Nucleotide Sequence of Aminoglycoside 6′-*N*-Acetyltransferase [AAC(6′)] Determinant from *Serratia* sp. 45

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Gene for aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)] from *Serratia* sp. 45 was cloned into *E. coli*. The enzyme produced in *E. coli* carrying the recombinant plasmid was compared to the *Serratia* enzyme. Both enzymes acetylated the 6′-C position of amikacin, dibekacin, tobramycin, sisomicin, gentamicin C_{1a} and kanamycin but effected gentamicin C_1, gentamicin C_2 and micronomycin minimally. No significant difference in optimal pH, isoelectric point or molecular weight was detected. The nucleotide sequence of the gene was determined. Initiating with a GTG codon for methionine, it was composed of 552 base pair coding for 184 amino acids. The molecular weight of the enzyme was about 20418. Comparison of the amino acid sequence of this AAC(6′) with the amino acid sequence of *aacA4* gene from *Serratia marcescens* (G. Tran Van Nhieu and E. Collatz, *J. Bacteriol.*, 169, 5708 (1987)) showed 98.3% homology.

Keywords 6′ aminoglycoside acetyltrasferase; *Serratia* sp.; cloning; nucleotide sequence

Introdution

Enzymatic inactivation of aminoglycoside antibiotics is one of the main mechanisms of resistance of both gram-positive and gram-negative clinical pathogens. The major aminoglycoside modification mechanisms include acetylation (AAC) of the amino group and phosphorylation (APH) and adenylylation (AAD) of the hydroxy groups of the antibiotics. Aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)] has been classified further into 4 subgroups, AAC (6′)-1, -2, -3 and -4,[1] based on its substrate profiles and the susceptibility of organisms to the drugs. The enzyme AAC(6′)-1 acetylates kanamycin-A (KM-A), kanamycin-B (KM-B) and neomycin (NM). The enzyme AAC(6′)-2 acetylates KM-A, KM-B, NM, gentamicin-C_{1a}(GMC_{1a}) and gentamicin-C_2(GMC_2). The enzyme AAC(6′)-3 acetylates KM-A, KM-B, NM, GMC_{1a}, GMC_2 and dibekacin (DKB). The enzyme AAC(6′)-4 acetylates KM-A, KM-B, NM, GMC_{1a}, GMC_2, DKB and amikacin (AMK). Recently new types of AAC(6′) were found among clinical isolates of *Staphylococcus aureus*,[2] *Serratia* spp.[3] and *Klebsiella pneumoniae*.[4] The enzyme produced by *S. aureus* acetylates GMC_{1a}, GMC_2, sisomicin (SISO), netilmicin (NT), KM-A, KM-B, DKB, tobramycin (TOB), AMK, ribostamycin (RSM), butirosin-A (BUT-A), neomycin-C (NM-C) and fortimicin (FTM) but not GMC_1, kanamycin-C (KM-C), paromomycin (PRM) or lividomycin (LV). The enzyme produced by *Serratia* spp. acetylates KM-A, KM-B, NM, GMC_{1a}, DKB and AMK but GMC_2 and 1-*N*-[(*S*)-3-amino-2-hydroxypropionyl]-gentamicin B (HAPA-B) minimally. These two AAC(6′)s are entirely different in their substrate profiles. We describe the cloning of the AAC(6′) gene from *Serratis* sp. 45, a wild strain producing this new type AAC(6′), in *E. coli* and compared the enzymatic properties of the recombinant plasmid with the donor. We present the nucleotide sequence of this new AAC(6′) gene from *Serratia* sp. 45 and additionally compared its nucleotide sequence to other acetyltransferases.

Materials and Methods

Bacterial Strain and Plasmid *Serratia* sp. 45 was isolated from clinical sources and maintained as a stock culture in this laboratory, *E. coli* C600 was used as the plasmids' host cell. Plasmids used and their derivation are listed in Table I.

Medium Heart infusion medium (HI; Difco), Heart infusion agar (HIA; Difco) and Mueller Hinton agar (MHA; Difco) were used for cultivation and the determination of bacterial drug resistance, respectively.

Antibiotics Susceptibility Test Minimum inhibitory concentrations (MICs) were determined by the agar dilution method wtih an inoculum of approximately 5×10^4 cells per spot.

Antibiotic and Chemicals KM, DKB, streptomycin (SM), TOB, AMK and ampicillin (AP) were obtained from Sigma Chemical Co., Ltd. HAPA-B, micronomycin (MCR), SS, and FTM were provided by the laboratories of Toyo Jozo Co., Ltd., Kyowa Hakko Kogyo Co., Ltd., Essex Nippon Co., Ltd., and Kyowa Hakko Kogyo Co., Ltd., respectively. GMC_1, GMC_{1a} and GMC_2 were prepared in our laboratories. Adnosine 5′-triphosphate (ATP), and acetylCoA were purchased from Sigma Chemical Co., Ltd. [8-^{14}C]ATP (56 mCi/mmol), [r-^{32}P]ATP (5000 mCi/mmol) and [1-^{14}C]acetylCoA (54 mCi/mmol) were purchased from the Radiochemical Center, Amersham, Japan. Restriction endonuclease, deoxyribonucleic acid (DNA) polymerase and alkaline phosphatase were purchased from Takara Syuzo Co., Ltd.

Conjugal Transfer of Resistance Plasmid transfers were performed by the following method. One tenth milliliter of overnight cultures of the recipient (*E. coli* X1037: *rif*) and donor (*Serratia* sp. 45) each were inoculated in 10 ml of fresh HI and shaken at 37 °C for 3 h. One part of the donor culture was mixed with four parts of recipient culture, and the mixture was passed through a millipore membrane filter (0.45 μm, Millipore Co., Ltd.). The filter was placed on drug free HIA. After incubation at 37 °C for 2 h, the millipore membrane filter was placed into 5 ml of phosphate buffered saline (PBS) and blended by a vortex mixer. One tenth milliliter of the mixed culture was placed on selective plates containing rifampicin (2000 μg/ml) and aminoglycoside antibiotics (AMK, DKB, TOB and KM).

Assay of Aminoglycoside-Modifying Enzymes Substrate specificity was determined by a cellulose phosphate binding assay.[5] For acetylation, the assay mixture contained 30 μl of a 105000 × *g* supernatant (S105 fraction), 10 μl of 1 mM antibiotic solution, 10 μl of 20 mM $MgCl_2$ solution and 10 μl of [1-^{14}C]acetylCoA (10 μCi/mmol) containing 1 mM acetylCoA solution, 60 μl in total. The reaction mixture was incubated at 37 °C for 15 min, and then heated at 100 °C for 3 min. Twenty microliters of supernatant, obtained by centrifugation at 15000 rpm for 3 min, was placed onto phosphocellulose paper (0.49 cm^2: Whatman p81), washed with a large volume of distilled water, dried and counted in a scientillator counter. For phosphorylation or adenylylation, the same reaction mixture was used, except that it contained [r-^{32}P]ATP (10 μCi/ml) or [8-^{14}C]ATP (2 μCi/ml) instead of acetylCoA solution, and was incubated for 60 min. The bioassay method was performed as described previously.[3] Protein concentration was measured by the method of Lowry *et al.*[6] using bovine serum albumin as the standard.

Optimum pH Tris–maleinic acid buffer (0.5 M: ranging from pH 6.0 to 7.5) and Tris HCl buffer (0.2 M: ranging from pH 8.0 to 8.5) were used to determine the optimal pH value. Enzyme activity was measured at each pH value by the bioassay method using AMK as the substrate.

Determination of the Isoelectric Point (pI) of the Enzyme Isoelectric focusing of enzyme was performed according to the method[7,8] with a 110 ml capacity electrofocusing column using carrier ampholytes having a pH range of 4.0 to 12.0 in solution. After electrophoresis for 48 h, the content of the column was collected in 2 ml fractions, and the enzyme activity and pH of the fractions were determined. Enzyme activity was

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measured by bioassay.

Determination of Enzyme Molecular Weight The molecular weights (M.W.) of the enzymes were determined by gel filtration using Sephadex G75.[9] Bovine serum albumin, ovalbumin, chymotrypsinogen A and ribonuclease A (Pharmacia Fine Chemicals) having M. Ws. of 67000, 43000, 25000 and 13700 were used as a standard.

Isolation of Inactivated AMK AMK was inactivated in a reaction mixture containing 70 ml of S-105 fraction, 200 mg acetylCoA, 200 mg of AMK and 0.2 M Tris HCl buffer (pH 8.0) containing 20 mM $MgCl_2$. After incubation for 18 h at 37 °C, the reaction was stopped by heating in boiling water for 5 min. The supernatant collected by centrifugation at 15000 rpm for 15 min. The reaction mixture (about 200 ml) containing inactivated AMK was passed through a column of IRC 50 (NH_4^+ form, 15 by 700 mm). After it had been washed with 500 ml of distilled water, the inactivated AMK was eluted with 500 ml of 1 N NaOH. The fraction which had a positive ninhydrin reaction was collected and neutralized with 1 N HCl. Inactivated AMK was subjected to CM-Sephadex C-25 (NH_4^+ form, 50 ml) column chromatography for purification. After washing with 1000 ml of distilled water, elution was carried out with a linear gradient of NH_4OH (0—0.1 N). Ninhydrin-positive fractions were collected and lyophilized to yield 30 mg of product. The chemical structure of the inactivated AMK was confirmed by mass spectroscopy and proton nuclear magnetic resonance (^{1}H-NMR) and carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy.

DNA Procedures Plasmid and chromosomal DNA were prepared by either the rapid alkaline extraction or CsCl–ethidium bromide equilibrium density gradient centrifugation as described previously.[10]

Digestion and ligation of DNA Endonuclease digestion and ligation reactions were performed according to the instructions of the manufacturer (Takara Syuzo Co., Ltd.). Restriction endonuclease were used with buffers prepared according to the recommendation of the supplier. The ligation reaction was carried out with excess T4 DNA ligase for 34 h at 4 °C. Restriction analysis and ligation with T4 ligase were performed using the enzymes recommended by the supplier.

DNA Nucleotide Sequence Determination The nucleotide saquence was determined by the dideoxy termination method[11] using restriction fragments cloned into M13 derivative mp18 and mp19. The nucleotide sequence data and search for sequence homolgies were analyzed by computer using the program of GENIAS system (Research Institute, Mitsui Knowledge Industry Co., Ltd.).

Results

Construction of Plasmid We examined conjugal transferability of aminoglycoside-resistance in *Serratia* sp. 45 using *E. coli* X1037 (*rif*) as the recipient. We were unable to acquire the transconjugant when the selection was made for AMK. DKB, TOB and KM. Additionally, we were unable to find plasmid DNA either by the rapid alkaline extraction method or the CsCl–ethidium bromide equilibrium density gradient centrifugation method. Therefore, chromosomal DNA from *Serratia* sp. 45 (5.04 μg) was digested by the restriction endonuclease *Eco*RI and ligated with an *Eco*RI digest from pACYC184 (3.55 μg). For transformation, 10 to 20 μl of plasmid DNA in ligation mixture was added to 300 μl of competent cells and kept on ice for at least 40 min. After incubation at 42 °C for 3 min, the mixture was added to 5 ml of HI and cultured at 37 °C for 60 min. The transformants were selected by HIA containing KM (25 μg/ml). Five colonies were found to be resistant to KM and tetracycline (TC) but sensitive to chloramphenicol (CM). All transformants carried the same plasmid, approximately an 8-kb fragment. Accordingly, we chose one DNA plasmid (pMT2) for further study. One recognition site for *Bam*HI was found on the inserted DNA fragment. pMT2 was cleaved by both *Eco*RI and *Bam*HI and ligated with pBR322. After transformation of the ligated DNA into *E. coli* C600, both KM and AP resistant transformations were selected. One of them, plasmic pMT22 carried a 2.5-kb insert. We tried futher subcoloning and obtained plasmid pMT222 by using *Bbe*I and *Hinc*II to digest pMT22 and pUC13 as the vector plasmid. Finally, a 1.2-kb of *Bbe*I–*Hinc*II fragment was cloned into pUC13 (Fig. 1).

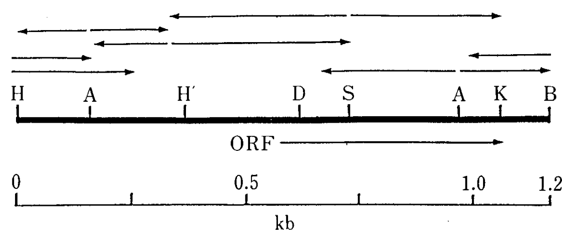

Fig. 1. Sequencing Strategy and Partial Restriction map of the AAC (6′) Resistance Determinant, Which Is Contained in a 1.2-kb(*Hinc*II–*Bbe*I) Fragment of pMT222

Arrows show the lengths of the fragments and the direction of sequencing. ORF as deduced from the nucleotide sequences shown in Fig. 2. Restriction sites are: H, *Hinc*II; A, *Ava*I; H′, *Hae*III; D, *Dde*I; S, *Sca*I; K, *Kpn*I; B, *Bbe*I.

Table I. Plasmids Constructed by *in Vitro* Recombination during This Study

Disignation	Size (kb)	Phenotype	Source and reference
pACYC184	4.0	CM TC	Chang *et al.* (1978)[22]
pBR322	4.4	AP TC	Boliver *et al.* (1977)[23]
pUC13	2.7	AP	Messing (1983)[24]
pMT2	12	AMK TC	8-kb *Eco*RI of *Serratia* sp. 45 chromosome cloned in pACYC184
pMT22	6.5	AMK AP	2.5-kb *Eco*RI/*Bam*HI of pMT2 cloned pBR322
pMT222	3.6	AMK AP	1.2-kb *Bbe*I/*Hinc*II of pMT22 cloned pUC13

Resistance Patterns of Aminoglycoside Antibiotics Table I shows the MIC of *Serratia* sp. 45, *E. coli* C600 carrying pMT22 or pMT222, *Serratia marcescens* TL-1 and plasmid-free *E. coli* C600 against 12 different aminoglycoside antibiotics. *Serratia* sp. 45, wild strain, was resistant to AMK, DKB, TOB, SISO, GMC_{1a} and KM but susceptible to GMC_1, GMC_2, HAPA-B, SM and FTM. The sensitivity to GMC_1, GMC_2 and MCR of the transformants (pMT22, pMT222) was 8 to 64 times higher than the wild strain, However, they showed similar resistance levels as the wild-type strain to AMK, DKB, KM, TOB and HAPA-B.

Identification of Acetyltransferase by *Serratia* sp. 45, pMT22 and pMT222 Each S-105 fraction from *Serratia* sp. 45 and transformants pMT22 and pMT222 was examined for activity of the three amino-glycoside-modification enzymes (AAC, APH and AAD) to most amino-glycosides. Strains harboring plasmid and wild type strain were found to contain AAC but no AAD nor APH (AAD, APH data not shown). Consequently, it was concluded that aminoglycosides were inactivated only be acetylation in these three strains. The substrate profiles of the AAC extracted from strains *E. coli* C (pMT22, pMT222) and *Serratia* sp. 45 were very similar (Table II). The transformants had roughly 3- to 9-fold higher AAC activity than the wild strain, and the transformant carrying pMT22 possessed almost the same AAC activity as the transformant carrying pMT222.

Some Properties of Each Enzyme The relationship

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between AAC activity and pH was determined by varying the pH of the reaction mixture using 0.5 M Tris–maleinic acid buffer (pH 6.0, 7.0 and 7.5) and 0.2 M Tris HCl buffer (pH 8.0, 8.5). The optimal pH for AMK inactivation was about 8.0 (data not shown). The inactivation decreased at pH values below 7.0 or over 8.5. No marked differences were observed between the two species. S-105 fractions were used as samples to determine the pI. AAC(6′)s from the wild strain and transformant pMT22 both had pIs of 4.6 (data not shown). The M. W. of different AACs was determined by gel filtration. The M. W. of AAC from the wild strain was about 20000 and the AAC from transformant pMT22 also was about 20000.

Table II. Resistance Patterns of *Serratia* sp. 45, *E. coli*C600 Carrying Recombinant Plasmids, *E. coli*C600 and *S. marscecens*TL-1

Drug	MIC (mcg/ml)				
	Serratia sp. 45	pMT22[a)]	pMT222[a)]	*E. coli*C600[b)]	*S. marscecens*-TL-1[c)]
HAPA-B	3.13	3.13	3.13	0.19	1.56
AMK	25	25	25	0.39	3.13
GMC_1	0.78	50	50	0.19	0.78
GMC_{1a}	25	50	50	0.78	1.56
GMC_2	1.56	25	25	0.19	0.78
MCR	3.13	25	25	0.19	1.56
DKB	100	100	100	0.39	6.25
TOB	25	25	25	0.10	6.25
SISO	25	25	25	0.10	0.78
KM	25	100	100	0.78	3.13
FTM	1.56	1.56	1.56	0.78	1.56
SM	0.78	0.78	0.78	0.78	0.78

a) These strains were transfered from recombinant plasmids into *E. coli*C600 as a recipient. *b)* This strain was used as the recipient of transformation. *c)* This strain was sensitive.

Identification of Inactivated AMK AMK was completely inactivated by the reaction described in Material and Methods. On thin layer chromatography (TLC) with the solvent of 10% CH_3COONH_4–CH_3OH (1 : 1), *Rf* values of the inactivated AMK and AMK were 0.26 and 0.04, respectively. The (^{1}H-NMR) spectrum of the inactivated AMK suggested the existance of a formed *N*-acetyl-group (2.20 ppm singlet) and in the ^{13}C-NMR spectrum of the same product, two other signals consistent with the presence of an acetate group were observed (data not shown). In view of the above experimental finding and the molecular ion peak in mass spectrum, the chemical structure of the inactivated AMK was 6′-*N*-acetyl-AMK. Both enzymes (wild strain and pMT22) acetylated the 6′-amino position.

Nucleotide Sequence of the AAC (6′) Gene The strategy for the nucleotide sequencing of the AAC(6′) gene region of pMT222 is shown in Fig. 2. The nucleotide sequence of the 1169-bp, *Hinc*II–*Bbe*I fragment was determined by the Sanger's dideoxy chain termination method. Possible open reading frame (ORF) on both strands of the DNA were examined by the GENIAS computer program (Mitsui

Fig. 2. Nucleotide Sequence of AAC (6′) Gene of pMT222

The nucleotide sequence is numbered from the first base of the initiation codon GTG. Amino acid sequence is shown under the nucleotide sequence. A probable Shine–Dalgarno sequence (GAGG, −14 to −11) and three putative promoters (−35 region and Pribnow box) are underlined. The alternative amino acid were those identified by G. Tran Van Nhieu *et al.*[19)]

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Knowledge Industry, Japan). ORF was found between the start codon GTG at positions +1 to +3 and the termination codon TAA at positions 553 to 555. This structure gene was composed of 552 base pair and 184 amino acid residues. Ten bases upstream from the GTG codon, there was a 4-base sequence, GAGG (−14 to −11), which may serve as the ribosome binding site (Shine–Dalgarno sequence[12]) for the translation of AAC (6′). This ORF codes for protein with a M. W. of 20418.

Discussion

The most common mechanism of aminoglycoside resistance in bacteria is enzymatic inactivation. Aminoglycoside antibiotics are inactivated by phosphorylation, adenylylation and acetylation. AMK has been an effective antimicrobial against bacteria resistant to DKB, GM, TOB and KM, but recently, several authors have reported strains of *Serratia marcescens* resistant to AMK.[13−16] Mitsuhashi classified AAC (6′) into four groups [AAC(6′)-1, -2, -3 and -4] on the basis of substrate profiles and drug susceptibility (1975).[1] AMK resistance is believed to be due primarily to AAC (6′)-4. Recently, a new enzyme has been found in a few clinical isolates of *Serratia* spp. Which is resistant to AMK, TOB, DKB and KM, but susceptible to GMC_2 and HAPA-B. This enzyme has been named AAC (6′).[3] We cloned the AAC (6′) gene from *Serratia* sp. 45 using vector plasmids (pACYC184, pBR322 and pUC13) and gene expression was confirmed in the recipient *E. coli* C 600. Recombinant plasmids pMT22 and pMT222 carried the natural AAC(6′) gene, having the 2.5-kb *Eco*RI–*Bam*HI and 1.2-kb *Bbe*I–*Hinc*II inserts, respectively. The enzyme produced from pMT22 was identical to the wild strain in terms of optimal pH, pI and M. W. Transformants carrying plasmids pMT22 and pMT222 produced levels of AAC (6′) activity 6- to 13-fold higher than the wild strain. This is believed to be due to the high copy numbers produced by plasmids pBR322 and pUC13. The nucleotide sequence of the AAC(6′) gene region revealed only one ORF encoding a protein composed of 184 amino acid residues. This ORF was preceded by a putative Shine–Dalgarno sequence[12] and by a potential promoter sequence.[17] Three putative promoters (−35 region and Pribnow box) are shown in Fig. 2; these are TTGACATAAGCCTGTTCGGTTCGTAAACT (−577 to −549), TTTCCAGATCGTGATGACGTTGATAA (−152 to −127) and TTGATCATTGGCAACTTTTGTTCTATA (−125 to −99). It has been reported that GTG is the initiation codon for KM nucleotide transferase encoded by plasmids pUB110 and pTB 913 in gram-positive bacteria.[18] The initiation codon of the AAC (6′) gene was GTG in our study. The AAC(6′) gene from *Serratia* sp. 45 was compared to other gram-positive and gram-negative bacteria. The sequence of another 6′-*N*-acetyltransferase, *aacA4* gene from *S. marcescens* has been determined.[19] It was confirmed that the whole nucleotide sequence was identical between both determinants of *aacA4* and pMT22 except for four bases. Cytosine at position +305, thymine (+546), guanine (+547) and adenine (+548) in structural gene in pMT222 were replaced by thymine, guanine, thymine, thymine in *aacA4* gene, respectively. The amino acid sequences of the two acetyltransferases differed by only three amino acids. This protein of 184 amino acids is seventeen N-terminal amino acids shorter than the coding potential for the *aacA4* gene. The homology with the amino acid sequence of this protein showed 98.3% with the *aacA4* gene. Moreover, this protein has been compared with AAC(6′)-II from *Pseudomonas aeruginosa*.[20] The number of amino acids in both the pMT222 and the AAC(6′)-II gene was the same (184 amino acids), but amino acid homologies were 77.7%. The amino acid sequences of the AAC(6′) gene from *Serratia* sp. 45 were compared to the amino acid sequences of AAC(6′)-APH(2″) from *S. faecalis*,[21] but the two sequences were not homologous. Whether the nucleotide sequence of each of the AAC(6′) subclasses is the same or not and whether there are different isoenzymes of AAC(6′) are interesting problems which require further study.

TABLE III. Incorporation of [^{14}C]Acetate into Various Aminoglycoside Antibiotics

Substrate	Incorporation of [^{14}C]acetylcoA into the drugs (dpm)		
	Serratia sp. 45	pMT22	pMT222
HAPA-B	3541	24579	25040
AMK	6311	45930	47388
GMC_1	601	1884	1984
GMC_{1a}	3080	27895	27893
GMC_2	1380	12106	12478
MCR	1310	8975	10495
DKB	4763	42780	46228
SISO	6010	45726	25219
FTM	0	0	0
SM	0	0	0

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References

1) S. Mitsuhashi, *Czecho slovak. Medical Press, Prague, Avicenum.*, **1975** 115.
2) T. Okubo, M. Inoue, M. Nagashima, M. Okii, T. Iida and S. Mitsuhashi, *J. Antibiot.*, **38**, 122 (1985).
3) T. Morohoshi, M. Toriya, S. Yokoiyama, K. Fujimoto, K. Hayano, S. Goto and A. Tsujiom, *J. Antibiot.*, **37**, 1687 (1984).
4) K. Nobuta, M. Tolmasky, L. M. Crosa and J. H. Crosa, *J. Bacteriol.*, **170**, 3769 (1988).
5) M. J. Hass and J. E. Dowding, *Methods Enzymol.*, **43**, 611 (1975).
6) O. H. Lowry, N. J. Rosebrough, A. L. Frarry and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
7) O. Vesterberg and H. Svensson, *Acta Chem. Scand.*, **20**, 820 (1966).
8) O. Vesterberg, *Methods Enzymol.*, **22**, 389 (1970).
9) P. Andrews, *Biochem. J.*, **91**, 222 (1964).
10) T. Maniatis, E. F. Fritsch and J. Sambrook, "Molecular Cloning: a Laboratory Manual," Cold Spring Harbor, New York, 1982, p. 150.
11) F. Sangaer, S. Nicklen and A. R. Coulson, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 5463 (1977).
12) J. Shine and L. Dalgarno, *Proc. Natl. Acad. Sci. U.S.A..*, **71**, 1342 (1974).
13) J. C. Arroyo, W. L. Milligam, B. Postic, J. Northey, E. Parker and C. S. Bryryam, *Infec. Control.*, **2**, 367 (1981).
14) F. J. Joseph, F. M. William, E. P. Kenneth and A. K. Peter, *Antimicrob. Agents Chemother.*, **21**, 587 (1982).
15) H. Knothe, *J. Infect. Dis.*, **134** (Suppl.), S271 (1976).
16) K. E. Price, P. A. Kresel, L. A. Farchione, S. B. Siskin and S. A. karpow, *Antimicrob. Agent Chemother.*, **8** (Suppl. A), 89 (1981).
17) D. Pribnow, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 784 (1975).
18) M. Matsumura, Y. Katkura, T. Imanaka and S. Aiba, *J. Bacteriol.*, **160**, 413 (1984).

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19) G. Tran Van Nhieu and E. Collatz, *J. Bacteriol.*, **169**, 5708 (1987).
20) K. Shaw, C. M. Rizzo, R. Mierzwa, K. Gewain, G. H. Miller and R. S. Hare, *Antimicrob. Agents Chemother.*, **33**, 2052 (1989).
21) J. F. Joseph, K. S. Gilmore and P. Courvalin, *J. Bacteriol.*, **167**, 631 (1984).
22) A. C. Chang and S. N. Cohen, *J. Bacteriol.*, **134**, 1141 (1978).
23) F. Bolovar, R. L. Roddrignez, P. J. Greene, M. C. Betlach, H. L. Heynecker and H. W. Boter, *Gene*, **2**, 95 (1977).
24) J. Messing, *Methods Enzymol.*, **101**, 20 (1983).

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