

blaI and *blaR1* Regulate β -Lactamase and PBP 2a Production in Methicillin-Resistant *Staphylococcus aureus*

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For *Staphylococcus aureus*, it is hypothesized that two genes located upstream of the β -lactamase gene, *blaZ*, are required for the inducible expression of β -lactamase. *blaR1* is predicted to encode a signal-transducing membrane protein, and *blaI* is predicted to encode a repressor protein. These same two genes may also regulate the production of penicillin-binding protein 2a (PBP 2a), a protein essential for expression of methicillin resistance. To confirm that these two genes encode products that can control both β -lactamase and PBP 2a production, *blaI*, *blaR1*, and *blaZ* with a 150-nucleotide deletion at the 3' end were subcloned from a 30-kb staphylococcal β -lactamase plasmid and three β -lactamase-negative strains of methicillin-resistant *S. aureus* were transformed with the recombinant plasmid containing that insert. The production of PBP 2a and a nonfunctional β -lactamase was detected by fluorography and by immunoblots with polyclonal antisera directed against each of the proteins. Whereas the parent strains did not produce β -lactamase and constitutively produced PBP 2a, PBP 2a and a truncated β -lactamase were now inducible in the transformants. Therefore, two plasmid-derived genes regulate the production of both PBP 2a and β -lactamase.

Resistance to β -lactam antibiotics in *Staphylococcus aureus* is primarily due to β -lactamase, an enzyme that is induced by β -lactam antibiotics (29). The β -lactamase gene (*blaZ*) is maintained on a plasmid, and two loci closely linked to *blaZ* are involved in the regulation of β -lactamase production (26, 29). Two open reading frames located upstream of and in the opposite orientation to *blaZ* have recently been sequenced and identified as *blaI* and *blaR1* (30, 38). Although the functions of the *blaI* and *blaR1* gene products have not been established, their regulatory roles have been inferred upon the basis of similarities to the genes that regulate β -lactamase production in *Bacillus licheniformis*. In that system, the chromosomally located regulatory genes *blaI* and *blaR1* are also encoded upstream of the β -lactamase gene (*blaP*), although in a reversed order (13). The deduced amino acid sequence of staphylococcal *blaI* is 37% identical and 58% similar (i.e., conservative substitutions) to *BlaI* from *B. licheniformis* (13, 30). Recombinant *BlaI* from *B. licheniformis* binds a specific operator site located upstream from *blaP* (39); by analogy, the staphylococcal *blaI* probably encodes a repressor protein that binds an operator site upstream of *blaZ*, thus preventing the RNA polymerase from binding to the promoter region and transcribing the gene. Like the *blaR1* in *B. licheniformis* (17, 18, 40), the deduced amino acid sequence of the staphylococcal *blaR1* predicts a membrane-spanning protein with an extracellular penicillin-binding domain and a cytoplasmic domain involved in signal transduction (38). This model of staphylococcal β -lactamase regulation predicts that when *BlaR1* is bound by the β -lactam antibiotic, a signal leading to *blaZ* transcription is transduced through the membrane into the cell via its cytoplasmic domain. The mechanism by which the repressor protein dissociates from the operator site, allowing *blaZ* transcription when cells are exposed to β -lactam antibiotics, is not known, but at least one additional

chromosomally derived element, *blaR2*, may be involved (35).

A second β -lactam antibiotic resistance mechanism in staphylococci is production of penicillin-binding protein 2a (PBP 2a), which is unique to methicillin-resistant staphylococci (11). Like β -lactamase, PBP 2a synthesis is induced by β -lactam antibiotics in strains containing a β -lactamase plasmid, and in most strains that have been examined, this inducibility of PBP 2a is lost when the β -lactamase plasmid is no longer present (16, 37). *mecA*, the gene encoding PBP 2a, has been cloned (23) and sequenced (34), and a region of dyad symmetry immediately upstream of the structural gene is proposed to be an operator site where a repressor protein binds. The inducibility of PBP 2a and β -lactamase and sequence similarities upstream of the *mecA* and *blaZ* structural genes suggest that the same regulatory system can control their transcription. Expression of both *blaZ* and *mecA* that is controlled at the transcriptional level has recently been demonstrated, supporting the proposed regulatory mechanism (31).

We have previously shown that *mecA* expression is regulated in *trans* by an unknown locus on a 30-kb β -lactamase plasmid (10), and *blaI* and *blaR1* are proposed to be the plasmid-encoded elements involved in the regulated expression of both *blaZ* and *mecA*. To test this hypothesis, a 3-kb fragment from this plasmid containing only *blaI*, *blaR1*, and a truncated *blaZ* was subcloned into a staphylococcal cloning vector. This recombinant plasmid was electroporated into three β -lactamase-negative strains of methicillin-resistant *S. aureus* (MRSA). Two of the three parent strains (COL and DU) constitutively produce PBP 2a, and in the third strain, 67-O, basal levels of PBP 2a are high and PBP 2a production is upregulated when the inducer is present. As predicted by the hypothesis, in all three transformed MRSA strains that contained *blaR1* and *blaI* the production of PBP 2a and β -lactamase was regulated and induced by β -lactam antibiotics.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>E. coli</i>		
HB101	Host for pBR322	4
XL-1 Blue	Host for pBluescript	Stratagene
<i>S. aureus</i>		
RN4220	Restriction-deficient MSSA ^a	20
COL	β -Lactamase-negative MRSA	24
COL ₅₅₄₂	COL with pRN5542	This work
COL ₆₃₁	COL with pCH631	This work
DU	β -Lactamase-negative MRSA	12
DU ₅₅₄₂	DU with pRN5542	This work
DU ₆₃₁	DU with pCH631	This work
67-O (β +))	67-O with β -lactamase plasmid	6
67-O (β -)	67-O without β -lactamase plasmid	6
67-O ₅₅₄₂	67-O with pRN5542	This work
67-O ₆₃₁	67-O with pCH631	This work
Plasmids		
pBR322	<i>E. coli</i> cloning vector	3
pBluescript SK ⁻	<i>E. coli</i> cloning vector	Stratagene
pRN5542	<i>S. aureus</i> cloning vector	25
pCH67-O	30-kb plasmid from 67-O	6
pCH631	pRN5542 with <i>bla</i> locus from pCH67-O	This work

^a MSSA, methicillin-susceptible *S. aureus*.

MATERIALS AND METHODS

Bacterial strains and plasmids used in this study are listed in Table 1. MRSA 67-O (β +) is a clinical isolate that contains pCH67-O, a 30-kb β -lactamase plasmid; strain 67-O (β -) has been cured of this plasmid (6). DNA manipulations were as described by Sambrook et al. (32) or Ausubel et al. (2). Chloramphenicol, ampicillin, penicillin, 2-(2'-carboxyphenyl)benzoyl-6-aminopenicillanic acid (CBAP), and lysostaphin were obtained from Sigma (St. Louis, Mo.). Restriction endonucleases and DNA-modifying enzymes obtained from GIBCO-BRL (Baltimore, Md.), New England Biolabs (Beverly, Mass.), or Promega (Madison, Wis.) were used according to manufacturers' instructions. Sodium clavulanate and [³H]benzylpenicillin were gifts of Beecham Corp. and Patrick Cassidy of Merck Corp., respectively.

Plasmid isolation and purification. For staphylococcal plasmids, log-phase organisms grown in Luria-Bertani (LB) medium were washed and suspended in 25% sucrose-50 mM Tris-1 mM EDTA (pH 8.0). After 1 h of incubation at 37°C with lysostaphin (100 μ g/ml), 0.1% Triton X-100 was added to lyse the cells and the plasmid DNA was purified by CsCl-ethidium bromide gradient centrifugation (2). To isolate plasmids from *Escherichia coli*, overnight cultures were lysed with 0.2 N NaOH-1% sodium dodecyl sulfate (SDS) and plasmid DNA was purified by CsCl-ethidium bromide gradient centrifugation (32).

Transformation of *E. coli* and *S. aureus*. *E. coli* XL-1 Blue and HB101 competent cells obtained from Stratagene and GIBCO-BRL, respectively, were transformed according to manufacturers' instructions. Transformants were selected on LB agar containing 50 μ g of ampicillin per ml. *S. aureus* strains were transformed with 0.1 to 1.0 μ g of DNA by electroporation (1). To prepare electroporation-competent cells, log-phase organisms were washed several times and

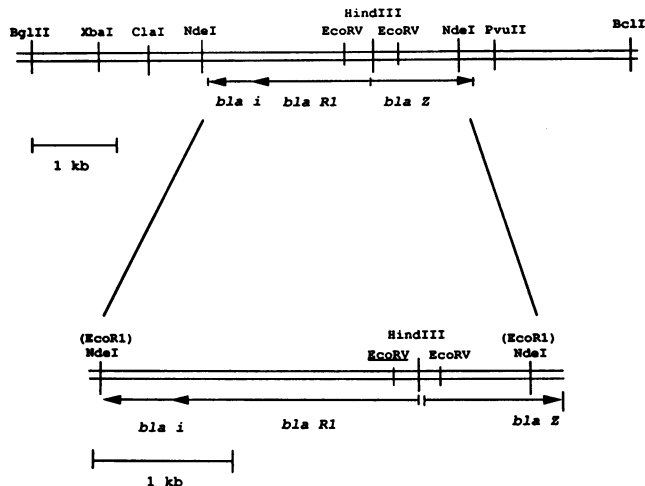


FIG. 1. A partial restriction map of pCH67-O, a 30-kb plasmid isolated from MRSA 67-O. The 3-kb *NdeI* fragment that was subcloned into *E. coli* contains *blaR1*, *blaI*, and *blaZ* with a 150-nucleotide deletion at the 3' end.

resuspended in 10% glycerol at 2×10^{10} CFU/ml and aliquots were frozen at -70°C . DNA was added to 50 μ l of cells in a 0.1-cm cuvette; electroporation parameters were 25 μ F, 10 kV/cm, and 100 Ω . Transformants were selected on B agar containing 10 μ g of chloramphenicol per ml.

Preparation of hybridization probes. An oligonucleotide specific for *blaZ* (5'-CTGTTTGAATTACATGCCT) (8) was synthesized at the Biomedical Resource Center, University of California, San Francisco, and radiolabeled at the 5' end with [γ -³²P]ATP (New England Nuclear Research Products, Boston, Mass.) with T4 kinase. A 700-bp *EcoRV* fragment that contained portions of the *blaR1* and *blaZ* genes (Fig. 1) was ligated to *EcoRI* linkers, subcloned into pBR322, and radiolabeled with [α -³²P]ATP and -TTP (NEN) by random priming (Multiprime DNA Labeling System; Amersham Corp., Northbrook, Ill.).

Southern blot hybridizations. Nucleic acids separated by agarose gel electrophoresis were transferred in 0.4 N NaOH to a nylon membrane filter (Hybond N; Amersham) (28). Hybridization (6 \times SSPE [1 \times SSPE is 0.18 M NaCl, 10 mM NaPO₄, and 1 mM EDTA {pH 7.7}]-100 μ g of heparin per ml-100 μ g of salmon testes DNA per ml-0.25% Sarkosyl-5 $\times 10^5$ cpm of probe per ml) was overnight at either 65°C (random-primed probe) or 37°C (5'-end-labeled oligonucleotide). Filters were washed twice with 6 \times SSPE-0.25% Sarkosyl (5 min at room temperature) and twice with either 0.3 \times SSPE-0.25% Sarkosyl (30 min at 65°C) for the random-primed probe or 6 \times SSPE-0.25% Sarkosyl (30 min at 37°C) for the radiolabeled oligonucleotide probe. The blots were autoradiographed with Kodak XAR-5 film.

DNA sequencing and analysis. The nucleotide sequence was determined by the dideoxy chain termination method (33). The preparation of double-stranded template DNA and the labeling reactions using Sequenase (United States Biochemical Corp., Cleveland, Ohio) and α -³⁵S-ATP (Amersham) were performed according to the manufacturers' instructions. Sequencing primers based upon an empirically determined sequence were synthesized as needed at the Biomedical Research Center. Analysis of nucleotide and deduced amino acid sequences was with software programs from DNA Strider 1.1 (22) and University of Wisconsin

Genetics Computer Group (University of Wisconsin Biotechnology Center, Madison, Wis.).

Detection of PBP 2a and β -lactamase. PBP 2a was detected by fluorography of radiolabeled membrane proteins as previously described (5). Briefly, membrane samples were prepared from exponential cultures grown in Trypticase soy broth with and without 7.5 μ M CBAP, a gratuitous inducer of β -lactamase (15) and PBP 2a. The cultures were harvested after 2 h of exposure to the inducer, and membranes were isolated by differential centrifugation. Membrane protein content was determined with Bio-Rad Protein Assay Reagent with bovine serum albumin as the standard. Membrane samples were preincubated (15 min at 37°C) with a non-radioactive β -lactam antibiotic (e.g., 10 μ g of nafcillin per ml or 200 μ g of clavulanic acid per ml) at a concentration that saturated the penicillin-binding sites of PBPs 1, 2, 3, and 4 but did not bind PBP 2a (6, 7). After preincubation with this competitive substrate, PBP 2a was specifically radiolabeled with 20 μ g of [³H]benzylpenicillin per ml.

Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (12% acrylamide–0.26% bisacrylamide) (21), and gels were stained with Coomassie blue to ensure that equal masses of protein were loaded in each sample. Relative amounts of β -lactamase and PBP 2a were determined by scanning densitometry (Quick Scan, Jr.; Helena Laboratories, Beaumont, Tex.) of Coomassie blue-stained gels or fluorographs, as previously described (6).

β -Lactamase activity was determined in whole cells with nitrocefin discs (Cefinase; BBL) and with a spectrophotometric assay using cephaloridine as the substrate (19).

Immunoblots. Affinity-purified PBP 2a (9) and β -lactamase from MRSA 67-O were used as antigens for the production of polyclonal antisera. Each protein was located and excised from a preparative SDS-polyacrylamide gel, mixed with Freund's adjuvant, and used to immunize New Zealand White rabbits. The antisera were screened with the Protoblot Alkaline Phosphatase Detection System (Promega).

RESULTS

Subcloning *blaI* and *blaRI* into *E. coli*. Upon the basis of a physical map of pCH67-O, a 7-kb *Bgl*III-*Bcl*I fragment containing the *bla* regulatory genes (Fig. 1) was gel purified (Gene-Clean; Bio 101, La Jolla, Calif.) and subcloned into the *Bam*HI restriction site of pBluescript SK⁻ to give pCH1712. This allowed the isolation of adequate amounts of DNA for subsequent cloning experiments.

The 3-kb *Nde*I fragment of pCH1712 that contained only *blaI*, *blaRI*, and *blaZ* with a 150-nucleotide deletion at the 3' end was ligated to *Eco*RI linkers and subcloned into the *Eco*RI site of pBR322 to give pCH863. The nucleotide sequence of this insert was identical to previously published *blaI*, *blaRI*, and *blaZ* sequences (30, 38).

Transformation of *S. aureus* with pCH631. The 3-kb *Eco*RI insert from pCH863 was ligated to the *Eco*RI site of the *S. aureus* cloning vector pRN5542 and electroporated into a restriction-deficient, modification-competent strain of *S. aureus*, RN4220. The recombinant plasmid isolated from this transformed strain, pCH631, was verified to contain the insert by restriction endonuclease analysis and Southern blot hybridization. The transformed strain was not resistant to penicillin, and hydrolysis of nitrocefin or cephaloridine was not detected with it, thus confirming that the inducible truncated β -lactamase was nonfunctional.

To test the hypothesis that *blaI* and *blaRI* are the plasmid-derived genes responsible for regulating the inducible ex-

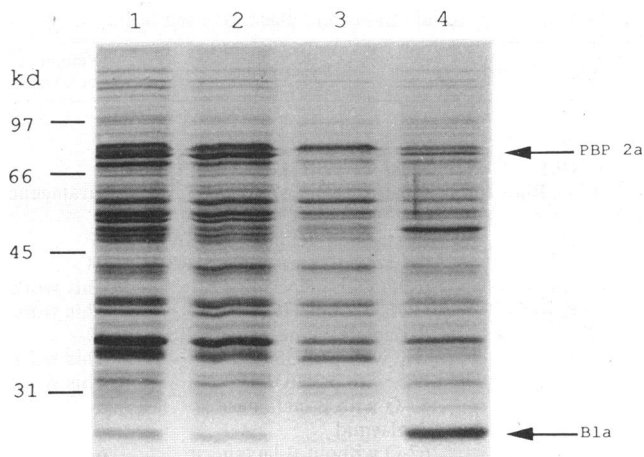


FIG. 2. Detection of inducible 76- and 26-kDa proteins in DU₆₃₁. Membrane proteins from the parent strain, DU, and the transformed strain, DU₆₃₁, were grown without (–) and with (+) CBAP, separated by SDS-PAGE, and stained with Coomassie blue. Lane 1, DU (–); lane 2, DU (+); lane 3, DU₆₃₁ (–); lane 4, DU₆₃₁ (+). Arrows indicate a 76-kDa protein (PBP 2a) and a 26-kDa protein (truncated β -lactamase) that were induced in DU₆₃₁.

pression of both PBP 2a and β -lactamase, pCH631 and the cloning vector pRN5542 were each electroporated directly into COL, DU, and 67-O, the three non- β -lactamase-producing MRSA strains.

Regulation of PBP 2a and β -lactamase expression in strains transformed with *blaI* and *blaRI*. The parent and transformed MRSA strains were examined for the presence of an inducible PBP 2a and a truncated β -lactamase. CBAP-inducible proteins were visible at 76 and 26 kDa in Coomassie blue-stained SDS-polyacrylamide gels of membrane proteins prepared from all three pCH631-transformed strains. Results for DU and DU₆₃₁ are shown as an example (Fig. 2). Whereas the parent strain DU constitutively produced a 76-kDa protein, production of this protein was repressed in DU₆₃₁ unless the strain was grown in the presence of the β -lactam inducer. On the basis of its low-affinity binding of β -lactam antibiotics, this inducible 76-kDa protein was shown by fluorography to be PBP 2a (Fig. 3). When the MRSA strains were transformed with pRN5542, PBP 2a production was the same as that in the parent strain. Therefore, the induction of PBP 2a in the pCH631-transformed strains was due to gene products derived from the 3-kb insert in the recombinant plasmid. COL₅₅₄₂ is shown as an example (Fig. 3A), and the same results were observed with DU₅₅₄₂ and 67-O₅₅₄₂.

The 26-kDa protein that was also induced in DU₆₃₁ (Fig. 2, lane 4) corresponded in size to that predicted for the truncated β -lactamase. This 26-kDa protein bound [³H]penicillin (Fig. 3) even though it did not hydrolyze β -lactam antibiotics (nitrocefin and cephaloridine).

A 50-kDa protein was also induced in DU₆₃₁ (Fig. 2, lane 4), but it did not bind [³H]penicillin, and it was not consistently detected in the transformed strains; therefore, we did not investigate it further.

Detection of an inducible PBP 2a and a truncated β -lactamase with anti-PBP 2a and anti- β -lactamase antisera. Polyclonal antisera directed against PBP 2a (Fig. 4A) and β -lactamase (Fig. 4B) also detected inducible 76- and 26-kDa proteins, respectively, in strains transformed with pCH631. Just as with the fluorograph, anti-PBP 2a antisera detected

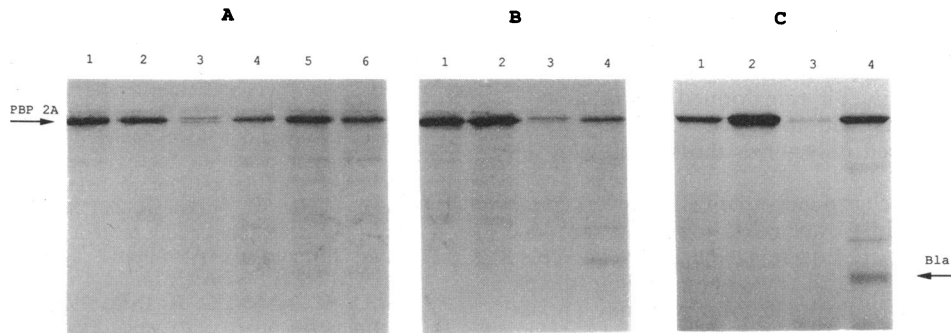


FIG. 3. Induction of PBP 2a production in the MRSA strains transformed with pCH631. Membrane proteins from bacterial cultures grown under noninducing (–) and inducing (+) conditions were radiolabeled with [3 H]penicillin as described in the text. PBPs were separated by SDS-PAGE and detected by fluorography. (A) COL strains. Lane 1, COL (–); lane 2, COL (+); lane 3, COL₆₃₁ (–); lane 4, COL₆₃₁ (+); lane 5, COL₅₅₄₂ (–); lane 6, COL₅₅₄₂ (+). Lanes 1 to 4 in panels B (DU strains) and C (67-O [β–] strains) are in the same order as for COL. The arrow indicates PBP 2a which was induced by CBAP in the pCH631-transformed strains. A truncated β-lactamase was also induced in these strains.

constitutive production of PBP 2a in DU and inducible production of PBP 2a in the transformed strain DU₆₃₁ (Fig. 4A). Although the 31-kDa functional β-lactamase was used as the immunogen, the anti-β-lactamase antisera detected an induced 26-kDa protein in DU₆₃₁ (Fig. 4B), thus confirming that the 26-kDa induced protein was a truncated β-lactamase. The other bands detected on the immunoblot were also detected with preimmune sera (data not shown), suggesting that the nonspecific antibodies were present in the rabbit from a preexposure to staphylococci.

Relative amounts of PBP 2a and β-lactamase produced in the pCH631-transformed strains increased by 3- to 14-fold when they were grown under inducing conditions, as quantitated by scanning densitometry (Table 2). The amounts of PBP 2a produced in the parent and transformed strains after induction were almost identical (80 to 130% relative densities); hence, the difference in their induction ratios was due to reduced gene expression in the transformed strains grown without an inducer. Therefore, pCH631 contains an element that is involved in negatively regulating the expression of both *mecA* and *blaZ*.

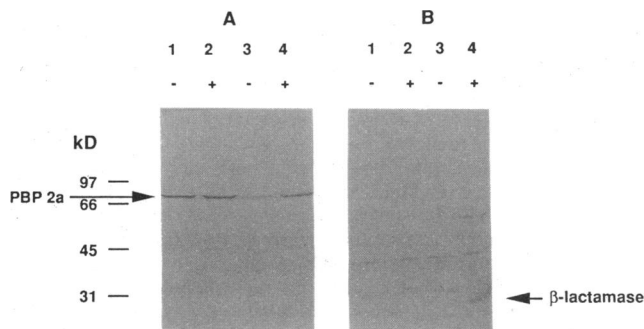


FIG. 4. Immunoblot detecting the induction of PBP 2a and a truncated β-lactamase (26 kDa) in DU₆₃₁. Membrane proteins from bacterial cultures grown without (–) and with (+) CBAP were separated by SDS-PAGE and transferred to nitrocellulose. Polyclonal antisera directed against PBP 2a (A) and β-lactamase (B) were used to detect each of these inducible proteins. Lane 1, DU (–); lane 2, DU (+); lane 3, DU₆₃₁ (–); lane 4, DU₆₃₁ (+).

DISCUSSION

These results confirmed that *blaI* and *blaR1* are required for the regulated expression of *blaZ*. When a recombinant plasmid containing *blaI*, *blaR1*, and *blaZ* (with a 150-nucleotide deletion at the 3' end) was introduced into non-β-lactamase-producing strains of *S. aureus*, there was induction of a truncated β-lactamase. The β-lactamase gene expression model predicts that after induction, the membrane protein BlaR₁ was bound by the inducer and this subsequently resulted in the dissociation of BlaI from the operator site and transcription of *blaZ*. Although *blaI* and *blaR1* are the only plasmid-derived genes essential for the induction of β-lactamase, these data do not suggest that they are the only components required for this regulatory system to function. The signaling events resulting in β-lactamase production probably involve at least one additional chromosomally derived gene product, BlaR₂ (35). This component, and perhaps others, provides the mechanism for transducing this induction signal into the cell. The specific functions of all the components in this staphylococcal signal transduction system remain to be characterized.

Previous studies have demonstrated that elements encoded on the β-lactamase plasmid are involved in the induction of PBP 2a, and it has been proposed that those elements are the β-lactamase regulatory genes. These experiments

TABLE 2. Induction of PBP 2a and β-lactamase in MRSA transformants containing pCH631^a

Strain	Induction ratio ^b for:	
	PBP 2a	β-Lactamase
COL ₆₃₁	11.3	3.4
COL	0.8	— ^c
DU ₆₃₁	6.6	13.5
DU	1.1	— ^c
67-O ₆₃₁	8.8	11.7
67-O (β–)	4.1	— ^c

^a The relative amounts of PBP 2a and β-lactamase produced were determined by scanning densitometry from fluorographs or from Coomassie blue-stained gels. Each number is an average of three measurements.

^b Ratio of density of protein band from culture grown with inducer to density of same band from culture grown without inducer.

^c β-Lactamase-negative strain.

provided the first direct evidence that *blaI* and *blaR1* are the plasmid-derived genes involved in the regulated expression of *mecA*. COL and DU are two MRSA strains that constitutively produce PBP 2a; yet in transformed strains containing a recombinant plasmid with these two regulatory genes, the protein was produced only when the strains were induced by CBAP.

Strain 67-O still produces an inducible PBP 2a when cured of its β -lactamase plasmid (6), and other strains that produce an inducible PBP 2a but are β -lactamase negative have also been identified recently (27, 36). Two open reading frames, *mecR1* and *mecI*, located upstream of *mecA* may encode proteins that can negatively regulate PBP 2a production in these strains (14). The derived amino acid sequences from *mecR1* and *mecI* are 34 and 61% homologous with the derived amino acid sequences from *blaR1* and *blaI*, respectively, strongly suggesting similar functions and/or origins for these regulatory genes (14). Although 67-O contains a *mecR* region upstream of *mecA* (36), whether it contains intact *mecR1* and *mecI* genes that encode functional regulatory proteins is not known and is currently being investigated. Basal levels of PBP 2a produced when 67-O (β^-) was grown without the inducer were higher than those found with 67-O₆₃₁ (Fig. 3C), suggesting that if *mecR1* and *mecI* are present in this strain, their gene products are not as effective as the *bla* regulatory genes at regulating the transcription of *mecA*. The complete repression of PBP 2a in 67-O₆₃₁ when the strain is grown without the inducer may be due to the high copy number of the recombinant plasmid and the therefore increased amounts of BlaI present in the cell (25), but that is unlikely since PBP 2a production in 67-O (β^+) is also repressed and this original isolate contains *blaI* and *blaR1* on a low-copy-number β -lactamase plasmid (5).

In summary, this study clearly demonstrated that the *blaI* and *blaR1* gene products regulate the expression of *blaZ* and *mecA*. Evidence with strain 67-O suggests that *blaI* and *blaR1* are the principal regulatory genes involved in PBP 2a expression even in strains that contain the *mec* regulatory genes, *mecR1* and *mecI*. Identification and characterization of the gene products that regulate *mecA* gene expression will make it possible to dissect the complex regulation of PBP 2a and determine its contribution, if any, to the expression of heterogeneous resistance in MRSA.

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