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Cloning and Sequencing of a Gene Cluster for the Resistance to Doxorubicin from *Streptomyces peucetius* subsp. *caesius* ATCC 27952

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The doxorubicin resistance locus from *Streptomyces peucetius* subsp. *caesius* (the doxorubicin producer, ATCC 27952) has been cloned. The sequence data over 4.4 kb regions reveals the presence of four possible open reading frames (ORFs). ORF2 and ORF3 would encode proteins containing 329 and 283 amino acids, respectively. The protein encoded by ORF2 has two almost identical ATP binding domains with p-glycoprotein, the product of a multidrug resistance gene from tumor cells, and that encoded by ORF3 has several hydrophobic domains suggesting that it is located in the bacterial membrane. These two remarkable similarities of the gene product to p-glycoprotein of mammalian tumor cells suggest that the two proteins may enable bacteria to extrude a variety of toxic agents, including daunorubicin and doxorubicin, by an ATP dependent efflux mechanism analogous to the multidurg resistance protein of cancer cells.

The cloning of antibiotic biosynthesis genes is a relatively new, but very active area in the study of gene organization, expression and regulation in the biosythesis of antibiotics. Since the first report on the cloning of a biosynthetic gene of actinorhodin from *S. coelicolor* (13), genes involved in the biosynthesis of more than 20 different antibiotics have been cloned (2). These reports provide much useful information for cloning antibiotic biosynthetic genes. For example antibiotic production genes appear to be clustered in the chromosome as continuous fragments of more than 20 kb (16); self-resistance genes are generally a part of the clusters (3, 5, 18); regulatory genes, either positive or negative, are often located in the production gene cluster (10); and some of the genes for related pathways, i.e. the polyketide synthase gene, show cross-hybridization with each other (14). Among the above characteristics of the gene clusters, the nature of the close linkage of self resistance and production genes offers a relatively easy straight forward method of cloning biosynthetic genes into a host such as *S. lividans* by initial selection of suitable resistant clones. *Streptomyces peucetius* subsp. *caesius* (ATCC 27952), which produces daunorubicin and doxorubicin, was chosen to isolate the doxorubicin/daunorubicin resistance gene.

The anthracycline antibiotics, daunorubicin and doxorubicin, are clinically and commercially important anticancer agents used to treat many types of cancer even though they are cummulatively cardiotoxic. The major drawback in the clinical application of doxorubicin is the appearance of resistant cancer cells that are caused by overexpression of p-glycoprotein, a product of the multidurg resistance gene (7). It has been suggested that many antibiotic resistance genes in pathogenic bacteria originate from organisms which produce the antibiotics. Therefore, the doxorubicin resistance gene of producing organism is of interest not only for studying the resistant mechanism in conjunction with regulation of doxorubicin and daunorubicin biosynthesis, but also for establishing a model system to unravel the complex mechanism of multidrug resistance in tumor cells.

In order to study the mechanism of doxorubicin resistance in the producing organism, we have cloned and

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characterized a 4.4 kb DNA fragment, which includes doxorubicin resistance genes from *S. peucetius* subsp. *caesius*. The sequence analysis of this 4.4 kb insert DNA revealed the presence of four possible open reading frames (ORF). The deduced protein of ORF2 has A- and B-type ATP binding domains that are common to the transport protein (8). The protein of ORF3 seems to be transcriptionally coupled to ORF2 and has a hydrophobic nature. These characteristics of the sequences suggest that the two proteins may function to allow the bacteria to extrude doxorubicin (daunorubicin) by an ATP-dependent efflux mechanism analogous to the multidrug resistance protein of cancer cells. The ORF2 and ORF3 domains turned out to have identical DNA sequences to those of the drr AB of strain ATCC 29050 reported by Guilfoile and Hutchinson (9). Also, the ric 1 fragment from strain 7600 reported by Colombo *et al.* revealed a restriction pattern similar to that of the 4.4 kb DNA fragment (4).

Materials and Methods

Chemicals

Doxorubicin and daunorubicin were obtained from Sigma chemical Co., St. Louis, Mo. Thiostrepton was obtained from Dr. D.W. Scott of E.R. Squibb & Sons. All other chemicals were obtained from Sigma.

Bacterial Strains and Plasmids

S. lividans 1326 and TK24 were obtained from David Hopwood of John Innes Institute and AFRC Institute of Plant Science, Norwich, United Kingdom. *S. peucetius* subsp. *caesius* ATCC 27952 and *S. galilaeus* ATCC 31133 were obtained from the American Type Culture Collection, Rockville, Md. *Steptomyces* sp. C5 was obtained from the Frederick Cancer Research Center. *Escherichia coli* strains JM109 and DH5α were used for M13 cloning. Streptomyces vectors pIJ 702 and pIJ 922 were obtained from David Hopwood. M13 phage derivatives mp18 were used for dideoxy DNA sequence analysis.

Media and Growth Conditions

Streptomyces strains were maintained on R2YE agar and *Streptomyces peucetius* subsp. *caesius* ATCC 27 952, grown in R2YE liquid at 28℃, was used for the preparation of protoplasts and the isolation of chromosomal and plasmid DNAs. *S. lividans* and recombinant strains were grown in YEME medium. Strepomyces plasmids (pIJ 702 and pIJ 922) were selected with thiostrepton at 25 μg/ml. *E. coli* was grown in LB medium at 37℃. M13 phages were propagated in 2×YT medium. Anthracycline production by *S. peucetius* was determined by HPLC analysis.

DNA Isolation and Manipulation

Chromosomal DNA was isolated from *S. peucetius* subsp. *caesius* by a modified method of Hopwood *et al.* (11). Small-scale plasmid DNA isolations from *E. coli* and *Streptomyces* were carried out as described by Maniatis *et al.* (15).

Enzymes were purchased from New England Biolabs and Promega Biotec., and used according to the manufacturers' specifications. DNA fragments were purified from gels using Geneclean II according to the manufacturer's protocol.

Transformation Procedure

Protoplasts of *S. peucetius* and *S. lividans* 1326 were prepared as described by Hopwood *et al.* (11). with the following modifications. Cells were grown in 10ml of R2YE medium in test tubes at 28℃ with shaking for 2 to 3 days. The culture was transferred to 100ml of R2YE containing 0.5% glycine in a 500 ml baffled flask and incubated for 18 to 24hours. The cells obtained were treated with 20 ml of P buffer containing 80 mg of lysozyme, and the resulting protoplasts were suspended in P buffer at a concentration of $\sim 10^9$/ml and frozen at −70℃. Streptomyces protoplasts were transformed as described by Hopwood *et al.* (11). by using 100 μl of protoplasts ($\sim 10^8$), 1 μg of plasmid DNA in 20 μl of TE buffer, and 500 μl of 25% PEG 1000 in P buffer. Samples (100 μl) were plated in 2.5 ml of 0.6% soft R2YE agar on R2YE regeneration plates. After incubation at 28℃ for 18 to 24 hours, the plates were overlaid with 2.5 ml of 0.6% soft R2YE agar containing 25 μg/ml of thiostrepton. Transformants were visible after incubation for an additional 3 to 5 days at 28℃. Competent *E. coli* JM109 cells were prepared and transformed by the calcium chloride procedure, as described by Maniatis *et al.* (15).

Southern Hybridization Analysis

DNA samples were run in 1% agarose gel and blotted to positively charged nylon membranes (Boehringer Mannheim) by capillary transfer. The probe was labeled with digoxigenin using the DNA Labeling Kit (Boehringer Mannheim). The DNA was prehybridized, hybridized and detected following the manufacturer's protocol for DIG labeled probe.

Analysis of Drug Resistance

S. lividans was tested by the gradient plate method using square plate (100 by 20 mm) and 25 ml layers of R2YE agar. Approximately 10^5 to 10^6 spores were applied in parallel lanes across a gradient of 0 to 100 μg of daunorubicin and 0 to 200 μg of doxorubicin per ml.

DNA Sequencing and Analysis

All DNA fragments were subcloned into M13 mp18. DNA sequencing was performed on single-stranded templates by the dideoxy method, using [α-^{35}S] dATP (1, 000 μci/mmol; Amersham) and Sequenase 2.0 (U.S.

Biochemicals) according to the instructions of the manufacturer. To reduce compression sequencing reactions were carried out with a 7-deaza-dGTP nucleotide mixture. Synthetic oligonucleotides (17 to 25 ıers) were used as sequencing primers. DNA sequencing reactions were separated by electrophoresis on 8% polyacrylamide −7 M urea wedge gradient gel. DNA and protein sequences were analyzed using the GENETYX (ver. 8.0), DNASIS (ver. 4.0) and FASTA computer programs.

RESULTS AND DISCUSSION

Cloning of the Doxorubicin Resistant Gene

Chromosomal DNA isolated from *S. peucetius* subsp *caesius* ATCC 27952 was partially digested with restriction endonuclease Sau3AI and then size-fractionated by sucrose gradient centrifugation. Fragments of 4 to 10 kb were ligated with *Bgl*II-cleaved and dephosphorylated vector pIJ 702. The ligation mixture was used to transform *S. lividans* 1326, which is sensitive to doxorubicin. Transformants were seleted with thiostrepton (25 μg/ml).

The resulting thiostrepton resistant transformants were screened for the doxorubicin resistant phenotype by replica-plating on doxorubicin containing plate (100 μg/ml). Three fast growing colonies were selected and the recombinant plasmids extracted from them were named pMC 1, pMC3 and pMC4, respectively (Fig. 1). All three plasmids were able to confer doxorubicin and daunorubicin resistance to *S. lividans* 1326 upon retransformation (Table 1).

Restriction Mapping of Cloned Fragments

Restriction maps of cloned fragments were obtained by digestion of the plasmids with various endonucleases. Comparison of the restriction maps of three clones indicated that pMC1, pMC3 and pMC4 shared a 2.1 kb-*Pst*I fragment and inserted DNAs of those plasmids had the same direction. All the results suggested that the

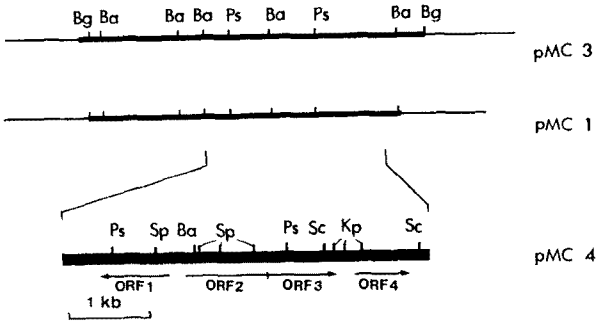

Fig. 1. Restriction maps of the pMC series.
Restriction enzyme abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; Ps, *Pst*I; Sc, *Sac*I; Sp, *Sph*I; Kp, *Kpn*I.

Table 1. Resistant level to Dnr and Dxr for strains cited in this work.

Strain	Plasmid	MIC (μg/ml)	
		Dxr	Dnr
S. lividans 1326	NO	−	−
	pIJ702	20	10
	pMC4	≥200	75
	pMC1	≥200	100
S. peucetius 27952	NO	40	−
	pIJ702	125	20
	pMC4	≥200	60
	pMC1	≥200	40

*Dxr: Doxorubicin, Dnr: Daunorubicin.

8.5 kb insert DNA of pMC3 contained the entire region of insert DNA of pMC1 and pMC4. This result was confirmed by Southern Hybridization analysis with a 2.1 kb-*Pst*I fragment as a probe. Therefore, the doxorubicin resistant gene is likely to be located within the 4.4 kb insert DNA of pMC4.

To further localize the resistant gene several subclones were made from the 4.4 kb insert of pMC4. The 2.1 kb *Pst*I fragement subcloned in pIJ 702 didn't confer resistance to the antibiotic, as in the deletion of 2.4 kb *Sph*I fragment. Deletion of the 1.3 kb *Sac*I fragment in pMC4, however, maintained resistance to the antibiotic, but at a reduced level. Further localization of the resistant gene was not successful due to a lack of suitable enzyme site.

Functional Analysis of Cloned Fragments

Both pMC1 and pMC4 increased the level of resistance to doxorubicin and daunorubicin in *S. lividans* 1326. As shown in Table 1 the level of resistance to doxorubicin was more than 10 fold greater than that obtained with the pIJ 702 vector as a control. Some partial resistance was observed in the host cells transformed with the vector alone and selection for thiostrepton resistance also increased doxorubicin resistance in *S. lividans* for unknown reasons. *S. lividans* is not significantly more sensitive to daunorubicin than to doxorubicin. However, both pMC1 and pMC4 apparently confer a higher level of resistance to doxorubicin than to daunorubicin in *S. lividans* transformants. It is difficult to explain this on the basis of ATP dependant efflux mechanism of self-resistance that will be discussed later. It is common for antibiotic producing species of Streptomyces to have more than one gene that confers resistance to the produced antibiotic, in some cases by different mechanisms (17). In the doxorubicin producer two more resistant genes have been cloned or detected (4, 19), but it is not clear whether one of them is specific for daunorubi-

cin resistance.

The effects of pMC1 and pMC4 in *S. peucetius* subsp. *caesius* 27952 were also confirmed by their ability to increase the resistance level to the antibiotics (Table 1).

Homology between Anthracycline Producers to the Resistant Gene

To determine whether other anthracycline producing species of *Streptomyces* contain the homologous region to the resistance gene, total DNA from two different species was isolated and digested with *PstI*. Fragments were separated by electrophoresis in 0.7% agarase gel, then transferred to a nylon membrane. The DNA was hybridized with a non-radiolabeled DIG probe of 2.1 kb *PstI* fragment of pMC4 that was part of the resistance gene (Fig. 2).

Total DNA of *S. lividans* 1326 was used as a control. The probe was hybridized to the same size fragment in *S. peucetius* subsp. *caesius* 27952 and to the 4.0 kb fragment in Streptomyces sp. C5 which produces ε-rhodomycinone and baumycin. This result suggests that strain C5 might have similar resistance gene. Surprisingly DNA fragment from *S. galilaeus* 31133, which produces aclacinomycin A, didn't hybridize with the probe. Aclacinomycin A, baumycin and doxorubicin have a common biosythetic step from hypothetical decaketide to aklavinone followed by diverging to either direct glycosylation for aclacinomycin A (21) or hydroxylation to C-11 of aklavinone prior to glycosylation for baumycin and doxorubicin, as shown in Fig. 3. Furthermore, the mode of action of aclacinomycin A is identical to that of doxorubicin. However, aclacinomycin A was equally effective for both doxorubicin resistant and sensitive p388 cell-lines while doxorubicin required a much higher dose for resistant cells than for sensitive cell (20). This difference in drug sensitivity is due to the multidrug resistance protein of the cell-line, which is likely to be analogous to the self-resistance mechanism of doxorubicin producers. This fact suggests that aclacinomycin A producer have a different self-resistance mechanism than doxorubicin producer. This result indicates that strain C5 is more closely related to strain 27952 than strain 31133.

Sequencing and Characterization of the 4.4 kb Fragment in pMC4

Due to the difficulty in localization the resistant gene we decided to sequence the 4.4 kb DNA fragment. The sequence was analyzed by a codon preference program (6) to locate a likely open reading frame by virtue of a high bias toward G or C in the third position of Streptomyces codons. Four putative ORFs within the sequence and the deduced amino acid sequences of ORF2 and ORF3 are shown in Figs. 4 and 5. The start site of the four ORFs were provisonally allocated by chosing the most upstream ATG or GTG preceded by a potential ribosome binding site. Based on this analysis the most

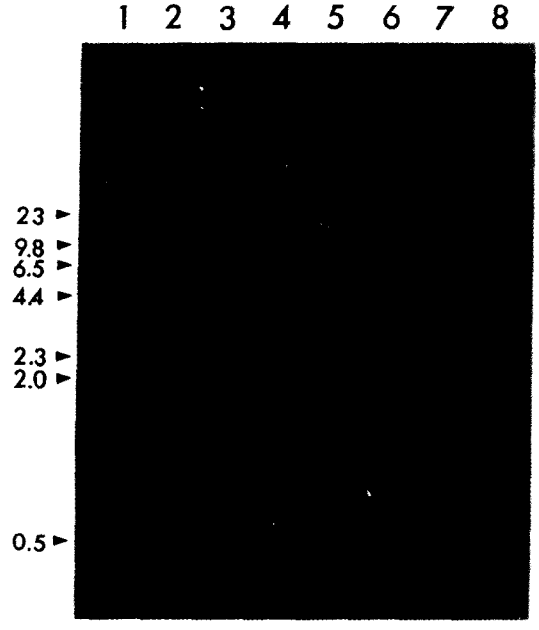

Fig. 2. Southern blot analysis of the strains with a non-radioactive labelled 2.1 kb-*PstI* fragment of pMC4. Lanes: 1. λ/*Hin*dIII, 2. ATCC 29050 DNA/*PstI*, 3. pMC1 /*PstI*, 4. ATCC 31133 DNA/*PstI*, 5. ATCC 27952 DNA/*PstI*, 6. C5 DNA/*PstI*, 7. *S. lividans* 1326 DNA/*PstI*, 8. pMC4/*PstI*.

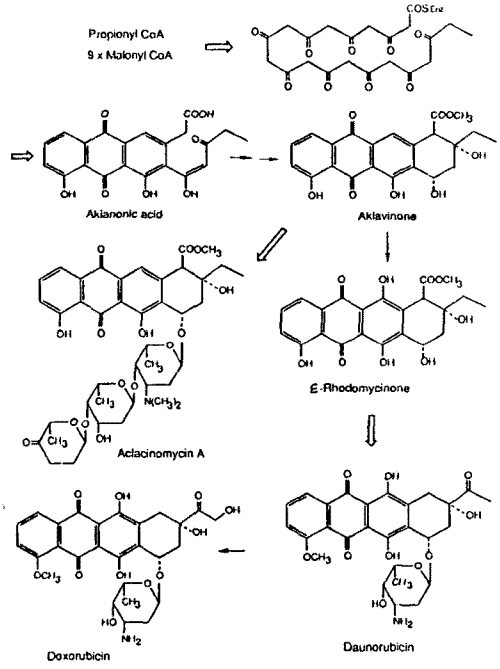

Fig. 3. Proposed biosynthetic pathway for doxorubicin and aclacinomycin A.

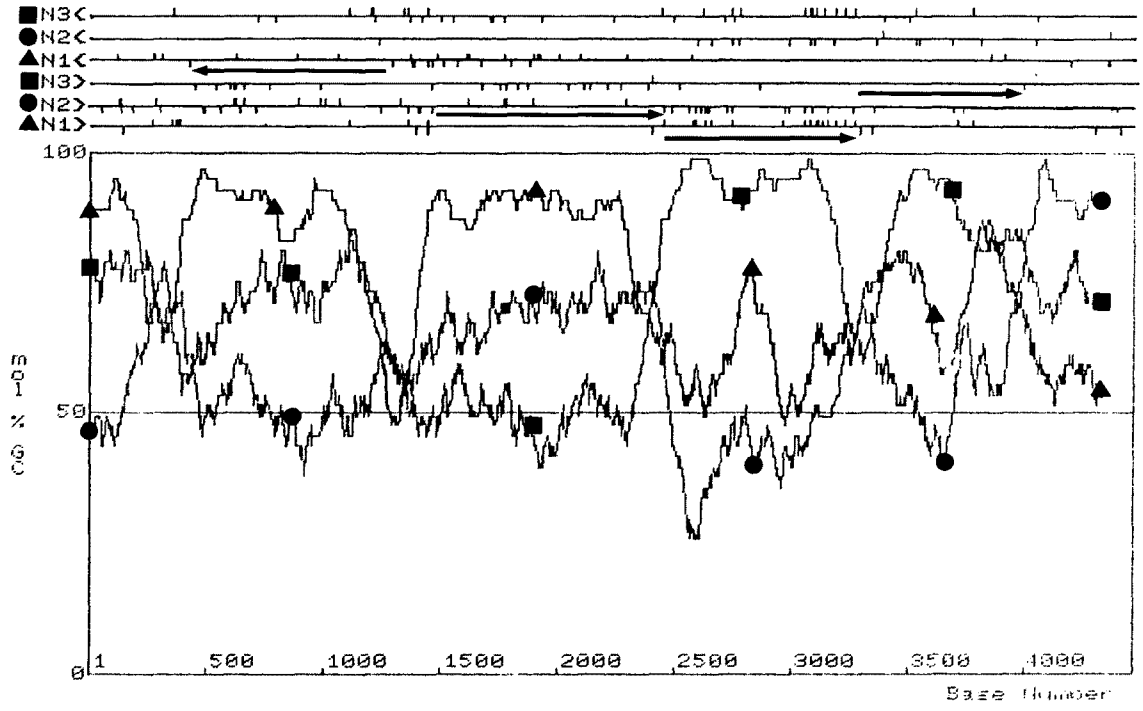

Fig. 4. Frame analysis of the 4,464 bp resistance region using an average span of 50 triplets.

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GAAGCTCTCCGCGAACTGTGCAGGAACGCGACCGCCCACGCGCTCCACGACCTTGATGGTGAAGTCG      67
CCCTGGGCGCCGCGGATGTCGTCCGTGGCCAGGATCTCGTCGGCCACGCCGCCGAAGCGGAACAGTT     134
CCATGGTGCGCGGGTTCTGGCCTGCCGCCTTGGGCAGGACCGAGGTGCTGGCATGCCGCTCCACCAG     201
CAGGACCCGCGCCCCCCGGCGGGCGAGGAACAGGGCGGTGGACAGCCCCCCGAGACCGCCGCCCACC     268
ACGAGGACATCGACATCCGGCTTCGTCAAGGCCACCTCAACCTCCTGAGTTCGCGGCGTCTGTTGCC     335
GCACCCCCAGCGTCGGGCGCCCCACTCGAAAGGGTCACGAGGCGCGGTGGAGCCCCGTACGAAAAAT     402
GGCTCGGCGAACGAAGCCATCGCGGCCATGAAGTGTCCTCATTGGGGGCTACGGTACTCAACGTGAA     469
CCCGATTGACCGAAATAACTTACAGTGCACGCTTTGTCCGCTCCATGTGACTACTGGGGGCGTTAG      535
 GTG AAC ACG CAG CCG ACA CGG GCC ATC GAA ACG TCC GGT CTC GTC AAG GT      585
 Met Asn Thr Gln Pro Thr Arg Ala Ile Glu Thr Ser Gly Leu Val Lys Va
C TAC AAC GGG ACG AGG GCG GTG GAC GGC CTG GAC CTC AAC GTC CCG GCC G     635
l Tyr Asn Gly Thr Arg Ala Val Asp Gly Leu Asp Leu Asn Val Pro Ala G
GT CTC GTC TAC GGG ATC CTG GGG CCG AAC GGC GCC GGC AAG TCC ACC ACC      685
ly Leu Val Tyr Gly Ile Leu Gly Pro Asn Gly Ala Gly Lys Ser Thr Thr
ATC CGC ATG CTG GCG ACG CTG CTG CGT CCC GAC GGC GGT ACG GCC CGG GT      735
Ile Arg Met Leu Ala Thr Leu Leu Arg Pro Asp Gly Gly Thr Ala Arg Va
C TTC GGT CAC GAC GTG ACG AGC GAA CCC GAT ACG GTC CGC CGC AGG ATC A     785
l Phe Gly His Asp Val Thr Ser Glu Pro Asp Thr Val Arg Arg Arg Ile S
GT GTC ACC GGC CAG TAC GCC TCC GTC GAC GAG GGC CTG ACC GGT ACG GAG      835
er Val Thr Gly Gln Tyr Ala Ser Val Asp Glu Gly Leu Thr Gly Thr Glu
AAC CTC GTC ATG ATG GGC CGG CTC CAG GGC TAC TCC TGG GCC CGG GCC AG      885
Asn Leu Val Met Met Gly Arg Leu Gln Gly Tyr Ser Trp Ala Arg Ala Ar
G GAG CGC GCC GCC GAA CTG ATC GAC GGC TTC GGA CTG GGC GAC GCC GCG C     935
g Glu Arg Ala Ala Glu Leu Ile Asp Gly Phe Gly Leu Gly Asp Ala Ala H
AC CGG CTC CTG AAG ACC TAC TCC GGT GGC ATG CGG CGG CGG CTC GAC ATC      985
is Arg Leu Leu Lys Thr Tyr Ser Gly Gly Met Arg Arg Arg Leu Asp Ile
GCC GCG AGC ATC GTC GTC ACC CCC GAC CTG CTG TTC CTG GAC GAA CCG AC     1035
Ala Ala Ser Ile Val Val Thr Pro Asp Leu Leu Phe Leu Asp Glu Pro Th
C ACC GGT CTC GAC CCG CGC AGC CGG AAC CAG GTC TGG GAC ATC GTG CGG G    1085
r Thr Gly Leu Asp Pro Arg Ser Arg Asn Gln Val Trp Asp Ile Val Arg A
CT CTG GTG GAC GCC GGC ACG ACG GTG CTG CTG ACG ACG CAG TAC CTC GAC     1135
la Leu Val Asp Ala Gly Thr Thr Val Leu Leu Thr Thr Gln Tyr Leu Asp
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(continued)

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GAG GCG GAC CAA CTG GCC GAC CGA TCG CGG TAC ATC GAC CAC GGC CGG GT    1185
Glu Ala Asp Gln Leu Ala Asp Arg Ser Arg Tyr Ile Asp His Gly Arg Va
G ATC GCG GAG GGC ACC ACC GGG GAG CTG AAG TCC TCG CTC GGT CCA ACG T  1235
l Ile Ala Glu Gly Thr Thr Gly Glu Leu Lys Ser Ser Leu Gly Pro Thr S
CC TGG CTG CGC CTC CAC GAC GCC CAG TCG CGT GCC GAG GCG GAA CGG CTG   1285
er Trp Leu Arg Leu His Asp Ala Gln Ser Arg Ala Glu Ala Glu Arg Leu
CTG AGC GCG GAA CTG GGC GTC ACG ATC CAC CGC GAC TCC GAT CCG ACG GC   1335
Leu Ser Ala Glu Leu Gly Val Thr Ile His Arg Asp Ser Asp Pro Thr Al
G CTG AGC GCG CGG ATC GAC GAC CCC CGG CAG GGC ATG CGG GCC CTG GCC G  1385
a Leu Ser Ala Arg Ile Asp Asp Pro Arg Gln Gly Met Arg Ala Leu Ala G
AG CTG TCG CGC ACG CAC TTG GAG GTA CGC AGT TTC TCC CTC GGC CAG TCC   1435
lu Leu Ser Arg Thr His Leu Glu Val Arg Ser Phe Ser Leu Gly Gln Ser
AGT CTC GAC GAG GTC TTC CTG GCG CTG ACC GGC CAC CCG GCC GAT GAC CG   1485
Ser Leu Asp Glu Val Phe Leu Ala Leu Thr Gly His Pro Ala Asp Asp Ar
G TCC ACG GAA GAA GCG GCG GAA GAG GAG AAG GTG GCA TGA CG ACG TCC CC  1535
g Ser Thr Glu Glu Ala Ala Glu Glu Glu Lys Val Ala ***
                                                  Met Thr Thr Ser Pr
C GGC ACC GTG GAA TCC ACG ACC CCT GTG AGC GGT CAG CTG CGG ACG GTG C   1585
o Gly Thr Val Glu Ser Thr Thr Pro Val Ser Gly Gln Leu Arg Thr Val L
TG TCC GCG GGT GAA CGG CCG GCC CGC GCG ACG GCG GTG TCC GCC ACC CTG   1635
eu Ser Ala Gly Glu Arg Pro Ala Arg Ala Thr Ala Val Ser Ala Thr Leu
ACC CAT CTG TGG CGG GCG ATG ATG GCG TTC AAG CAC TTC CCG GTG CAG CT   1685
Thr His Leu Trp Arg Ala Met Met Ala Phe Lys His Phe Pro Val Gln Le
G ATC GAC ATC GTC CTG ATG CCG TTG ATC TTC CTG CTG ATG TTC ACG TAC C  1735
u Ile Asp Ile Val Leu Met Pro Leu Ile Phe Leu Leu Met Phe Thr Tyr L
TG TTC GGC GGG GCG TTC GCG GAC TCC ACC GAG GAG TAC CTG CAG TTC TAC   1785
eu Phe Gly Gly Ala Phe Ala Asp Ser Thr Glu Glu Tyr Leu Gln Phe Tyr
CTC CCG GGC GTG ACG GTG CAG GCG GTC GTC ATG ATG ACG GTC TAC ACC GG   1835
Leu Pro Gly Val Thr Val Gln Ala Val Val Met Met Thr Val Tyr Thr Gl
C ACC TCG CTC AAC ACG GAC ATC CAC AAG GGC GTA TTC GAC CGT TTC CGG A  1885
y Thr Ser Leu Asn Thr Asp Ile His Lys Gly Val Phe Asp Arg Phe Arg T
CG CTC CCC TTC TGG CAG CCG GCG ACG CTC GCG GGC AGT CTC CTC GGT GAC   1935
hr Leu Pro Phe Trp Gln Pro Ala Thr Leu Ala Gly Ser Leu Leu Gly Asp
GTG CTC CGG TAC GTC GTC GCC CTG GCG ACC ACG GTG TCC CTC GGG CTG CT   1985
Val Leu Arg Tyr Val Val Ala Leu Ala Thr Thr Val Ser Leu Gly Leu Le
G CTG GGC TTC CGC GCC GAC GGC GGT TTC CTC GGC GTG GTC GGC GCG ATG C  2035
u Leu Gly Phe Arg Ala Asp Gly Gly Phe Leu Gly Val Val Gly Ala Met L
TG GTG CTG ATC GTC TTC GGG TTC AGC GTG AGC TGG ATC TTC GCC GCC CTG   2085
eu Val Leu Ile Val Phe Gly Phe Ser Val Ser Trp Ile Phe Ala Ala Leu
GGC GTG GTG GCC AGC GAG CCC GAA CGG TTC TCC GGG ACC AGC ATG ATC GT   2135
Gly Val Val Ala Ser Glu Pro Glu Arg Phe Ser Gly Thr Ser Met Ile Va
G CTG TAT CCG CTG CTG TTC ATG AGC AAC ATC TTC GTC ATG CCG GAG ACG A  2185
l Leu Tyr Pro Leu Leu Phe Met Ser Asn Ile Phe Val Met Pro Glu Thr M
TG CCC GGC TGG ATG CAG GCC ATC GTC GAC GCC AAC CCG ATG AGC CAC GCG   2235
et Pro Gly Trp Met Gln Ala Ile Val Asp Ala Asn Pro Met Ser His Ala
GCG ACG GCC TCC CGC GAG CTC ATG CAC GGC ACG GCC GGC TTC TGG GAC GT   2285
Ala Thr Ala Ser Arg Glu Leu Met His Gly Thr Ala Gly Phe Trp Asp Va
G GGC CTG GTC CTG TGC ATG TCG GCA GGG CTG GTG GCC GTC TTC GCA CCG C  2335
l Gly Leu Val Leu Cys Met Ser Ala Gly Leu Val Ala Val Phe Ala Pro L
TG ACC ATG CGT CTG TAC CGC AAC AAG AAC GCC CAC TGA CACAGGTACCGGAGTG  2388
eu Thr Met Arg Leu Tyr Arg Asn Lys Asn Ala His ***
CCGCTCGCACCGCGCTGGAGCGTCGTTGAGGGGGTGTGAGCGGCGCACCTACTGTCCCCGGAGACAC  2455
ATCCCGGAGATTTCACAGACACCTGGAAGGGGCGTCTCCCGTGGCCCGGTACCCACAGGCTCCCTCG  2522
GCACCGCACGCACAGGTCCGGCCGTCCTCCGTCGTCGGCCGGCACGATCCCCGTTACCCCGATCTGG  2589
TCGGCCGCGGCATCAACGCCCGCTTCACCCCCGACCCCGACACCGTCGGGTGGTGGCCACCGCGGAG  2656
CAGGCCGTGCGGGCGGTGCAGGACAGCGTGCGCGACGGTACCCGGCTGGCGGTGCGCACGGGGGGGC  2723
ACTGCTTCGAGTCGCTCGTGGACGACCCCGCCGTCACCACGGTGATCGACGTGTCCGAGATGCGGTC  2790
GGTGTACTTCGACGGGGAACTGAACGCGTTCTCCGTCGACTCGGGAGCCACCCTCGGCACCATGTAC  2857
CGGAGCCTGTACCTCGGGTGGGACGTCACCGTCCGGCCGGACGCTGCCCCGAGGTCGGGTCGGCGGC  2924
CACATCGCGGGCGGCGGGGGCGGACGGCTGTCGCCGACGTACGGACTGTCGGTCGACCATCTCCACG  2991
GAGTCGAGGTCGTGGTGGTG                                                  3011
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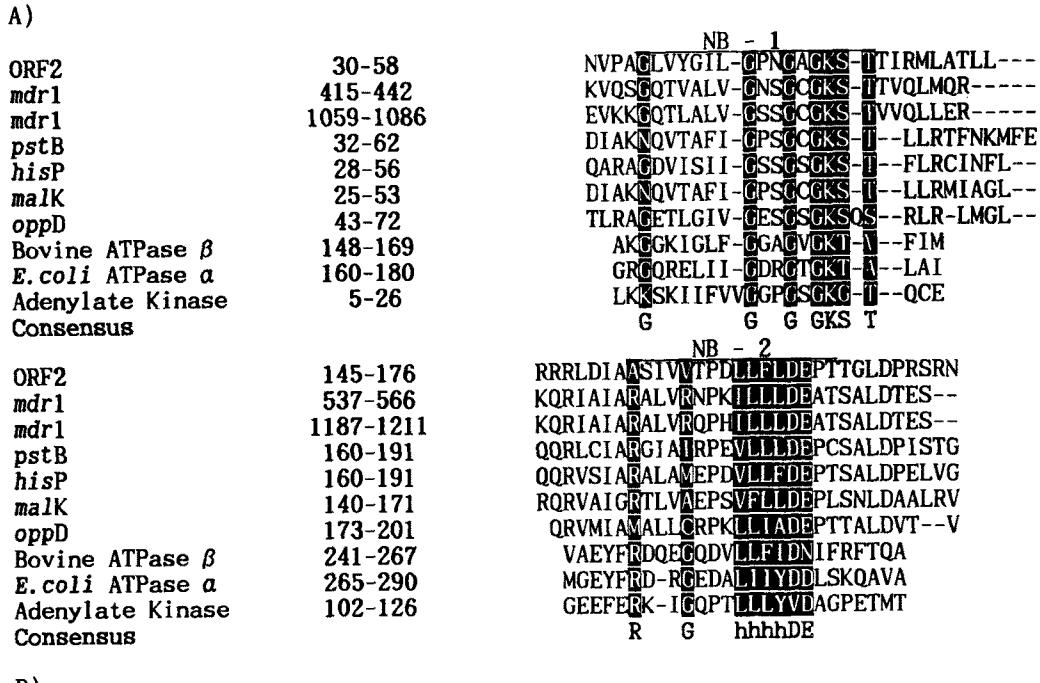
Fig. 5. DNA sequence of the 3,011 bp region containing ORF2 and ORF3.

The putative ribosome binding site and translational start sites for ORF2 and ORF3 are singly and doubly underlined, respectively.

A)



B)

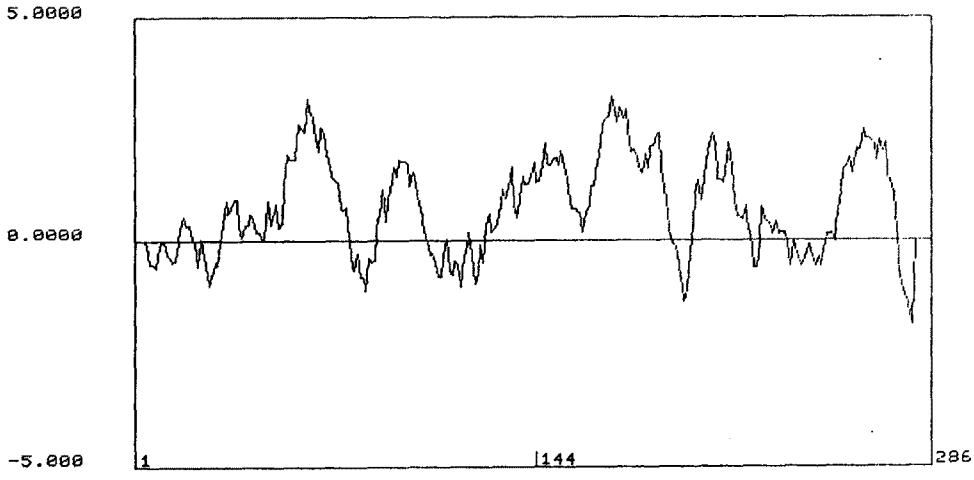


Fig. 6. (A) Alignment of amino acid sequences of consensus nucleotide binding sites (NB-1 and NB-2). (B) Hydrophobicity analysis of the deduced product of ORF3 using the program GENETYX.

probable translational start site for ORF2 is GTG at position 536-538 and the stop site is TGA at position 1523-1525. The sequence GTG at position 536 preceded by ribosome binding site (RBS) GGGGG which shows a high degree of complementarity to the 3' end of *S. coelicolor* A3 (2) 16S rRNA (5'-ACCUCCUUUCU-OH 3') (1). Another ORF 3 is likely to start at the position 1522-1524 and stop at position 2373. These ORF2 and ORF3 are likely to be translationally coupled with overlapping stop and start codons.

In computer analysis using the FASTA program sequences of many proteins are as homologous to that of the protein encoded by ORF2. Among others, histidine permease inner membrane receptor protein, hemolysin B of *E. coli* nodulation protein of *Rhizobium leguminosa*, ribose transport protein and the multidrug resistant protein of tumor cells are of interest because most of them belong to a ATP-binding transport protein. The deduced amino acid sequence of ORF2 contains both an A-type and a B-type nucleotide binding consensus sequence, and an alignment illustrating the homology between the many ATP binding domains from several

proteins is shown in Fig. 6(A). In contrast, no protein similar to an ORF3 product was found in this same data-base. However, hydrophobicity analysis (12) indicated that the protein encoded by ORF2 is hydrophilic and the ORF3 product could be hyrophobic, suggesting a hydrophobic integral membrane protein (Fig. 6(B)).

The human multidrug resistance protein GP170 is composed of two main domains. One is a predicted transmembrane domain, and the other is the intra-cytoplasmic part of the protein containing two ATP binding regions (8). The multidrug transport protein is known to use the energy of ATP to extrude a variety of toxic drugs from cells. The presence of a highly conserved ATP binding domain in ORF2, the hydrophobic nature of ORF3, and the translationally coupled gene organization of the two ORFs suggest a great similarity between the two deduced proteins and the mdr protein GP170. Therefore, it is likely that these two proteins act jointly to confer doxorubicin and daunorubicin resistance in the producing organism by ATP dependant efflux of antibiotics as proposed in P-glycoprotein of the multidrug resistant phenotype.

Further study to identify the doxorubicin binding protein in this transport process is required.

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