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Molecular cloning and nucleotide sequence determination of the regulator region of *mecA* gene in methicillin-resistant *Staphylococcus aureus* (MRSA)

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Molecular cloning and nucleotide sequence analysis were performed for the identification of the regulator genes of methicillin resistance in the genome of a MRSA strain N315. Two open reading frames (orfs) were identified in the 5'-flanking region of the *mecA* gene. Predicted amino acid sequences of these orfs showed extensive homology to the co-inducer and the repressor protein of the penicillinase (PCase) production in *Staphylococcus aureus* as well as in *Bacillus licheniformis*. These orfs are considered to encode putative co-inducer and repressor proteins specific for the regulation of methicillin resistance in MRSA.

mecA; Repressor; Co-inducer; Methicillin resistance; *Staphylococcus aureus*

1. INTRODUCTION

MRSA expresses methicillin resistance by producing the specific penicillin-binding protein, PBP2′, that has a decreased binding affinity to β-lactam antibiotics [1–3]. Although the structure gene, *mecA*, coding for PBP2′ is located on the chromosome [4], it is shown to be regulated by the PCase plamid which is present in most of the MRSA strains [5]. Elimination of the PCase plasmid from the cell changes the inducible mode of PBP2′ production to the constitutive one in those MRSA strains [5]. Recently, however, Tesch et al. have isolated from a clinical strain of *S. epidermidis* a genomic clone carrying the *mecA* gene, and have identified the genomic region responsible for down-regulation of PBP2′ towards the upstream of the *mecA* gene [6]. On the other hand, we have identified a group of MRSA strains, represented by a strain N315, which retains inducible production of PBP2′ even after elimination of the PCase plasmid from the cell [7]. In this study, we looked for the regulator genes of the *mecA* gene using the chromosomal DNA of N315 as a source for cloning as well as for direct genomic nucleotide sequencing.

2. EXPERIMENTAL

2.1. *Bacterial strains*

Three strains were used for nucleotide sequence determination. The stain N315 is a MRSA strain isolated in Japan in 1982, and was described previously [7]. N315P-ZR is a highly methicillin-resistant mutant of N315 [7], PBP2′ production of which is derepressed (unpublished data). MR108 is a MRSA strain whose production of PBP2′ is inducible but becomes constitutive when its PCase plamid is eliminated [8].

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2.2. *Molecular cloning*

The N315 genomic DNA library was constructed as follows. The DNA of the strain N315 was extracted as described previously [8]. DNA was partially digested with *Sau*3AI, and subjected to electrophoresis in an 0.8% agarose gel. The fragments larger than 4-kb were eluted from the gel, and ligated into the *Bam*HI-cleaved vector plasmid pACYC177. Resultant recombinant plasmids were used to transform *E. coli* MC1061. The library was screened by colony hybridization using the ^{32}P-labelled 4.0-kb *Hin*dIII insert of pMR111 as a probe. The insert of the plasmid pMR111 is a genomic DNA fragment derived from MR108, and contains the whole *mecA* gene [8]. One of the obtained clones, pES1, contained a 6.0-kb genomic fragment whose restriction map is presented in Fig. 1A.

2.3. *DNA sequencing*

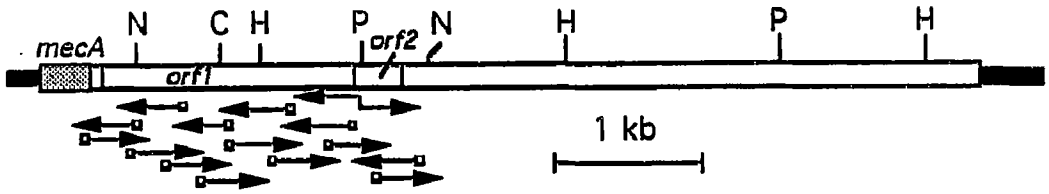
The nucleotide sequences of the 5'-flanking region of the *mecA* gene carried by the plasmids pES1 and pMR111, and the corresponding regions of genomic DNAs of N315, N315P-ZR and MR108 were determined by the dideoxynucleotide-termination method of Sanger et al. [9] using synthesized oligonucleotides as primers (Fig. 1A). In order to eliminate possible cloning artifacts represented on the plasmid clones, direct sequencing of the genomic DNAs was carried out using the PCR-amplified DNA fragments as templates for the sequence reaction after elution and purification of these by agarose gel electrophoresis.

3. RESULTS

3.1. *Nucleotide sequence of the 5'-flanking region of mecA gene*

Fig. 1A shows the sequencing strategy of plasmid pES1. Nucleotide sequence of a total of 2471 bases

A



B

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AAATTTTTATCTTCAATTGCATCAATAGTATTATTAATTTCTTTATCTTTCGAAGCATAAAAATATATACCAAACCCCGACAACTACAACTATTAAAATAAGTGGAACAATTTTTATCTTT 120
MecA                                  -35                -10          SD                orf1
TTCATCAATATCCTCCTTATATAAGACTACATTTGTAGTATATTACAAATGTAGTATTTATGTCAAAATAATGTTATAATTTTTGTGATATGGAGGTGTAGAAGGTGTTATCATCTTTTT 240
  GTA     AGGAGG                    TGATAT                ACAGTT                                        MetLeuSerSerPheL
            SD                      -10                   -35
TAATGTTAAGTATAATCAGTTCATTGCTCACGATATGTGTAATTTTTTTAGTGAGAATGCTCTATATAAAATATACTCAAAATATTATGTCACATAAGATTTGGTTATTAGTGGTCGTCT 360
euMetLeuSerIleIleSerSerLeuLeuThrIleCysValIlePheLeuValArgMetLeuTyrIleLysTyrThrGlnAsnIleMetSerHisLysIleTrpLeuLeuValLeuVals

CCACGTTAATTCCATTAATACCATTTTACAAAATATCGAATTTTACATTTTCAAAAGATATGATGAATCGAAATGTATCTGACACGACTTCTTCGGTTAGTCATATGTTAGATGGTCAAC 480
erThrLeuIleProLeuIleProPheTyrLysIleSerAsnPheThrPheSerLysAspMetMetAsnArgAsnValSerAspThrThrSerSerValSerHisMetLeuAspGlyGlnG

AATCATCTGTTACGAAAGACTTAGCAATTAATGTTAATCAGTTTGAGACCTCAAATATAACGTATATGATTCTTTTGATATGGGTATTTGGTAGTTTGTTGTCCTTATTTTATATGATTA 600
lnSerSerValThrLysAspLeuAlaIleAsnValAsnGlnPheGluThrSerAsnIleThrTyrMetIleLeuLeuIleTrpValPheGlySerLeuLeuCysLeuPheTyrMetIleL

AGGCATTCCGACAAATTGATGTTATTAAAAGTTCGTCATTGGAATCGTCATATGTTAATGAACGACTTAAAGTATGTCAAAGTAAGATGCAGTTCTACAAAAAGCATATAACAATTAGTT 720
ysAlaPheArgGlnIleAspValIleLysSerSerSerLeuGluSerSerTyrLeuAsnGluArgLeuLysValCysGlnSerLysMetGlnPheTyrLysLysHisIleThrIleSerT

ATAGTTCAAACATTGATAATCCGATGGTATTTGGTTTAGTGAAATCCCAAATTGTACTACCAACTGTCGTAGTCGAAACCATGAATGACAAAGAAATTGAATATATTATTCTACATGAAC 840
yrSerSerAsnIleAspAsnProMetValPheGlyLeuValLysSerGlnIleValLeuProThrValValValGluThrMetAsnAspLysGluIleGluTyrIleIleLeuHisGluL

TATCACATGTGAAAAGTCATGACTTAATATTCAACCAGCTTTATGTTGTTTTTAAAATGATATTCTGGTTTAATCCTGCACTATATATAAGTAAAACAATGATGGACAATGACTGTGAAA 960
euSerHisValLysSerHisAspLeuIlePheAsnGlnLeuTyrValValPheLysMetIlePheTrpPheAsnProAlaLeuTyrIleSerLysThrMetMetAspAsnAspCysGluL

AAGTATGTGATAGAAACGTTTTAAAAATTTTGAATCGCCATGAACATATACGTTATGGTGAATCGATATTAAAATGCTCTATTTTAAAATCTCAGCACACATAAATAATGTGCCAGCACAAT 1080
ysValCysAspArgAsnValLeuLysIleLeuAsnArgHisGluHisIleArgTyrGlyGluSerIleLeuLysCysSerIleLeuLysSerGlnHisIleAsnAsnValAlaAlaGlnT
                                                                                                                          A
                                                                                                                          S
ATTTACTAGGTTTTAATTCAAATATTAAAGAACGTGTTAAGTATATTGCACTTTATGATTCAATGCCTAAACCTAATCGAAACAAGCGTATTGTTGCGTATATTGTATGTAGTATATCGC 1200
yrLeuLeuGlyPheAsnSerAsnIleLysGluArgValLysTyrIleAlaLeuTyrAspSerMetProLysProAsnArgAsnLysArgIleValAlaTyrIleValCysSerIleSerL

GCTTCACATGAAACAGCTAAAGAAGCTT
erPheThr***
TTTTAATACAAGCACCGTTACTATCTGCACATGTTCAACAAGACAAATATGAAACAAATGTATCATATAAAAAATTAAATCAACTAGCTCCGTATTTCAAAGGATTTGATGGAAGTTTTG 1320
euLeuIleGlnAlaProLeuLeuSerAlaHisValGlnGlnAspLysTyrGluThrAsnValSerTyrLysLysLeuAsnGlnLeuAlaProTyrPheLysGlyPheAspGlySerPheV

TGCTTTATAATGAACGGGAGCAAGCTTATTCTATTTATAATGAACCAGAAAGTAAACAACGATATTCACCTAATTCTACTTACAAAATTTATTTAGCGTTAATGGCATTCGACCAAAATT 1440
alLeuTyrAsnGluArgGluGlnAlaTyrSerIleTyrAsnGluProGluSerLysGlnArgTyrSerProAsnSerThrTyrLysIleTyrLeuAlaLeuMetAlaPheAspGlnAsnL

TACTCTCATTAAATCATACTGAACAACAATGGGATAAACATCAATATCCATTTAAAGAATGGAACCAAGATCAAAATTTAAATTCTTCAATGAAATATTCAGTAAATTGGTATTACGAAA 1560
euLeuSerLeuAsnHisThrGluGlnGlnTrpAspLysHisGlnTyrProPheLysGluTrpAsnGlnAspGlnAsnLeuAsnSerSerMetLysTyrSerValAsnTrpTyrTyrGluA

ATTTAAACAAACATTTAAGACAAGATGAGGTTAAATCTTATTTAGATCTAATTGAATATGGTAATGAAGAAATATCAGGGAATGAAAATTATTGGAATGAATGTTCATTAAAAATTTCTG 1780
snLeuAsnLysHisLeuArgGlnAspGluValLysSerTyrLeuAspLeuIleGluTyrGlyAsnGluGluIleSerGlyAsnGluAsnTyrTrpAsnGluSerSerLeuLysIleSerA

CAATAGAACAGGTTAATTTGTTGAAAAATATGAAACAACATAACATGCATTTTGATAATAAGGCTATTGAAAAAGTTGAAAATAGTATGACTTTGAAACAAAAAGATACTTATAAATATG 1900
laIleGluGlnValAsnLeuLeuLysAsnMetLysGlnHisAsnMetHisPheAspAsnLysAlaIleGluLysValGluAsnSerMetThrLeuLysGlnLysAspThrTyrLysTyrV

TAGGTAAAACTGGAACAGGAATCGTGAATCACAAAGAAGCAAATGGATGGTTCGTAGGTTATGTTGAAACGAAAGATAATACGTATTATTTTGCTAGACATTTAAAAGGCGAAGACAATG 1920
alGlyLysThrGlyThrGlyIleValAsnHisLysGluAlaAsnGlyTrpPheValGlyTyrValGluThrLysAspAsnThrTyrTyrPheAlaThrHisLeuLysGlyGluAspAsnA
                                                  SD                orf2
CGAATGGCGAAAAAGCACAACAAATTTCTGAGCGTATTTTAAAAGAAATGGAGTTAATATAATGGATAATAAAACGTATGAAATATCATCTGCAGAATGGGAAGTTATGAATATCATTTG 2040
laAsnGlyGluLysAlaGlnGlnIleSerGluArgIleLeuLysGluMetGluLeuIle***
                                                                MetAspAsnLysThrTyrGluIleSerSerAlaGluTrpGluValMetAsnIleIleTr
                                                                                                                          A
CATGAAAAAATATGCAAGTGCGAATAATATAATAGAAGAAATACAAATGCAAAAGGACTGGAGTCCAAAAACCATTCGTACACTTATAACGAGATTGTATAAAAAGGGATTTATAGATCG 2160
pMetLysLysTyrAlaSerAlaAsnAsnIleIleGluGluIleGlnMetGlnLysAspTrpSerProLysThrIleArgThrLeuIleThrArgLeuTyrLysLysGlyPheIleAspAr
                                                                                                                          Hi
TAAAAAAGACAATAAAATTTTTCAATATTACTCTCTTGTAGAAGAAAGTGATATAAAATATAAAACATCTAAAAACTTTATCAATAAAGTATACAAAGGCGGTTTCAATTCACTTGTCTT 2280
gLysLysAspAsnLysIlePheGlnTyrTyrSerLeuValGluGluSerAspIleLysTyrLysThrSerLysAsnPheIleAsnLysValTyrLysGlyGlyPheAsnSerLeuValLe
s
AAACTTTGTAGAAAAAGAAGATCTATCACAAGATGAAATAGAAGAATTGAGAAATATATTGAATAAAAAATAAAATTGTTGTGTTTACAACAATACATAGAAAACAGAGGAAACAATCAA 2400
uAsnPheValGluLysGluAspLeuSerGlnAspGluIleGluGluLeuArgAsnIleLeuAsnLysLys***

GTCCTGAATATTTGCTGTGTTTTTTAGTTGAAAAAAATTAACCGAAAGCCTGAATGCAAGTCTTGATTAAA                                                    2471
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Fig. 1. (A) Restriction map of pES1 and sequencing strategies. N, *Nde*I; C, *Cla*I; H, *Hin*dIII; P, *Pst*I. The positions of primers are designated by open boxes. (B) Nucleotide sequences of the 5′-flanking region of the *mecA* gene. The nucleotide and predicted amino acid sequences of N315 are fully spelled out. The different nucleotide sequence of MR108 and its amino acid translation is shown above the N315 sequence. Sequences of MR108 beyond the position 1228 have not been determined yet. Possible Shine–Dalgarno sequences and promoter sequences (−10, −35) are shown in italics. The positions of inverted repeats are underlined. These sequence data will appear in the EMBL Nucleotide Sequence Data Library under the accession number X63598.

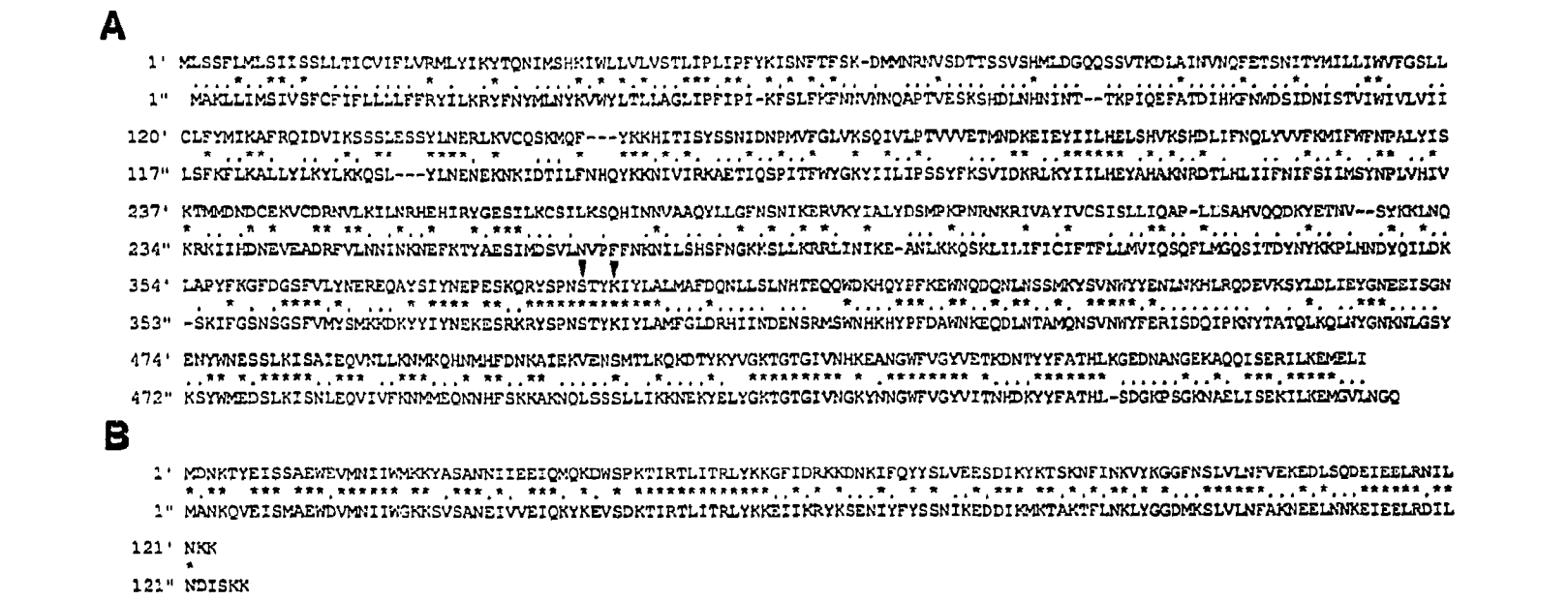


Fig. 2. Comparison of amino acid sequences. (A) Predicted amino acid sequence of *orf1* vs. BlaR1. Arrowheads indicate the presumed penicillin-binding motif. (B) Predicted amino acid sequence of *orf2* vs. BlaI. The upper sequences are the predicted ones of N315 in both panels. Asterisks show identical amino acids. Dots show the substitution of amino acids by those of the same nature.

containing 125 bases of the 5′ end of *mecA* gene and its 5′-flanking region was determined (Fig. 1B). The nucleotide sequence was free from cloning artifacts because nucleotide sequencing of the corresponding region of the N315 genomic DNA showed complete agreement with that of pES1. Two orfs were found in the direction of transcription which is opposite to that of the *mecA* gene (shown as *orf1* and *orf2* in Fig. 1B). The nucleotide sequencing of N315P-ZR, however, revealed one base substitution from guanine to adenine at position 2160 in *orf2* of N315 which caused amino acid substitution from arginine to histidine in the predicted protein (Fig. 1B). In the case of MR108, a more drastic difference was observed; the nucleotide sequence was identical with that of N315 until position 1199 in the *orf1*, beyond that point, however, it was replaced by a totally different sequence, causing the *orf1* of MR108 terminate prematurely at position 1208 (Fig. 1B). Moreover, the DNA region corresponding to the *orf2* was not present in the genome of MR108 which was shown by Southern hybridization using a PCR-amplified *orf2* DNA as a probe (data not shown).

3.2. *Predicted proteins encoded by the* orf1

The predicted protein encoded by *orf1* consists of 585 amino acids (predicted molecular weight = 68,501.92). The protein is 34% homologous to BlaR1 of *S. aureus* [10], and is 31% homologous to PenJ (or BlaR1) of *B. licheniformis* [11,12], both of which are known as the co-inducer protein involved in the regulation of penicillinase production in the respective bacterial species (Fig. 2). Especially, the predicted amino acids corresponding to the penicillin-binding site of BlaR1 with a motif of [serine-X-X-lysine] (indicated by arrowheads) shows strong homology. Hydropathy profile further supported similarity of the predicted protein to BlaR1 (Fig. 3). The predicted protein shared the characteristic five hydrophobic peaks (indicated by arrows) and an increased hydrophylicity towards the carboxyl terminus.

3.3. *Predicted protein encoded by the* orf2

The second orf is predicted to encode a protein composed of 123 amino acids (predicted molecular weight = 14,789.74), which characteristically contains a high percentage of basic amino acids; it contains 18 lysine (14.63%) and 4 arginine residues (3.25%). The amino acid sequence of the predicted protein was very homologous to BlaI [10] of *S. aureaus* (61%) and to PenI [13] of *B. licheniformis* (43%); both of these are repressor proteins of PCase regulation. The amino acid compositions of these three proteins were also very similar to each other.

4. DISCUSSION

Fig. 4 summarizes the genomic organization of the *mec* region (containing the *mecA* gene and its 5′-flanking region) of N315 in comparison with that of the *bla* region of the *S. aureus* PCase plasmid pI258 [10]. It is noted that both the sizes and pattern of arrangement of the two orfs are evidently similar to those of *blaR1* and *blaI*. Based on the comparison of the nucleotide sequence of the *mecA* gene and its operator region with

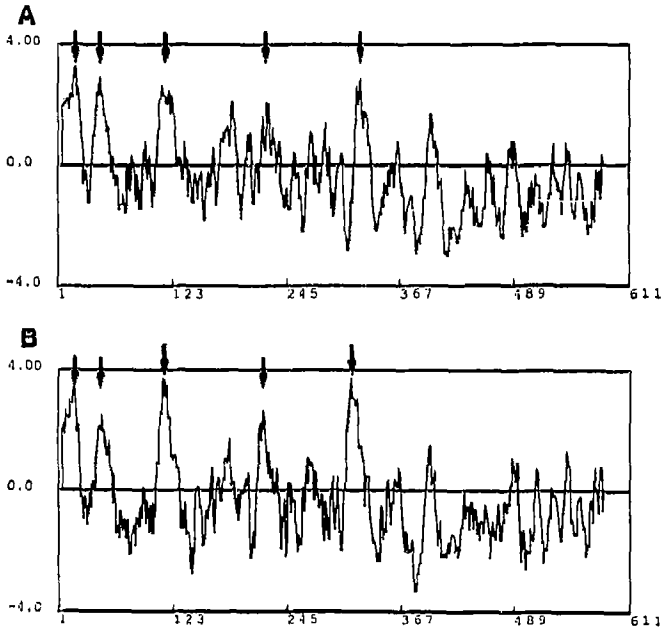


Fig. 3. Hydropathy profile of the predicted *orf1*-encoded protein (A) and of BlaR1 (B). The program of Kyte and Doolittle was used [15]. The mean hydropathy values of a moving segment of 9 amino acid residues are plotted at the midpoint of each segment. Arrows show conserved five hydrophobic peaks among two proteins.

those of *E. coli* PBP genes and a *S. aureus* PCase gene, Song et al. have proposed the idea that the 5′ end and the operator region of the *mecA* structure gene is derived from the β-lactamase gene and the rest of the structure gene derived from the PBP gene [14]. This idea is further substantiated by the similarity of size, arrangement, and encoded proteins of the two orfs with those of the *bla* regulator genes (Fig. 4).

Although further studies on the gene products are required, it is very likely that the two orfs encode the co-inducer (MecR1) and the repressor (MecI) proteins which are specifically involved in the regulation of methicillin resistance. Premature termination of the *orf1* and the absence of *orf2* in the genome of MR108 coincides well with this view as the expression of the *mecA* gene product is constitutive in MR108 after elimination of its PCase plasmid from the cell [8]. Most MRSA clinical isolates in recent years, represented by MR108, are constitutive producers of PBP2′ in the absence of the PCase plasmid. In these cells, the genomic fragment containing *orf2* seems to be absent. Tesch et al. also reported that the genomic region carrying an element for down-regulation of PBP2′ production (probably identical with the *orf2* according to the similarity of restriction enzyme map) is absent in the constitutive PBP2′ producer strains [8]. The amino acid substitution identified in the *orf2* of N315R-ZR may also be responsible for the observed de-repressed transcription of the *mecA* gene by partially inactivating the repressor function of the *orf2*-encoded protein.

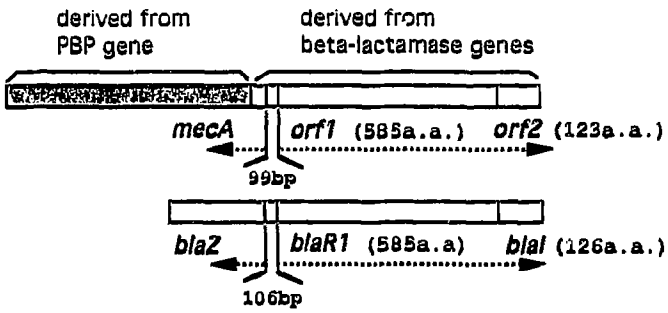


Fig. 4. Genomic organization of the *mec* region in comparison with the *bla* region. The stippled box shows the part of the *mecA* structure gene homologous to *E. coli* PBP genes [14]. Open boxes show DNA segments homologous to the corresponding segments of the *bla* region. The number of base pairs show the length of the operator of each region. Arrows indicate the direction of transcription.

It seems that N315 expresses the original sets of regulator proteins for methicillin resistance which are lost in the recent MRSA strains by deletion or other genetic alterations and are replaced by the regulator proteins of the PCase plasmid. It is conceivable that better inducibility and a higher resistance level towards various β-lactam antibiotics are achieved by the latter regulator system than the original one; thus, the selective expansion of the MRSA strains with the latter regulation system was brought about in the hospital environment.

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