laboratory Standards (29). The incubation period used in the disk diffusion tests was extended to 48 h. The values for the inhibition zones are given for 24 and 48 h of incubation. The MIC for *S. epidermidis* WT55 corresponds to that for a resistant strain, but in the disk diffusion test, Mc^r colonies appeared in the inhibition zone only after 48 h.

The inducible methicillin resistance phenotype seen in strain WT55 was observed in some but not all staphylococcal strains carrying *mecR*, as exemplified by the different behaviors found for two clinical isolates in an epidemiological study currently being done in our laboratory. Both isolates were shown to contain *mecA* and *mecR* by Southern blot hybridization. Interestingly, *S. aureus* EK710 had the same delayed methicillin resistance phenotype as strain WT55

TABLE 2. Test results for the strains used in this study

Strain	β-Lactamase[a]	*mecA*[a]	*mecR*[a]	MIC of methicillin (μg/ml) on the following medium[b] at the indicated time:				Zone diam (mm) for oxacillin at the following time:	
				CAMHB		CAMHB–2% NaCl			
				24 h	48 h	24 h	48 h	24 h	48 h
BB270	−	+	−	8	256	16	128	6	6
BB565	+	+	−	8	256	16	128	6	6
BB255	−	−	−	1	1	4	4	26	28
WT55[c]	+	+	+	16	32	32	256	21	6
EK876	+	+	+	2	4	4	8	14	6
EK710[c]	+	+	+	4	8	8	32	18	15

[a] +, presence; −, absence.
[b] CAMHB, cation-supplemented Mueller-Hinton broth (29).
[c] These strains showed growth on methicillin-containing plates only after prolonged incubation.

when tested by disk diffusion, whereas the other isolate, *S. aureus* EK876, did not (Table 2).

DISCUSSION

The staphylococcal *mecA* gene codes for PBP2′, which confers resistance to β-lactam antibiotics. The global response of certain Mc^r staphylococcal strains to methicillin is known to be dependent on environmental factors, such as pH, temperature, and osmolarity, and on the acquisition of genes controlling the resistance mechanism (7, 15). The control of gene expression could take place at any of several stages, namely, transcription, translation, and processing. We present evidence that *mecA* is regulated at the level of transcription and that its expression can be one of the following: constitutive, immediately inducible, or delayed inducible, in correlation with the presence of no regulatory elements or of penicillinase plasmid pI524 or *mecR*.

In *S. aureus* BB270 lacking β-lactamase plasmids and the *mecR* locus, $mecA_{BB270}$ is transcribed constitutively during the exponential growth phase. At the onset of the stationary growth phase, we observed a decrease in the level of detectable mRNA. The generally short half-life (3 to 4 min) of prokaryotic mRNA during exponential growth, as was observed for *mecA* as well, has been suggested to allow cells to respond rapidly to environmental changes (21). Our finding of increased *mecA* mRNA synthesis during exponential growth accounts for the necessity of PBP2′, especially in dividing cells. As soon as BB270 was transformed by penicillinase plasmid pI524, which carries the *bla* operon, $mecA_{BB565}$ became inducible by β-lactam antibiotics, and considerable amounts of transcripts were detectable immediately after induction. This result is in accordance with the hypothesis that *mecA* is regulated by a gene(s) encoded by the penicillinase plasmid. The gene(s) is probably encoded by the *bla* operon, since the *mecA* promoter has palindromic sequences similar to those of the *blaZ* promoter (33, 39). However, $mecA_{BB565}$ under the control of pI524 was not fully repressed, and a small but measurable amount of $mecA_{BB565}$ was synthesized during the mid-exponential growth phase.

In *S. epidermidis* WT55, the *mecA* gene is linked to its own regulatory locus (*mecR*), able to negatively control the synthesis of $mecA_{WT55}$ mRNA. In this strain, the regulation of $mecA_{WT55}$ is chromosomally dependent, and the gene in uninduced cells is strongly repressed. The time course of $mecA_{WT55}$ induction in strain WT55 was delayed compared with that in strain BB565. This finding was also reflected in the phenotypic expression of methicillin resistance in the two strains. Strain WT55 had a repressed methicillin resistance phenotype and developed full resistance only after 48 h of incubation on methicillin-containing plates, as opposed to strain BB565, which immediately showed full resistance. The weak methicillin resistance phenotype correlated with the presence of the *mecR* locus. The first evidence for the activity of *mecR* was reported in *S. carnosus* WT79 bearing $mecA_{WT55}$-$mecR_{WT55}$ on plasmid pWT79 (41). In this recombinant strain, we observed total repression of the methicillin resistance phenotype, due primarily to the negative control by *mecR* of the transcription of *mecA*. In agreement with this result, Tesch and coworkers (41) also detected *mecR* in *S. aureus* E67-0, which had a phenotype similar to that of WT55 (13). In addition, by using the same criterion of delayed methicillin resistance, we also detected *mecR* in clinical isolate *S. aureus* EK710 in an epidemiological survey currently being done in our laboratory. In a disk diffusion test, isolate EK710 showed the same delayed methicillin resistance expression that was observed in *S. epidermidis* WT55 and indeed carried the *mecR-mecA* locus. Staphylococcal strains with a phenotype similar to that described in the present paper have been reported by different authors (1, 16). We propose that some of these isolates bear *mecR* and show delayed induction of the methicillin resistance phenotype. It has been shown that the inducibility of PBP2′ is also dependent on the kind of β-lactam antibiotic used as an inducer (11, 30). We tested different β-lactam antibiotics to rule out a specific effect of methicillin on the induction of *mecA*. At the transcriptional level, we found no differences in the abilities of various β-lactams to induce *mecA* in our strains. The different induction efficiencies of β-lactam antibiotics must therefore be a strain-specific trait.

In addition, it has been reported that altered rates of mRNA decay are an additional means for cells to control the amount of a specific protein (6). We analyzed the overall stability of *mecA* mRNA by using the rifampin assay. However, the half-lives of *mecA* mRNA were equivalent for all the strains analyzed. The results indicate that the induction of PBP2′ is not regulated by an alteration in *mecA* mRNA stability. Furthermore, the results are in agreement with the observation that the degree of methicillin resistance expression (poorly resistant versus highly resistant strains) is not necessarily correlated with the total amount of PBP2′ present in the cells (9, 10, 12, 22).

The ability of Mc^r staphylococcal strains to escape standard clinical resistance testing is a well-known problem. We propose that the presence of the *mecR* locus in some genetic backgrounds transiently affects the expression of the resistance phenotype. Our observations indicate that the regulation of methicillin resistance is actually more complicated than presumed and is definitely dependent on the strain-specific genetic background in which the *mec* genes are embedded. More and more complex regulation pathways are beginning to be identified in Mc^r staphylococcal strains involved in the emergence of worldwide clinical infections. Major efforts are currently under way to understand the genetic and molecular nature of this resistance, and the study of these multifarious resistance mechanisms is essential to prevent confusion in susceptibility testing of staphylococci.

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