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Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion

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A new β-lactam-inducible penicillin-binding protein (PBP) that has extremely low affinity to penicillin and most other β-lactam antibiotics has been widely found in highly β-lactam(methicillin)-resistant *Staphylococcus aureus* (MRSA). The gene for this protein was sequenced and the nucleotide sequence in its promoter and close upstream area was found to show close similarity with that of staphylococcal penicillinase, while the amino acid sequence over a wide range of the molecule was found to be similar to those of two PBPs of *Escherichia coli*, the shape-determining protein (PBP 2) and septum-forming one (PBP 3). Probably the MRSA PBP (M_r 76462) evolved by recombination of two genes: an inducible type I penicillinase gene and a PBP gene of a bacterium, causing the formation of a β-lactam-inducible MRSA PBP.

Methicillin resistance; Penicillin-binding protein; Penicillinase; DNA sequence; Induction; β-Lactam; (*Staphylococcus aureus*)

1. INTRODUCTION

Recently, the occurrence of highly β-lactam-resistant strains of *Staphylococcus aureus*, called MRSA (methicillin-resistant *S. aureus*) strains, has become a serious problem in clinical microbiology. The major cause of β-lactam resistance in MRSA is attributed to the production of a new penicillin-binding protein (PBP) with extremely low binding affinities to most β-lactam antibiotics (M_r estimated as 75000 on SDS-polyacrylamide gel) [1–4]. Staphylococcal cells usually do not have this PBP (named MRSA PBP) and proliferate by using normal PBPs for forming cell walls that are composed of peptidoglycan as proved in *Escherichia coli* [5]. However, in the presence of β-lactam antibiotics, MRSA cells produce this unique PBP in excessively large amounts [1,3] and can still proliferate, while all the normal PBPs are inactivated. This reversible switching ability of PBP formation seems to be advantageous for survival of the cells.

Ubukata et al. [1] concluded that the induction of this PBP by β-lactam is caused by a gene located on the penicillinase plasmid in one of the MRSA strains, from the finding that removal of this plasmid caused constitutive formation of MRSA PBP in excessively large amounts. Thus, there may be a common mechanism for the induction of penicillinase and of MRSA PBP and we suppose that the gene of the MRSA PBP may be a hybrid of a pencillinase gene and a PBP gene of some bacteria, the regulatory region involving the pro-

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moter area being derived from the former, and the major part of the coding frame from the latter.

2. EXPERIMENTAL

Nucleotide sequencing of the MRSA PBP gene from strain TK784 [1] was carried out according to the dideoxy method [6] using the sequence vector pUC19 and synthetic primers for primer extension [7]. A 4 kilobase (kb) *Hin*dIII fragment of chromosomal DNA that contained the MRSA PBP gene was obtained by recloning from the 18.5 kb plasmid pSM5 [8] and used to isolate smaller DNA fragments for sequencing. Southern blot hybridization was carried out at 65°C.

3. RESULTS AND DISCUSSION

Fig. 1 shows the nucleotide sequence of the promoter area and 42 base pairs (bp) at the head of the coding frame of the MRSA PBP gene compared with that of staphylococcal penicillinase [9]. High base homologies could be seen in the total sequences shown in this figure. Moreover, both DNAs contained a couple of large palindrome sequences among which that indicated by thick lines could be a candidate for the repressor-binding site, overlapping a putative −10 promoter sequence. The base homologies between these two palindrome sequences were high. The same areas also contained promoter-like and Shine-Dalgarno (SD)-like sequences in opposite directions followed by a start codon for another open reading frame. This open reading frame extended for at least 250 bp in MRSA PBP (only 32 bp being shown in fig.1), while it ended at 24 bp in the sequence of the staphylococcal penicillinase gene reported [9]. A similar open reading frame in the opposite direction has been observed in cephalosporinases of *Enterobacter cloacae* [10] and *Citrobacter freundii* [11] and type I β-lactamases of *Proteus vulgaris (blaA;* Okuguchi, M., Nakada, H., Nakajima, N. and Sugimoto, K., personal communication) and *Bacillus licheniformis* [12] and is supposed to be involved in regulation of these inducible β-lactamase genes.

The total base sequence of the coding frame of MRSA PBP is shown in fig.2 and the amino acid sequence of the PBP (M_r calculated to be 76462) deduced from the base sequence of the gene is compared with those of staphylococcal penicillinase [9,13] and *E. coli* PBPs 2 [14] and 3 [15] as seen in fig.3. The amino acid alignment is based on the homologous sequences with at least 9 identical amino acids in a maximum sequence of 25 amino acids. High levels of homology were observed over wide ranges of both the putative transglycosylase (N-terminal) and transpeptidase (C-terminal) domains [5,14] of the three PBP molecules. The homology between MRSA PBP and the penicillinase was much lower. Two different sets of conserved sequences containing Ser-X-X-Lys for penicillin binding were found in MRSA PBP, the first being located around Ser-25 (showing homology with the penicillin-binding domain of the penicillinase) and the second around Ser-405 (showing homology with the penicillin-binding domain of *E. coli* PBPs 2 and 3). Probably the Ser-405 residue is the penicillin-binding site of MRSA PBP, but the sequence around Ser-25 (YASKDK) could be a relic of the evolution of this domain from a penicillinase gene. Clearly, a deletion has occurred between the N-terminal 10–20 amino acid sequence and this YASKDK sequence.

Southern-blot hybridization experiments (fig.4) indicated the presence of base homology between the region upstream to the head of the coding frame for MRSA PBP (including the head) and a

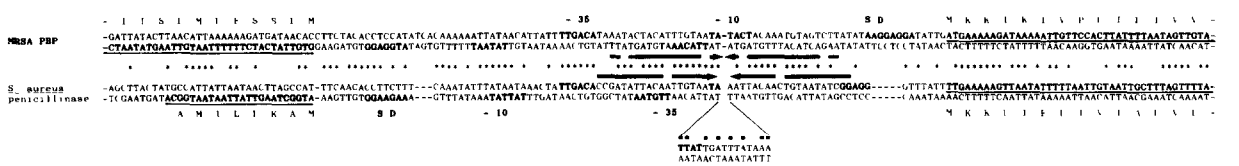

Fig.1. Nucleotide sequence around the proposed regulatory region of the MRSA PBP gene [8] compared with that of an *S. aureus* penicillinase gene [9]. A pair of palindrome sequences (▬) overlapping putative promoter sequences and identical residues (*) are indicated. Open reading frames indicated by underlined letters and putative −35 and −10 promoter sequences and SD sequences are shown by bold-face letters in both strands of DNA.

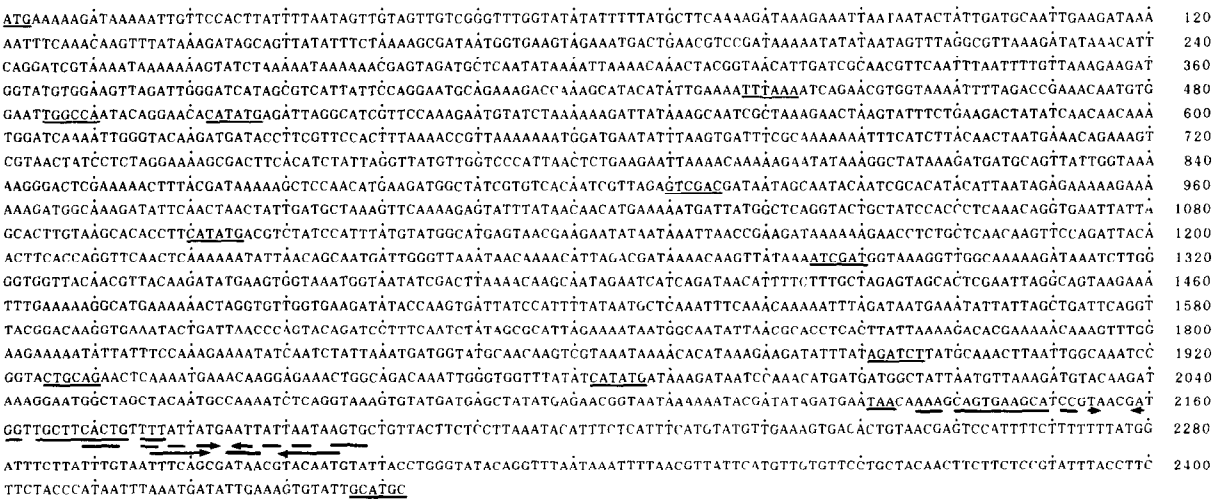

Fig.2. Nucleotide sequence of the coding frame for the MRSA PBP gene. Underline indicates the start codon ATG, termination codon TAA, recognition sites by the restriction enzymes *Aha*III (TTTAAA), *Bal*I (TGGCCA), *Nde*I (CATATG), *Hinc*II (GTCGAC), *Cla*I (ATCGAT), *Bgl*II (AGATCT), *Pst*I (CTGCAG) and *Sph*I (GCATGC) and palindrome sequences after the termination codon.

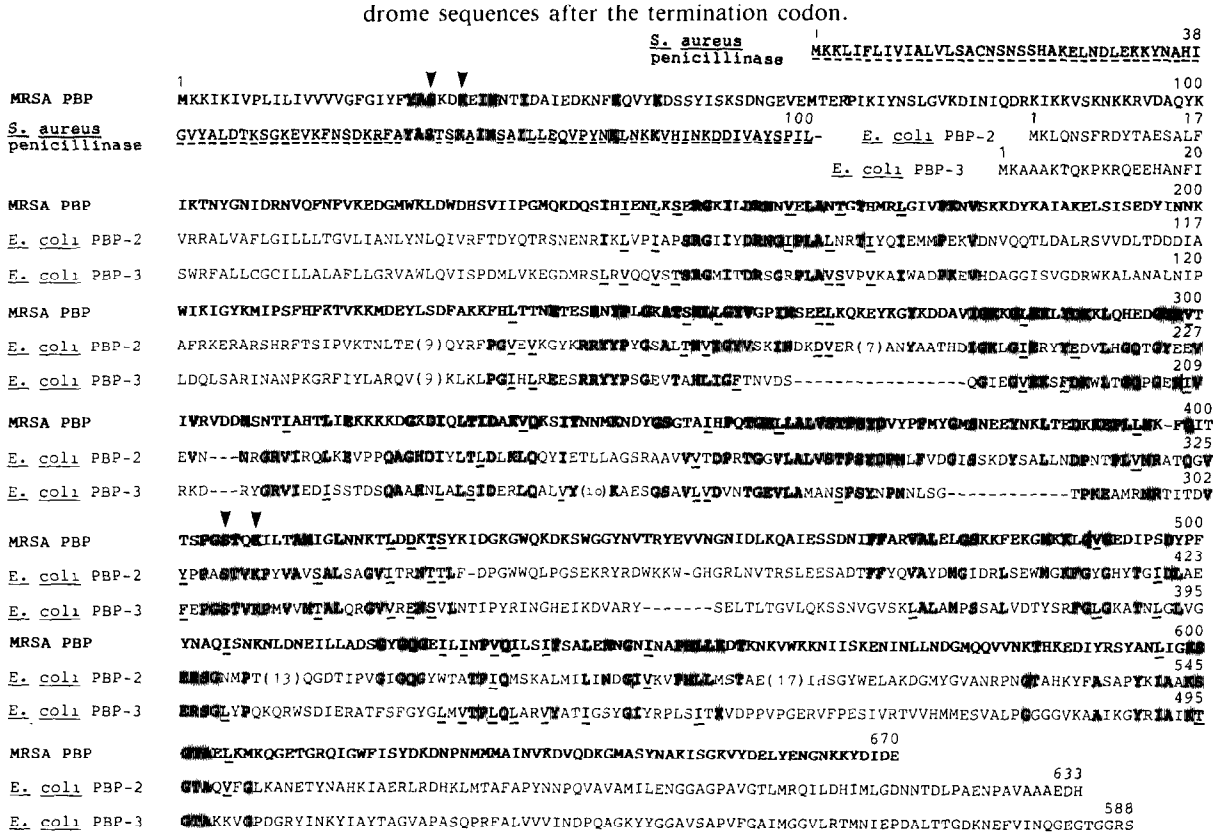

Fig.3. Amino acid alignment of MRSA PBP with an *S. aureus* penicillinase [9,13] and *E. coli* PBPs 2 [14] and 3 [15]. *Homology boxes containing at least 9 identical amino acids in a maximum sequence of 25 amino acids (36% homology)* are shown by shaded amino acids (single-letter notation). Structurally related residues in each box are also indicated by underlining. Conserved serine and lysine residues in the penicillin-binding sites are denoted by arrows. For aligning amino acids, several appropriate short fragments have been deleted from the sequences of *E. coli* PBPs. These are shown as the number of amino acid residues deleted in parentheses.

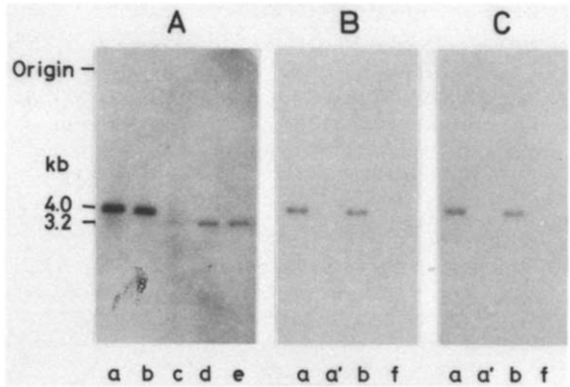

Fig.4. DNA homology of the MRSA PBP gene (promoter region and coding frame) with staphylococcal penicillinase plasmid and MRSA chromosome. The ^{32}P-labeled probes used were: (A) the 600 bp *Hin*cII Sau3A fragment of the MRSA PBP gene containing a 250 bp portion of the inverted open reading frame, the promoter region and a 243 bp portion of the coding frame from Met-1 to Asp-82; (B) the 800 bp *Hae*III-*Cla*I fragment of the MRSA PBP gene containing the coding region from Ala-163 to Asp-430; and (C) the 520 bp *Cla*I-*Pst*I fragment of the MRSA PBP gene containing the coding region from Asp-430 to Ala-603. The probe was labeled by primer extension [7] (A) or the multi-primer method [16] (B,C) using [α-^{32}P]dCTP. Samples tested by Southern blot hybridization were: (a) *Hin*dIII digest of MRSA strain TK784 total DNA; (a') *Hin*dIII digest of β-lactam-sensitive revertant strain TK784E total DNA; (b) *Hin*dIII digest of MRSA strain TK731 total DNA; (c) *Hin*dIII fragment of a 3.2 kb penicillinase plasmid from MRSA strain TK731; (d) *Bam*HI digest of the same plasmid as (c); (e), *Eco*RI digest of the same plasmid as (c,d); (f), *Kpn*I-*Bgl*II-digested total DNA from an *E. coli* K-12 strain containing the plasmid pIT101A carrying the PBP 2 gene. Similar results were obtained by hybridization at 37°C.

penicillinase plasmid from an MRSA strain TK731 [1] (fig.4A, lanes c–e). In contrast, major parts of the coding frame for MRSA PBP only hybridized with the chromosome of MRSA strains (lanes a,b in fig.4B,C), and not with that of a sensitive strain which has a complete set of normal PBPs (lanes a' in fig. 4B,C). Therefore, normal staphylococcal PBPs could not be direct ancestors of MRSA PBP. The latter probes also did not hybridize with the *E. coli* chromosome (lanes f in fig.4B,C).

We are now attempting to find the DNA fragments in various bacteria that show the most

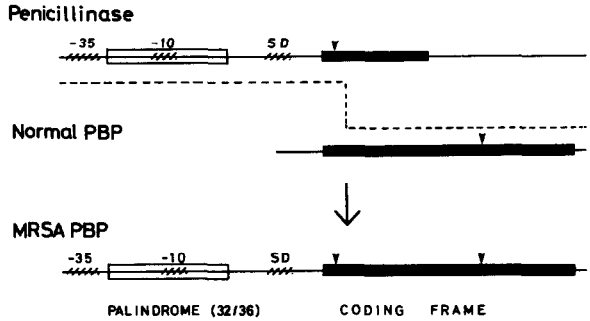

Fig.5. Proposed scheme of the evolution of MRSA PBP by recombination of a penicillinase and a normal PBP gene. Conserved sequences of penicillin-binding sites are marked by an arrow.

complete homology with one of the two regions of the MRSA PBP gene, the promoter region and the major part of the coding frame. If we find such DNA fragments, we can conclude that these genes fused to form a new gene coding for an inducible PBP of MRSA (fig.5). By sequencing these fragments we may find the sites at which gene recombination took place and how the protein also acquired resistance to β-lactam compounds.

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