Biochem. J. (1990) 268, 671–677 (Printed in Great Britain)

Cloning of aminoglycoside phosphotransferase (*APH*) gene from antibiotic-producing strain of *Bacillus circulans* into a high-expression vector, pKK223-3*

Purification, properties and location of the enzyme

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The aminoglycoside phosphotransferase gene from a butirosin-producing strain of *Bacillus circulans* was cloned in a high-expression vector (pKK223-3) to give the recombinant plasmid pMS5. *Escherichia coli* harbouring the plasmid, *E. coli* JM103[pMS5], was characterized, and several features of the expression of the phosphotransferase were studied. The phosphotransferase activity was best expressed in a medium lacking glucose, and the highest levels of the enzyme were found between 12 and 24 h of growth. The induction of the phosphotransferase expression with isopropyl β-D-thiogalactopyranoside (inducer) was found to be undesirable as the overproduction of the enzyme led to the killing of the bacteria. The subcellular location of the phosphotransferase, and also the site *in vivo* of the phosphorylation of neomycin, was found to be in the cytoplasm. The phosphotransferase was purified to homogeneity in good yield (17 mg of purified protein/3 litres of culture) and was shown to be a monomer of M_r 30000–32000. The *N*-terminal amino acid sequence was in agreement with that predicted from the gene sequence and confirmed the absence of any signal sequence. The regiospecificity of the phosphotransferase reaction was studied by m.s. and by ^{1}H-, ^{13}C- and ^{31}P-n.m.r. using ribostamycin as the substrate, and it was found that the antibiotic was phosphorylated at the 3′-hydroxy group.

INTRODUCTION

Aminocyclitol antibiotics are produced mostly by differentiating strains of *Streptomyces* and in a few cases by *Bacillus*. These organisms are Gram-positive bacteria, and an unusual problem facing these antibiotic-producing micro-organisms is to avoid being killed by their own antibiotic. It has been hypothesised that the mechanism used by antibiotic-producing micro-organisms to protect themselves are similar to those found in clinical isolates of antibiotic-resistant strains (reviewed by Demain, 1974; Davis & Smith, 1978; Foster, 1983; Cundliffe, 1984). This postulate was originally examined by us in Southampton (Herbert *et al.*, 1983, 1986; Akhtar & Sarwar, 1988) and the Biogen group in Geneva (Thompson & Gray, 1983) by studying an aminocyclitol-antibiotic-inactivating enzyme, namely aminoglycoside phosphotransferase. For this purpose we selected a butirosin-producing strain of *Bacillus circulans*, and the Biogen group used *Streptomyces fradiae*, which biosynthesizes neomycin. Aminoglycoside phosphotransferase genes (*APH*) from these organisms were cloned and sequenced. Comparison of the deduced protein sequences of these phosphotransferases with those from antibiotic-resistant bacteria showed a remarkable degree of similarity, suggesting (but not proving) that antibiotic-producing organisms may be the original source of the resistance determinants in clinical isolates (Herbert *et al.*, 1986).

In the present paper we have studied the expression in *Escherichia coli* of the phosphotransferase gene from the butirosin-producing strain of *B. ciculans*. It should be noted from the outset that since butirosin is not commercially available, two related antibiotics, neomycin and ribostamycin (for structures see Fig. 1), were used as alternative substrates.

MATERIALS AND METHODS

Materials

[α-^{32}P]dATP, [α-^{35}S]dATP (each having 400 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham International, Amersham, Bucks., U.K. Ribostamycin and neomycin as their sulphate salts, and Sepharose 4B-200, were obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. DNA polymerase Klenow fragment, phage-T_4 DNA ligase and all restriction endonucleases were obtained from BCL–Boehringer Corp., Lewes, East Sussex, U.K. All other chemicals were from BDH, Poole, Dorset, U.K. or Sigma Chemical Co. The affinity-chromatography matrix, agarose—O—C(=NH)NH·$(CH_2)_3$—NH—CO—$(CH_2)_2$-CO-neomycin, was synthesized as described by Sarwar (1989). Sequencing primer was manually synthesized by using standard phosphotriester chemistry (Sarwar, 1989).

Media

The following media were used: LB medium, which consisted of Bacto-Tryptone (10 g/l), Bacto-Yeast extract (5 g/l) and NaCl (5 g/l), and LB rich medium, which consisted of Bacto-Tryptone (10 g/l), Bacto-Yeast extract (5 g/l), NaCl (10 g/l) and glucose (10 g/l).

Bacterial strains and plasmids

The following strains were used in this work: *E. coli* HB101-[pCH5] (*E. coli* HB101 harbouring plasmid pCH5), *E. coli* JM103[pMS5] (*E. coli* JM103 harbouring pMS5) and *E. coli* JM103[pKK223-3] (*E. coli* JM103 harbouring pKK223-3). The

Abbreviations used: IPTG, isopropyl β-D-thiogalactopyranoside; *APH*, aminoglycoside phosphotransferase gene.

*This is a contribution from the SERC Centre for Molecular Recognition, University of Southampton.

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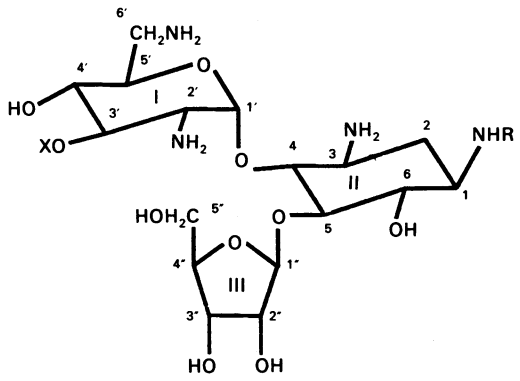
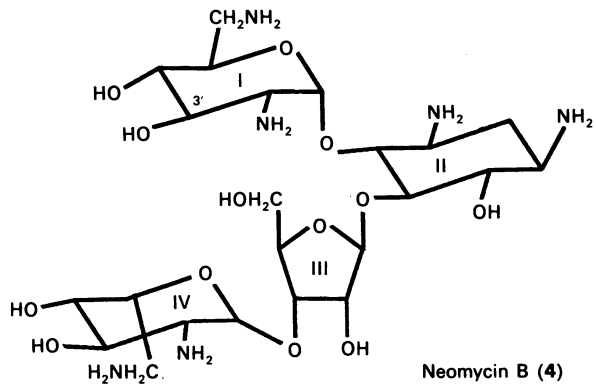


Ribostamycin (R=H, X=H; **1**)

Ribostamycin 3'-phosphate (R=H, X=PO_3^{2-}; **2**)

Butirosin B (R=CO·CHOH·CH_2·CH_2·NH_2, X=H; **3**)



Neomycin B (**4**)

Fig. 1. Structures of antibiotics

plasmids have the following characteristics: pCH5 is a recombinant derivative obtained by inserting 2.7 kb *SalI APH* fragment into pBR322 (Herbert *et al.*, 1986). In this construct the *APH* gene is under the control of its original promoter. pMS5 is a recombinant derivative obtained by inserting a 1232 bp APH fragment into PKK223-3. In this construct (pMS5) the *APH* gene is placed under the control of the *tac* promoter. pKK223-3 is a high-expression cloning vector derived from pBR322 and was obtained from P-L Biochemicals Ltd., Northampton, U.K.

Plasmid preparation

LB medium (500 ml) containing ribostamycin (25 μg/ml) was inoculated with an overnight culture of *E. coli* HB101[pCH5] (10 ml) and grown at 37 °C with shaking until the A_{600} was less than 1. Then solid chloramphenicol was added to a final concentration of 170 μg/ml and the culture was grown for 18 h. The cells were harvested by centrifugation at 5000 rev./min for 10 min at 4 °C, the plasmid (pCH5) was isolated by the method of Clewell & Helinski (1970) and purified on a CsCl/ethidium bromide density gradient as described by Maniatis *et al.* (1982).

Isolation of 1229 bp *DdeI APH* fragment from pCH5

pCH5 (20 μg) was digested with *SalI* (20 units) and *DdeI* (20 units) for 1 h. The digest was resolved by electrophoresis on 0.7% agarose gel, and the 1229 bp *DdeI APH* fragment was identified as being the largest of all the fragments obtained by the double digestion. The fragment was excised and the DNA extracted from the agarose by the freeze–thaw-disruption method (Smith, 1980) using a 2 ml sterile syringe having two glass-microfibre GF-C (Whatman) filters at the bottom of the barrel. The DNA extract (500 μl) was mixed with butan-2-ol (500 μl) and the aqueous layer (100 μl) was extracted with phenol and diethyl ether. Finally the DNA was precipitated by the addition of 0.1 vol. of 3 M-sodium acetate, pH 5.5, and 2.5 vol. of ethanol at −70 °C for 30 min.

The vector, pKK223-3, was prepared from *E. coli* JM103[pKK223-3] and then digested with *Eco*RI. The digest was subjected to electrophoresis, and the *Eco*RI-cut DNA isolated from the agarose as described above.

Filling in of recessed termini and blunt-end ligation

The 'sticky' ends of the vectors and the 1229 bp *APH* fragment were converted into blunt-ended DNA by using DNA polymerase I (Klenow fragment) and nucleotide triphosphates. The reaction mixture consisted of the 1229 bp *DdeI* fragment DNA (5 μl, 1 μg), *Eco*RI-cut vector pKK223-3 (5 μl, 0.2 μg), 2 μl (1 mM) of each dNTP (dATP, dCTP, dGTP, dATP) and 2 μl of 10 × ligase buffer [200 mM-Tris/HCl (pH 7.5)/100 mM-$MgCl_2$/100 mM-dithiothreitol). To the latter, DNA polymerase I (Klenow fragment, 1 μl, 1 unit/μl) was added. The mixture was incubated at 30 °C for 1 h, and then ATP was added to give a final concentration of 0.6 mM. Finally, 1 unit of phage-T_4 DNA ligase was added and the mixture was incubated at 13 °C overnight. The mixture was then used to transform *E. coli* JM103 competent cells (Davis *et al.*, 1980). After heat shock at 42 °C, 1 ml of LB medium was added to the transformation mixture, which was incubated at 37 °C for 1 h to permit the expression of the gene. The transformed bacteria were spread on minimal-salt plates containing glucose, thiamin and ribostamycin (25 μg/ml) in the presence or absence of isopropyl β-D-thiogalactopyranoside (IPTG). After incubation of the plates at 37 °C there was no bacterial growth on the plates containing IPTG, but 18 colonies grew on plates without IPTG. The plasmid DNA was isolated and analysed.

Sequencing of pMS5

For plasmid sequencing, the DNA samples were prepared (Wallace *et al.* 1981) and sequenced using the dideoxy-chain-termination method of Sanger *et al.* (1977).

Expression of the phosphotransferase in *E. coli* JM103[pMS5]

The bacteria were grown in LB-rich medium containing 25 μg of ribostamycin/ml (2 × 1 litre) to an A_{600} of 0.95, and to one of the cultures IPTG was added to give a final concentration of 1 mM and the bacteria were grown for a further 1.5 h. The control flask received no addition. Samples of the culture (50 ml) were removed at various time intervals, and the bacteria were pelleted by centrifugation. The pellet was subjected to osmotic shock, and the phosphotransferase activity was measured by a radiochemical assay (Haas & Dowding, 1975).

Purification of aminoglycoside phosphotransferase

E. coli JM103[pMS5] were grown in LB medium (3 × 1 litre) containing ribostamycin (25 μg/ml) at 37 °C with shaking to an A_{600} of 2.0. The bacteria were harvested by centrifugation at 5000 rev./min ($r_{av.}$ 26 cm) for 5 min and the cells washed with ice-cold buffer [10 mM-Tris/HCl (pH 7.6)/10 mM-$MgCl_2$/50 mM-NH_4Cl/2 mM-β-mercaptoethanol] and resuspended in the same buffer (50 ml) containing DNAase I (4 μg/ml). The suspension was cooled (NaCl/ice bath) and subjected to 30 s bursts from an MSE Soniprep-150 sonicator at 10 μm amplitude. Bursts were alternated with 90 s cooling periods. In total about eight bursts were sufficient to obtain complete breakage of cells. The sonicated

cell suspension was centrifuged at 40000 rev./min ($r_{av.}$ 7.7 cm) for 90 min at 4 °C. The supernatant was dialysed overnight in buffer [10 mM-Tris/HCl (pH 7.6)/10 mM-$MgCl_2$/50 mM-NH_4Cl/2 mM-β-mercercaptoethanol].

To the sonicated material solid $(NH_4)_2SO_4$ was added to 60% saturation (39 g/100 ml of solution) over a 45 min period at 0 °C and the mixture was allowed to stir for another 15 min. The precipitate was removed by centrifugation at 18000 rev./min ($r_{av.}$ 8.5 cm) for 15 min. The supernatant was adjusted to 90% $(NH_4)_2SO_4$ saturation by a slow addition of a further 22.7 g of solid $(NH_4)_2SO_4$/100 ml of supernatant over a period of 30 min. The precipitate was collected by centrifugation at 18000 rev./min ($r_{av.}$ 8.5 cm) at 4 °C for 15 min and dissolved in 5 ml of buffer A [20 mM-Tris/HCl (pH 7.2)/10 mM-magnesium acetate/60 mM-KCl/100 mM-1,4-dithiothreitol]. It was then dialysed overnight against two changes of buffer A.

An affinity column (2 cm × 12 cm) containing neomycin bound to Sepharose 4B was equilibrated with buffer A. The dialysed 60–90% -$(NH_4)_2SO_4$ fraction (5.5 ml containing abut 130 mg of protein) was loaded on to the column at 4 °C, which was washed with buffer A (200 ml) until the eluant showed no absorbance at 280 nm. The enzyme adsorbed on the column was eluted with a linear gradient developed from 100 ml each of buffer A and buffer A containing 1.5 M-NaCl at a flow rate of 15 ml/h at 4 °C. Fractions (2.2 ml) were collected, and enzyme activity was determined in each fraction. The whole of the phosphotransferase activity was eluted with 0.4–0.65 M-NaCl.

Aminoglycoside phosphotransferase assay

The phosphotransferase activity in crude extracts was determined by a radiochemical method (Haas & Dowding, 1975), whereas the activity of purified enzyme was determined by using a coupled spectrophotometric assay (Thompson *et al.*, 1982). One unit is defined as the amount of enzyme which phosphorylates 1 μmol of the ribostamycin or neomycin/min at 30 °C.

Protein assay

Protein assays were performed by a modified Lowry method (Bensadoun & Weinstein, 1976), with BSA as standard.

N-Terminal sequence of the phosphotransferase

The protein (approx. 3 mg) was attached to phenylene di-isothiocyanate-activated aminopropylaminoethyl-glass and sequenced by the solid-phase method of Laursen *et al.* (1972).

Isolation of periplasmic and cytoplasmic fractions from *E. coli* strains

The method was a modified version of that described by Weiss (1976). The bacteria were grown in LB medium (1 litre), containing neomycin (25 μg/ml) to an A_{600} of ~ 1. The bacteria were harvested by centrifugation at 5000 rev./min for 10 min. The bacteria (1g wet wt.) were suspended in 8.5 ml of 20 mM-Tris/HCl, pH 7.5, containing 0.75 M-mannitol. Lysozyme (110 μl of a 10 mg/ml solution) was added slowly at 4 °C and the suspension was gently agitated at 4 °C for 15 min. Ten 0.1 ml aliquots of 100 mM-EDTA, pH 7.5, were added in 1 min intervals. After incubation for 5 min at 4 °C, 0.5 ml of $MgCl_2$ (1 M) was added. The mixture was incubated for a further 2 min and then centrifuged at 12000 rev./min for 20 min at 4 °C to pellet the sphaeroplasts. The supernatant (which contained the periplasmic material) was removed. The sphaeroplasts were then lysed by suspending them in 10.5 ml of 20 mM-Tris/HCl, pH 7.5. The suspension was stirred for 10 min at 4 °C, and then centrifuged at 45000 rev./min for 1 h. The supernatant (which contained cytosolic fraction) was removed, and the pellet containing the membrane fraction was discarded.

The fractions were assayed for phosphotransferase (Haas & Dowding, 1975), glucose-6 phosphate dehydrogenase (Deutsch, 1983) and β-lactamase activity (Waley, 1974).

Subcellular site for phosphorylation of neomycin in *E. coli* JM103[pMS5]

The bacteria were grown in LB medium (2 × 1 litre) in the presence or absence of neomycin (300 μg/ml) at 37 °C with shaking to an A_{600} of about 1.0 and, after harvesting, the periplasmic and cytoplasmic fractions were isolated as described above. The two fractions (10.5 ml of cytoplasmic and 10.5 ml of periplasmic extract) were separately loaded on to Amberlite CG50 (NH_4^+ form) columns (30 ml). The columns were washed with distilled water (1 litre) and the antibiotic was eluted with 2 M-NH_3 (100 ml). The eluates were dried, then dissolved in 1 ml of water. A portion of this was quantified by the furfural test (Dutcher *et al.*, 1953) and another (20 μl) was spotted on a silica t.l.c. plate alongside neomycin (as its sulphate salt) and neomcyin phosphate (produced enzymically as described for ribostamycin below). A 2 mg portion of neomycin phosphate was obtained from 1 g of *E. coli* JM103[pMS5] grown in the presence of neomycin (30 mg).

Large-scale phosphorylation of ribostamycin

Phosphotransferase buffer [67 mM-Tris/maleic acid (pH 7.1 at 4 °C)/42 mM-$MgCl_2$/400 mM-NH_4Cl/2 mM-1,4-dithiothreitol; 3 ml] was mixed with 50 mM-ATP, pH 7.2 (3 ml), and 50 mg of ribostamycin sulphate, and the pH adjusted to 7.2–7.4. After equilibration to 35 °C, four successive 1 ml aliquots (1 unit) of the phosphotransferase were added to the mixture at 15 min intervals. After the last addition the reaction was allowed to proceed for another 1 h, the time at which t.l.c. analysis revealed optimal formation of the phosphorylated product. The reaction mixture was then loaded on to a column (2.5 cm × 15 cm) packed with Amberlite CG 50 resin (NH_4^+ form). The column was washed exhaustively with distilled water (1 litre) and the antibiotic was eluted with a linear gradient developed from 350 ml each of 0.05 M- and 0.27 M-NH_3 at a flow rate of 100 ml/h. Fractions (10 ml each) were collected and analysed by t.l.c. The fractions containing phosphorylated ribostamycin were pooled and dried. About 90% yield of ribostamycin phosphate (structure **2**) was obtained. When the samples were run on silica-gel t.l.c. plates using methanol/NH_3 (5:2, v/v), ribostamycin phosphate was more mobile (R_r 0.397) than ribostamycin (as its sulphate salt) (R_F 0.22).

Fast-atom-bombardment m.s. was performed on a VG TS-250 mass spectrometer and gave a molecular ion for ribostamycin phosphate at 535 $[C_{17}H_{35}O_{13}N_4P(534)+H]^+$, whereas ribostamycin as its salt ($2H_2SO_4$) gave three ions at 455, 553, 651. These were assigned to ribostamycin $[C_{17}H_{34}O_{10}N_4(454)+H]^+$, ribostamycin with one sulphate group $[C_{17}H_{34}O_{13}N_4 \cdot H_2SO_4(552)+H]^+$ and ribostamycin with two sulphate groups $[C_{17}H_{34}O_{10}N_4 \cdot 2H_2SO_4(650)+H]^+$.

N.m.r. spectra were recorded on a Bruker AM360 instrument in ^2H_2O at pH 7.2 using hexamethyldisiloxane as internal standard. The ^{31}P spectrum of ribostamycin phosphate showed the presence of a single resonance at 2.5 p.p.m., corresponding to one phosphate group. Data for ^1H and ^{13}C chemical shifts are given in Table 1.

RESULTS AND DISCUSSION

Insertion of the protein coding region of *APH* into pKK223-3

We have previously shown that the *B. circulans APH* gene is located on a 2.7 kb *Sal*I fragment. This fragment has been inserted into *Sal*I-cut pBR322 to give a recombinant plasmid,

Table 1. ^{3}H (*a*) and ^{13}C (*b*) chemical shifts (p.p.m.) of ribostamycin 3′-phosphate (structure 2)

(*a*) The ^{1}H assignments to the various hydrogen atoms in ring I of ribostamycin were made from published data (Yagisawa *et al.*, 1972; Matsuhashi *et al.*, 1977; Reid & Gajjar, 1987). (*b*) The resonances to the various carbon atoms of ribostamycin were assigned from information on neomycin (Reid & Gajjar, 1987; Rhinehart *et al.*, 1974)

(*a*)	Chemical shift (p.p.m.)	
Proton	Ribostamycin	Ribostamycin 3′-phosphate
H-1′	5.85	5.85
H-2′	3.22	3.22
H-3′	3.85	4.26
H-4′	3.50	3.54
H-5′	3.98	3.98
H-6′a	3.33	3.31
H-6′b	3.14	3.14

(*b*)	Chemical shift (p.p.m.)	
Carbon atom	Ribostamycin	Ribostamycin 3′-phosphate
C-1	52.94	53.07
C-2	32.87	34.34
C-3	51.42	51.41
C-4	80.15	81.11
C-5	87.71	87.95
C-6	73.63	73.64
C-1′	97.83	97.59
C-2′	56.55	56.25
C-3′	71.82	74.67
C-4′	71.34	71.12
C-5′	71.70	71.84
C-6′	42.99	42.91
C-1″	112.76	112.45
C-2″	75.71	76.49
C-3″	77.80	77.86
C-4″	85.15	85.03
C-5″	63.76	63.72

pCH5, which was subsequently used to transform *E. coli* HB101 to give the strain *E. coli* HB101[pCH5] (Herbert *et al.*, 1986). The sequences of the coding as well as the regulatory region of the *APH* gene are now known, and these contain a *Dde*I site 86 bp upstream of the codon for translational initiation (ATG) and another 357 bp downstream of the translational termination codon (TTT). The digestion of pCH5 with *Dde*I is expected to produce a 1229 bp fragment containing the *APH* coding sequence, another fragment of 1269 bp and several smaller fragments. In order to achieve a clear separation between the 1229 bp and 1269 bp fragments, pCH5 was digested with *Dde*I and *Sal*I and the desired 1229 bp fragment, which was the largest of the digestion products, could now be readily separated and isolated. The fragment was mixed with *Eco*RI-cut pKK223-3 and the recessed ends of the two DNA species filled in using dNTPs and DNA polymerase I (Klenow fragment). The resulting mixture was then supplemented with phage-T_4 DNA ligase to accomplish the blunt-end ligation and the mixture used to transform *E. coli* JM103. The bacterial growth was accomplished on plates using media containing ribostamycin (25 μg/ml) with or without IPTG. The same plasmid (pMS5 = pKK223-3 containing a 1232 bp fragment of the *APH* gene of *B. circulans*) was isolated from all these colonies and was shown to have the expected restriction profile. When the purified pMS5 was used to transform *E. coli* JM103 once again, the resulting strain, *E. coli* JM103[pMS5], grew well on up to 300 μg of ribostamycin/ml, but no growth could be detected in the presence of IPTG alone or IPTG + ribostamycin. This observation was unexpected, since if the *APH* gene had been placed under the control of the *tac* promoter, its expression should be dramatically increased by the presence of IPTG. That this was not the case necessitated the examination of the sequence of the recombinant plasmid, particularly in the *tac* promoter region. For this purpose a primer 3′TGTTTACTTGCTTTCA5′ that was complementary to the non-coding (sense) strand was synthesized and used to obtain the sequences between −22 and −349 nucleotides upstream (towards the 5′ end of the molecule) of the codon for protein initiation. The results showed that the *tac* promoter was intact and correctly positioned in the recombinant plasmid (Sarwar, 1989).

Factors affecting the expression of the *APH* gene

It was found that the new construct, *E. coli* JM103[pMS5], produced at least 10-fold more enzyme than did *E. coli* HB101[pCH5], in which the phosphotransferase expression was under the control of the parent *Bacillus* promoter (Fig. 2; cf. lanes 3 and 4). The increased production of the enzyme in *E. coli* JM103[pMS5] occurred in the absence of the inducer IPTG. In the presence of the latter, although there was a transient burst of phosphotransferase, this phase was short-lived and was followed

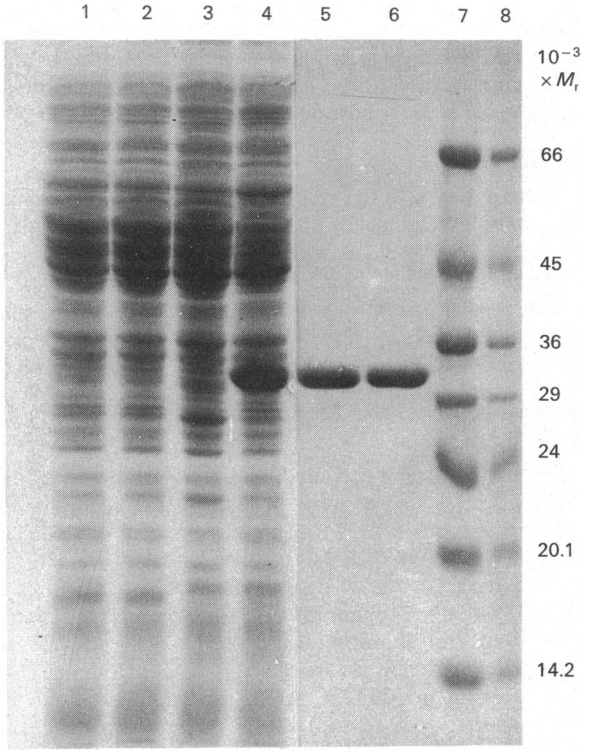


Fig. 2. SDS/PAGE of bacterial supernatants and of the purified phosphotransferases

Aliquots (20 μl containing 75–120 μg of protein) were electrophoresed on an SDS/12%-(w/v)-polyacrylamide gel in the presence of β-mercaptoethanol (Laemmli, 1970). Lane 1, supernatant from *E. coli* JM103; lane 2, *E. coli* JM103 harbouring the vector pKK223-3; lane 3, *E. coli* HB101[pCH5]; lane 4, *E. coli* JM103[pMS5]; lanes 5 and 6, purified phosphotransferase; lanes 7 and 8, M_r markers. For details of the strains, see the Materials and methods section.

by an apparent cessation of cellular protein synthesis, which was subsequently attributed to the lysis of the bacterial cells (Sarwar, 1989). This conclusion is based on the measurement of viable cell count, which after the exposure of *E. coli* JM103[pMS5] to IPTG, was reduced by more than 90% (Sarwar, 1989). The observation highlights the hazards of overproduction of foreign proteins in *E. coli*. Other examples of this phenomenon have been recorded in literature (Little, 1979; Brosius, 1984; Yudkin, 1986). The time course of the development of the phosphotransferase activity showed that it continued to increase for at least 12 h in the stationary phase and the increase was dependent on the absence of glucose from the growth medium.

Purification and properties of aminoglycoside phosphotransferase

For the large-scale purification of the cloned aminoglycoside phosphotransferase, bacteria were grown under conditions which were shown from the above experiments to give the optimal production of the enzyme (see above and Sarwar, 1989). Initially the enzyme was released from the bacterial pellet by osmotic shock, since such a procedure had originally be described by Haas & Dowding (1975) to isolate aminoglycoside phosphotransferase from an antibiotic-resistant strain of *E. coli*. This method of the release of the enzyme was based on the belief that the enzyme was located in the periplasmic space. Subsequent studies showed that the original assumption about the subcellular location of the phosphotransferase was not applicable to the strain under investigation and at least 8-fold higher enzyme activity was released by sonication compared with osmotic shock.

The purification of the phosphotransferase was achieved from the two extracts using an initial precipitation with $(NH_4)_2SO_4$, followed by affinity chromatography. Both extracts gave similar extents of protein purification; however, as expected, the overall yield was 8-fold higher when the enzyme was released by sonication. A 17 mg yield of purified enzyme were obtained from 6 g of bacterial paste (Table 2). The specific activity of crude extract varied from 1.2 to 1.9 units/min per mg of protein, whereas the specific activity of pure phosphotransferase was 12–15 units/min per mg of protein. The subunit M_r of the phosphotransferase found by SDS/PAGE was 30000–32000 (Fig. 2), which was very close to the value of 29917 predicted from the DNA sequence. The phosphotransferase elution profile from the gel-filtration column shows all the activity in one single peak corresponding to a protein of M_r 31000. Therefore the native enzyme must exist as a monomer.

Structure of the phosphorylated product

^{31}P.n.m.r. studies on the ribostamycin phosphate produced by the cloned enzyme revealed that it contains only one phosphate group in the molecule. This conclusion was confirmed by fast-atom-bombardment m.s. The regiospecificity of phosphorylation was examined by using ^{1}H and ^{13}C n.m.r., and the results are presented in Table 1. Yagisawa *et al.* (1972) and Matsuhashi *et al.* (1977) observed that, in the conversion of a related antibiotic, butirosin A, into a phosphorylated derivative by the phosphotransferase from an antibiotic-resistant isolate of *E. coli* JR66/W67 and *B. circulans*, the 3-^{1}H signal of the antibiotic at δ4.1 shifted to δ4.5. This shift of 0.4 p.p.m. was attributed to the location of the phospho group at the 3′-position of the ring I. A similar shift was observed in the present work when a signal centred at 3.85 p.p.m. in ribostamycin was found at 4.26 p.p.m. (Table 1) in the phosphorylated derivative, thus suggesting that in this case also the phospho group is located at the 3′-hydroxy group.

The 3′-position of the phospho group was further confirmed by comparing the ^{13}C spectra of ribostamycin and ribostamycin phosphate. By using the existing information on neomycin (Rinehart *et al.*, 1974; Reid & Gajjar, 1987) the assignment of ^{13}C signals to 17 C-atoms of the antibiotic are tabulated in Table 1. The data show that the most spectacular change is in the resonance due to C-3′. This carbon resonates at 71.82 ppm in ribostamycin and at 74.67 p.p.m. in the phosphorylated derivative. Another position showing a shift of δ1.47 is C-2 for which at present no satisfactory explanation is available. Notwithstanding this the cumulative data are consistent with the conclusion that the phospho group in the product was present at the C-3′ position, thus showing that cloned enzyme had the same specificity as the enzyme from the donor strain of *B. circulans* (Matsuhashi *et al.*, 1977). The cloned enzyme may therefore be designated as 3′-aminoglycoside phosphotransferase.

Subcellular location of the phosphotransferase in the engineered organism

The subcellular location of the phosphotransferases have previously been investigated in antibiotic-resistant strains of *E. coli*, and it was reported that the phosphotransferases are periplasmic enzymes (Ozanne *et al.*, 1969; Goldman & Northrop, 1976; Matsuhashi *et al.*, 1976). This aspect was examined for the organism under discussion by using the method of Weiss (1976), with modifications, and the results are summarized in Table 3. Glucose-6-phosphate dehydrogenase is a cytoplasmic enzyme, whereas β-lactamase is a periplasmic enzyme. Most of the glucose-6-phosphate dehydrogenase and the phosphotransferase were found in the cytoplasmic fraction, whereas β-lactamase was released in the periplasmic fraction. From the knowledge of the behaviour of these two marker enzymes it is obvious that phosphotransferase in *E. coli* JM103[pMS5] is a cytoplasmic enzyme. This assignment is broadly consistent with the fact that the *N*-terminal amino acid sequence of the phosphotransferase obtained by sequencing of the protein was the same as that

Table 2. Purification of phosphotransferase from *E. coli* JM103[pMS5]

The enzyme was prepared from 6 g (wet wt.) of bacteria as described in the Materials and methods section.

	Total volume (ml)	Protein (mg/ml)	Total activity (units*)	Specific activity	Yield (%)
Cell-free sonicated material	50	6	594.7	1.981	100
$(NH_4)_2SO_4$ fraction 0–60% satn.	5	30	103.9	0.693	17.5
60–90% satn.	5.5	23	347.2	2.75	58.4
Phosphotransferase eluted from the column	22	0.832	234.0	12.79	42.9

* One unit is defined as the amount of enzyme which phosphorylates 1 μmol of the antibiotic/min at 30 °C.

Table 3. Comparison of the activities of 3′-phosphotransferase, β-lactamase and glucose-6-phosphate dehydrogenase in periplasmic and cytoplasmic fractions

A 1 g (wet wt) portion of bacteria was used and processed as detailed in the Materials and methods section.

Enzyme	Activity (%)		Activity (μmol/min per g of bacteria)		Specific activity (μmol/min per mg)	
	Periplasmic	Cytoplasmic	Periplasmic	Cytoplasmic	Periplasmic*	Cytoplasmic*
Glucose-6-phosphate dehydrogenase	12.52	87.48	1.1044	7.7155	0.035	0.099
β-Lactamase	92.67	7.33	702.77	55.55	22.589	0.714
Aminoglycoside phosphotransferase	11.64	88.36	5.764	43.764	185.28	562.68

* Total protein in the periplasmic fraction was 31.1 mg, whereas that in the cytoplasmic fraction was 77.7 mg.

Table 4. *N*-Terminal amino acid sequence of aminoglycoside phosphotransferase

The sequence was determined as described in the Materials and methods section. The amino acid released as phenylthiohydantoin derivatives at each cycle was as shown.

Cycle	Amino acid [amount (nmol)]	Predicted from DNA sequence (Herbert *et al.*, 1986)
1	– (–)	Met
2	Asn (45)	Asn
3	Gln (58)	Gln
4	– (–)	Ser
5	Thr (13)	Thr
6	Arg (24)	Arg
7	Asn (34)	Asn
8	– (–)	Trp
9	Pro (8)	Pro
10	Glu (31)	Glu
11	Glu (33)	Glu
12	Leu (35)	Leu

deduced from the DNA sequence (Table 4). Generally the periplasmic enzymes have an extended *N*-terminal amino acid sequence (Blobel *et al.*, 1979; Talmadge *et al.*, 1981; Benson *et al.*, 1985; Heijne, 1988; Verner & Schatz, 1988) which is lost at some stage during its transport to the periplasmic compartment.

The deduced sequences of several aminoglycoside 3′-phosphotransferases are now available, and in none of these is there any evidence of the presence of signal sequences characteristic of periplasmic or secretary proteins (Herbert *et al.*, 1986). The subcellular location of an aminoglycoside phosphotransferase from an antibiotic-resistant isolate of *E. coli* has been determined previously, and it was suggested that the enzyme is located in the periplasmic compartment, a compartment that is generally believed to have antibiotic-modifying enzymes (Ozanne *et al.*, 1969; Matsuhashi *et al.*, 1976; Goldman & Northrop, 1976). We have, however, now shown that, consistent with the location of the phosphotransferase of *E. coli* JM103[pMS5] in the cytoplasmic fraction, growth of the organism in the presence of neomycin results in the antibiotic being exclusively found in the cytoplasmic fraction and in a phosphorylated form; no free neomycin was detected within the bacterial cell.

We thank the Government of Pakistan for a scholarship and for a CVCP ORS award to M.S. We gratefully acknowledge Dr. I. G. Giles for helpful discussions. We also thank Dr. R. P. Sharma and Dr. D. L. Corina for their help in n.m.r. spectroscopy and m.s. We thank Dr. M. G. Gore and Mr. L. Hunt of the Protein Sequencing Unit of the Institute of Biomolecular Sciences, SERC Centre for Molecular Recognition, University of Southampton, for protein sequence information. Mass spectra were taken using VG TS-250 mass spectrometer, purchased by a grant from the Wellcome Trust. Part of this work is taken from a thesis submitted by M.S. to the University of Southampton in partial fulfilment of the requirements for a Ph.D. degree (Sarwar, 1989).

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Received 4 October 1989/8 December 1989; accepted 8 January 1990