終 わ り に

以上，概説したように，現在，多種多様なβ-ラクタマーゼを産生する細菌が出現し，患者材料から実際に分離されている。この様な状況下で，一部ではあるが，例えばESBL産生菌による感染症であるにもかかわらず，抗菌薬の院内投薬プロトコルに従って，盲目的にCAZなどの第三世代セフェム薬を投与し続けたり，あるいは，メタロ-β-ラクタマーゼ産生菌による感染症の患者に対し，広域抗菌スペクトルを期待して，漫然とセファマイシンやカルバペネムを投与し，その結果，病院（病棟）自体がこれらの耐性菌を“増幅”する“培養装置”と化している施設も見受けられる。このような事態を避けるためには，臨床分離されるβ-ラクタム耐性菌が，単にβ-ラクタマーゼを産生しているか否かという点に留まらず，さらに，どの種類のβ-ラクタマーゼを産生しているのかを十分に考慮しつつ，適正な抗菌薬を選択するというきめ細かな化学療法の実施が必要となっている。

2001年のDRG/PPSの導入後，薬剤耐性菌による院内感染症や術後感染症のコントロールに失敗した医療施設は，経営的に大きな困難に遭遇する事態も懸念されている。したがって，全ての臨床家は専門の如何を問わず，薬剤耐性菌による感染症への対策や抗菌薬の適正使用などの諸問題を避けて通れない状況に直面していると言えよう。

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Cloning and Genetic Analysis of the UV Resistance Determinant (*uvr*) Encoded on the *Enterococcus faecalis* Pheromone-Responsive Conjugative Plasmid pAD1

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The conjugative pheromone-responsive plasmid pAD1 (59.6 kb) of *Enterococcus faecalis* encodes a UV resistance determinant (*uvr*) in addition to the hemolysin-bacteriocin determinant. pAD1 enhances the UV resistance of wild-type *E. faecalis* FA2-2 and *E. faecalis* UV202, which is a UV-sensitive derivative of *E. faecalis* JH2-2. A 2.972-kb fragment cloned from between 27.7 and 30.6 kb of the pAD1 map conferred UV resistance function on UV202. Sequence analysis showed that the cloned fragment contained three open reading frames designated *uvrA*, *uvrB*, and *uvrC*. The *uvrA* gene is located on the pAD1 map between 28.1 and 29.4 kb. *uvrB* is located between 30.1 and 30.3 kb, and *uvrC* is located between 30.4 and 30.6 kb on the pAD1 map. The *uvrA*, *uvrB*, and *uvrC* genes encode sequences of 442, 60, and 74 amino acids, respectively. The deduced amino acid sequence of the *uvrA*-encoded protein showed 20% homology of the identical residues with the *E. coli* UmuC protein. Tn*917* insertion mutagenesis and deletion mutant analysis of the cloned fragment showed that *uvrA* conferred UV resistance. A palindromic sequence, 5′-GAACNGTTC-3′, which is identical to the consensus sequence found within the putative promoter region of the *Bacillus subtilis* DNA damage-inducible genes, was located within the promoter region of *uvrA*. Two *uvrA* transcripts of different lengths (i.e., 1.54 and 2.14 kb) which terminate at different points downstream of *uvrA* were detected in UV202 carrying the deletion mutant containing *uvrA*. The longer transcript, 2.14 kb, was not detected in UV202 carrying the deletion mutant containing both *uvrA* and *uvrB*, which suggests that *uvrB* encodes a terminator for the *uvrA* transcript. The *uvrA* transcript was not detected in any significant quantity in UV202 carrying the cloned fragment containing *uvrA*, *uvrB*, and *uvrC*; on the other hand, the 1.54-kb *uvrA* transcript was detected in the strain exposed to mitomycin C, which suggests that the UvrC protein functions as a regulator of *uvrA*.

Certain conjugative plasmids of *Enterococcus faecalis* transfer at high frequency in broth mating, at a frequency of 10^{-3} to 10^{-1} per donor cell within a few hours (8, 9, 13). This transfer is related to the plasmid response to a specific peptide sex pheromone secreted by the potential recipient (8, 9, 13). The sex pheromone induces the synthesis of an aggregation substance on the donor cell surface that facilitates the formation of a mating aggregate (8, 9, 13). Of these plasmids, the pheromone-related conjugation systems of plasmids pAD1 (8, 9, 11, 21, 40, 48, 49, 58), pCF10 (7, 14, 29, 30, 41), and pPD1 (17, 18, 35, 51, 62), which respond to the sex pheromones cAD1, cCF10, and cPD1, respectively, have been studied in detail. Determinants encoded on the pheromone-responsive plasmids include hemolysin, bacteriocin, drug resistance, and UV light resistance. The conjugative plasmid pAD1 encodes hemolysin-bacteriocin (Hly-Bac) proteins mediated by the same genetic determinant (27) and a UV resistance gene (*uvr*) (6, 8, 22, 25, 28) and belongs to the incHly group (11, 22). Hly-Bac is believed to contribute to virulence in the opportunistic pathogen (6, 8, 22, 25, 28). Uvr contributes to the protection of *E. faecalis* strains carrying pAD1 against UV light or DNA damage. Of these two phenotypes, the mechanisms of Hly-Bac production and expression have been analyzed in detail (3, 19, 20, 24, 27, 44). Most (over 90%) of the Hly-Bac plasmids identified from clinical isolates are identical, exhibit extensive homology to pAD1, respond to cAD1, and are classified as members of the incompatibility group incHly (12, 23). pAD1 is representative of this group.

The UV resistance genes (*uvr*) or DNA damage-inducible genes have been analyzed in detail for *Escherichia coli* (34, 60). These genes are included in the SOS regulatory system, are induced by a variety of agents that cause DNA damage, and function in DNA repair and mutagenesis of the bacterial chromosome (2, 34). SOS-like DNA repair systems have been reported for other bacterial species (37, 43, 47, 52). In gram-positive bacteria, the SOS response is well conserved in *Bacillus subtilis*. The conjugative plasmid pAD1 conferred UV resistance function on an *E. faecalis* host. UV resistance activity is observed when the plasmid is introduced into *E. faecalis* UV202 (10, 61). *E. faecalis* UV202 is a UV-sensitive derivative of *E. faecalis* JH2-2 and is deficient in recombination (61). When pAD1 is introduced into *E. faecalis* UV202, it enhances the UV resistance of *E. faecalis* UV202 (10). Tn*917* insertion mutants of pAD1 show that the *uvr* determinants are located in the 28.2- to 28.6-kb region of the pAD1 map (10, 50). In this report, we describe the cloning of the *uvr* determinant and the regulation of the *uvr* gene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. faecalis* strains were grown in Todd-Hewitt broth (THB) (Difco Laboratories, Detroit, Mich.) or N2GT broth (nutrient broth no. 2; Oxoid Ltd., London, England) supplemented with 0.2% glucose and 100 mM Tris-HCl (pH 7.5). N2GT broth was also used in the mating experiments. *E. coli* strains were grown in Luria-Bertani medium. Agar plates

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
E. faecalis		
DS16	*tet* pAD1(Hly/Bac) pAD2[Sm Km Em(Tn*917*)]	54
UV202	*rif fus*; UV-sensitive mutant of JH2-2	61
E. coli		
DH5α	*supE44ΔlacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
DH1	*supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	42
ME8412	*umuC*::Tn*10 thr-1 leuB6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE44*	National Institute of Genetics, Genetic Stock Research Center, Mishima, Japan
ME8368	F⁻ umuC::Tn*5 thr-1 leuB6 thi-1 lacY1 galK2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 rpsL31 tsx-33 supE44*	National Institute of Genetics, Genetic Stock Research Center, Mishima, Japan
Plasmids		
pAD1	*uvr hly/bac*; 59.6-kb conjugative plasmid from DS16	11
pAM2011E	*uvr* Em(Tn*917*); miniplasmid derived from pAD1	57
pAM401	*cat tet*; *E. coli-E. faecalis* shuttle vector	59
pMW119	*bla lacZ*	Nippon Gene Co., Ltd.

were prepared by adding 1.5% agar to broth media. All bacterial strains were grown at 37°C. Antibiotics were used at the indicated concentrations: ampicillin, 100 μg/ml; erythromycin, 10 μg/ml; chloramphenicol, 20 μg/ml; fusidic acid, 25 μg/ml; rifampin, 25 μg/ml; tetracycline, 10 μg/ml; streptomycin, 500 μg/ml; kanamycin, 500 μg/ml; gentamicin, 500 μg/ml. Rifampin and fusidic acid were kindly provided by Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan, and LEO Pharmaceutical Products, Ballerup, Denmark, respectively. Streptomycin was provided by Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan, and Sankyo Pharmaceutical Co. Ltd., Tokyo, Japan.

Isolation and manipulation of plasmid DNA. Plasmid DNA was isolated by the alkaline lysis method (42). Plasmid DNA was treated with restriction enzymes and submitted to agarose gel electrophoresis for analysis of DNA fragments, etc. Restriction enzymes were obtained from Nippon Gene (Toyama, Japan), New England Biolabs, Inc. (Beverly, Mass.), and Takara (Tokyo, Japan) and were used in accordance with the supplier's specifications. Agarose was obtained from Wako Chemicals, Osaka, Japan. Gels with a 0.8% agarose concentration were used for size determination of large DNA fragments (greater than 0.5 kb), and 1.2% agarose gels were used to determine the sizes of smaller fragments (less than 0.5 kb) (17). A glass milk kit (Gene Clean II kit; Bio 101, Inc., La Jolla, Calif.) or low-melting-point agarose and β-agarase I (Nippon Gene) were used for the elution of the DNA fragments from agarose gels. The eluted fragments were ligated to dephosphorylated, restriction enzyme-digested vector DNA with T4 DNA ligase and were then introduced into *E. coli* by electrotransformation (16). Transformants were selected on Luria-Bertani medium agar containing suitable antibiotics.

Transposon mutagenesis. Tn*917* insertion mutants were generated as described previously (27). Exponentially growing DS16 cells were exposed to erythromycin (0.5 μg/ml) for 4 h. The cells were washed once with N2GT and mated with UV202 in N2GT broth for 4 h. Transconjugants were selected on THB agar plates containing erythromycin, rifampin, and fusidic acid. Of the erythromycin-resistant transconjugants obtained, streptomycin- and kanamycin-sensitive transconjugants were selected to exclude transconjugants carrying pAD2, which confers resistance to erythromycin, streptomycin, and kanamycin. The isolated transconjugants were assumed to carry pAD1::Tn*917* only. Forty microliters of plasmid-free UV202 and UV202(pAD1::Tn*917*) cultures was streaked onto well-dried THB agar plates. After the liquid was adsorbed, the cells were irradiated with a germicidal lamp for 0, 3, 6, 9, 12, and 15 s by progressive movement of a piece of cardboard over the plate to reveal the streaks. Irradiation was carried out with a Panasonic GL-15 germicidal lamp at a distance of 30 cm. After overnight incubation at 37°C, the UV sensitivity was determined. Strains able to grow in the least irradiated area and which were as sensitive as plasmid-free UV202 were used for further study.

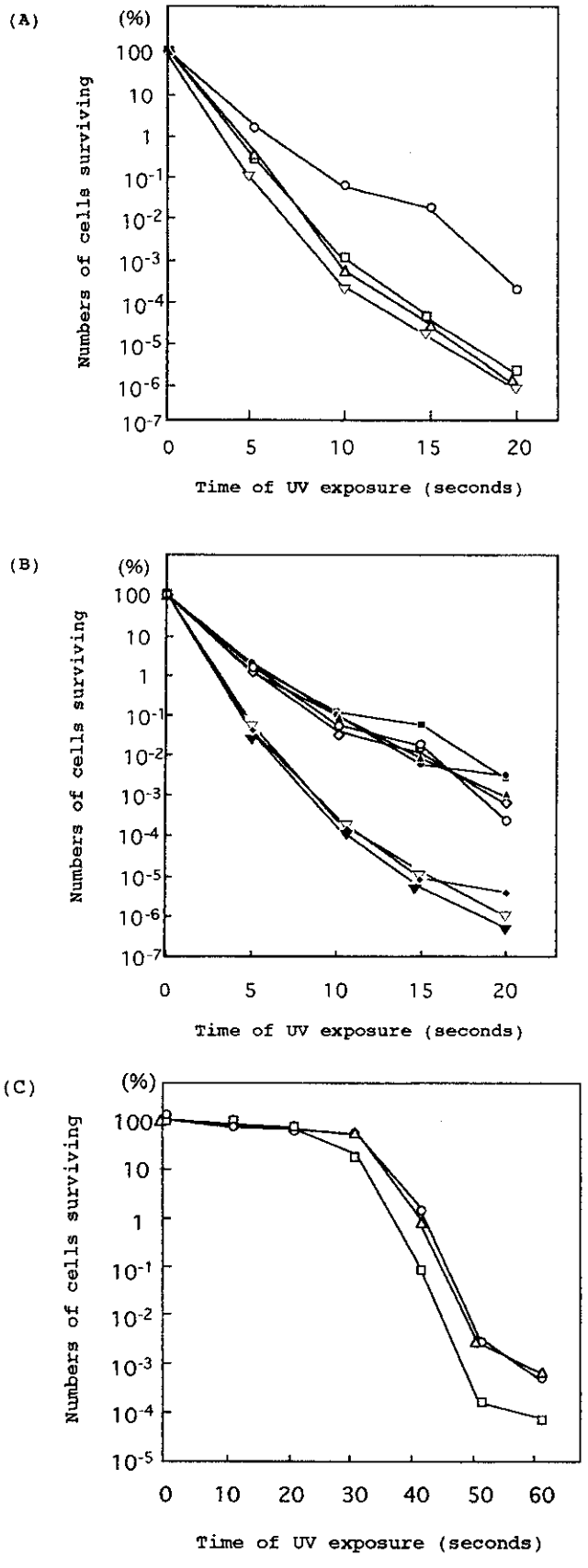
Survival curve with UV light. Five-milliliter aliquots of overnight culture in petri dishes (9 cm in diameter) were irradiated for various periods with UV light with a germicidal lamp at a distance of 30 cm. The amount of UV radiation used in the assay was 1.14 J/m²/s. The bacterial cultures were appropriately diluted and plated on THB agar plates, and the number of survivors was counted after overnight incubation at 37°C.

DNA sequence analysis. The nucleotide sequence of the *uvr* gene of pAD1 was determined from a series of nested deletion mutants with the ABI 373A automated DNA sequencer (Applied Biosystems, Perkin-Elmer, Foster City, Calif.). The nested deletion kit was purchased from Nippon Gene. The cloned plasmid DNAs were digested with *Sph*I and *Bam*HI and treated with exonuclease III for various periods of time. This treatment was followed by incubation with mung bean nuclease and then Klenow fragment and self-ligation with T4 DNA ligase. Transformation of DH1 or DH5α with the ligation product was carried out by electroporation (16). The M13 reverse dye primer and the *Taq* dye primer cycle sequencing core kit (Applied Biosystems, Perkin-Elmer) were used for sequencing of the *uvr* gene. The *Taq* Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems, Perkin-Elmer) and an 18-bp primer (TCCTAAACACTT AAGAGA) which annealed to a site 112 bp upstream of the Tn*917* left end were used to map the Tn*917* insertion point (45). All sequencing reactions were performed according to the manufacturer's protocol.

Analysis of transcripts. The extraction of RNAs by two different methods was compared. RNAs were extracted either with the FastPrep FP120 Instrument of Bio 101 or by the method described below. The results of analysis of transcripts obtained by these two methods were not essentially different. Thus, we used the method described in this section throughout this work. RNAs were prepared according to the method described previously with some modifications (45, 49). Overnight cultures of *E. faecalis* UV202 carrying the plasmids of interest were diluted to 10% in 30 ml of fresh N2GT medium, and the cells were grown for 3 h. Mitomycin C was then added to the cultures at a concentration of 2 μg/ml to determine the effects of exposure. At certain points of the time course, chloramphenicol (200 μg/ml) and sodium azide (20 mM) were added to stop cell growth. The cells were chilled on ice, harvested, and suspended in 2 ml of 25% sucrose in Tris-HCl (pH 8.0)–100 μl of 20-mg/ml lysozyme. The cells were maintained on ice for 30 min, pelleted, and resuspended in 300 μl of lysis buffer (20 mM Tris-HCl [pH 8.0], 3 mM EDTA, 200 mM NaCl). Then, 300 μl of lysis solution (1% sodium dodecyl sulfate in lysis buffer) was added, and the mixture was incubated at 100°C for 2 min, with occasional mixing. Six hundred microliters of hot phenol saturated with lysis buffer (65°C) was added, and the mixture was kept at 65°C for 5 min with occasional inversion of the tube. After centrifugation, the aqueous phase was transferred to a new tube and extracted once with phenol-chloroform and then twice with ether. The RNA was precipitated by adding a 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol at −80°C for 10 min and then centrifuged. The pellet was suspended in 100 μl of DNase I buffer (100 mM sodium acetate, 5 mM $MgCl_2$ [pH 5.0]), and the DNA was digested with DNase I (RNase free; Boehringer Mannheim Biochemicals, GmbH)for 2 h at 37°C. The RNA was recovered by ethanol precipitation. The RNA species (135 μg) were then separated by electrophoresis in a 1.4% agarose–MOPS [3-(*N*-morpholino)propanesulfonic acid]–formaldehyde system (1). Northern hybridization was carried out at 42°C in the presence of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50% formamide–50 mM sodium phosphate buffer (pH 7.0)–7% sodium dodecyl sulfate–2% blocking reagent–0.1% lauroylsarcosine–50 μg of yeast total RNA per ml. The *Sal*I-*Hin*dIII fragment (1.1 kb), which is contained in the *uvrA* gene, was used as probe 1. The 219-bp PCR product of the sequence downstream of *uvrA* was used as probe 2. The *Eco*RI-*Nco*I fragment (306 bp) of pAM401, which is contained in vector pAM401, was used as probe 3. Each probe was labeled with digoxigenin-dUTP and used for Northern blot analysis. The corresponding transcript was detected by enzyme immunoassay (DIG DNA labeling and detection kit [nonradioactive] and PCR DIG probe synthesis kit; Boehringer Mannheim Biochemica).

PCR. The PCR was performed with a PCR DIG labeling kit (Boehringer Mannheim Biochemica) and a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, Conn.) to make probes from the downstream region of *uvrA* for Northern blotting. The primers used are shown in Fig. 3.

Nucleotide sequence accession number. The nucleotide sequence data reported in this article will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession no. AB007844.



RESULTS

Tn*917* insertion mutagenesis. Insertion of Tn*917* into pAD1 was achieved as a result of erythromycin-induced transposition (45, 54) from the nonconjugative pAD2 plasmid in *E. faecalis* DS16, by the procedure described in Materials and Methods. pAD1::Tn*917* derivatives were examined for UV sensitivity as described in Materials and Methods. Twenty-one UV202 (pAD1::Tn*917*) derivatives exhibiting UV sensitivity were isolated from independent experiments. The locations of the Tn*917* insertions were determined by *Eco*RI and *Sal*I enzyme digestions of the plasmids as described previously (21, 27, 41), and the precise location of Tn*917* insertion was determined by DNA sequence analysis (see Fig. 3). The 21 insertions were mapped to two different positions: 9 of the 21 mutants were mapped to 28.2 kb and 12 of the 21 mutants were mapped to 28.6 kb of the pAD1 map.

The survival curves of *E. faecalis* UV202 carrying the pAD1::Tn*917* derivatives were examined as described in Materials and Methods. pMG500 and pMG501 were used as typical UV-sensitive derivatives. The number of UV202 cells carrying pMG500 or pMG501 which survived was about 2 orders of magnitude lower than that of UV202 cells carrying the wild-type pAD1 (Fig. 1A). These results indicated that insertions at 28.2 or 28.6 kb of the pAD1 map gave rise to altered UV resistance.

Cloning of the *uvr* determinant. The 28.2- and 28.6-kb positions of the pAD1 map were determined to lie within a 2.9-kb fragment between a *Kpn*I site (27.7 kb of the pAD1 map) and an *Eco*RI site (30.6 kb of the pAD1 map) of *Eco*RI fragment B. This suggested that the *Eco*RI-*Kpn*I (2.9-kb) fragment might encode the UV resistance determinant. The following experiments were carried out to clone the 2.9-kb *Kpn*I-*Eco*RI fragment. pAM2011A is a pAD1 derivative containing a Tn*917*(Em)-*lac* insertion in the pAD1 *Eco*RI fragment B at approximately 17.8 kb of the pAD1 map in the *traE* gene (31, 57). The pAD1 *Eco*RI B fragment contains the genes necessary for DNA replication (21, 57). The miniplasmid pAM2011E, which consists of the entire *Eco*RI fragment B, has been previously constructed from pAM2011A (57). The miniplasmid pAM2011E was cloned into the *Eco*RI site of the vector pMW119 to construct pMW119::pAM2011E; this plasmid was designated pMG502.

pAM2011E has one *Kpn*I site located 2.9 kb from one of the fragment B *Eco*RI sites. pMW119 contains a *Kpn*I site 18 bp from an *Eco*RI site. pMG502 DNA was digested with *Kpn*I, and the digested DNA was cloned into the *Kpn*I site of pMW119. Two clones (pMW119::*Kpn*I fragment [2.984 kb]) which possessed the 2.984-kb insert in opposite orientations were obtained. The two clones were designated pMG503-1 and pMG504-1 (Fig. 2). The clones contained the *Eco*RI-*Kpn*I (2.972-kb) fragment originating from *Eco*RI fragment B plus the *Eco*RI-*Kpn*I (18-bp) fragment originating from the pMW119 multicloning site.

pMG504-1 was digested with *Bam*HI and was then cloned into the *Bam*HI site of pAM401, which is an *E. coli-Streptococcus* shuttle vector (59), and the resulting plasmid pAM401::pMG504-1 was designated pMG504. The *E. faecalis*

FIG. 1. UV survival curves of *E. faecalis* strains. The UV survival curves were obtained as indicated in Materials and Methods. (A and B) ○, UV202(pAD1); △, UV202(pMG500); □, UV202(pMG501); ▽, UV202; ◇, UV202(pAM2011E); ●, UV202(pMG504); ▲, UV202(pMG505); ■, UV202(pMG506); ▼, UV202(pMG507); ◆, UV202(pMW119::pAM401). (C) ○, FA2-2(pAD1); △, FA2-2(pMG506); □, FA2-2.

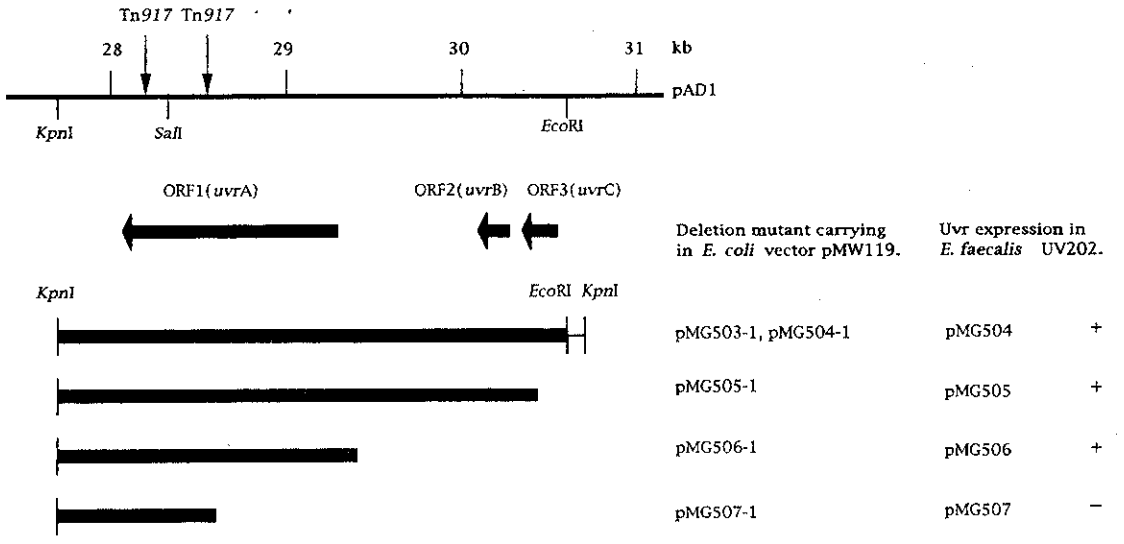


FIG. 2. Physical maps of the *Eco*RI-*Kpn*I fragment (2.972 kb) of pAD1 (which is carried on pMG503-1 or pMG504-1 in opposite orientations) and deletion mutants of pMG503-1. Thick horizontal arrows indicate ORFs on the pAD1 physical map and the direction of ORF transcription. Vertical arrows indicate the positions of Tn*917* insertions of UV-sensitive mutants of pAD1. Thick lines represent the cloned pAD1 fragment. Thin lines represent vector DNA. The vertical bars at the ends of the designated plasmid segments indicate the restriction sites used for construction; the absence of a bar indicates that the end was generated by a nested deletion. pMG504, pMG505, pMG506, or pMG507 is the recombinant plasmid between *E. coli-E. faecalis* shuttle vector pAM401 and pMG504-1, pMG505-1, pMG506-1, or pMG507-1, respectively.

UV202 strain was transformed by pMG504 (16) and was examined for UV resistance. As shown in Fig. 1B, UV202 (pMG504) showed the same level of UV resistance as UV202(pAD1) and UV202(pAM2011E). The number of UV-resistant cells surviving UV exposure was about 2 orders of magnitude greater than those of the UV-sensitive strains UV202 and UV202 carrying the vector plasmid (Fig. 1B). These results indicate that the 2.9-kbp *Kpn*I-*Eco*RI fragment contained the entire UV resistance determinant encoded on pAD1.

DNA sequencing of UV resistance determinant region. pMG503-1 and pMG504-1 were used for DNA sequencing. The two clones were digested with *Bam*HI and *Sph*I and trimmed with a nuclease, as described in Materials and Methods for DNA sequencing analysis. The resulting constructs were sequenced as described in Materials and Methods. The result is shown in Fig. 3. Computer analysis revealed the presence of three open reading frames (ORFs) in the 2.9-kbp region spanning map positions 27.7 to 30.6 kb of the clockwise orientation of the pAD1 (59.6-kb) map. Figure 3 shows the ORFs which had a consensus ribosome binding site in a region 20 bases upstream of the predicted start codon.

ORF1, which was designated *uvrA*, encoded a 442-amino-acid protein with a molecular mass of 49.6 kDa. The ATG start codon was preceded by a putative potential ribosome binding site (GAAGG) located 7 bp upstream. There was an apparent promoter sequence upstream of the start codon. Comparison of the deduced amino acid sequence of the *uvrA* protein showed 20% homology of the identical residues with the UmuC protein, which is encoded by an *E. coli* DNA damage-inducible gene (Fig. 4) (34, 39, 60). A palindromic sequence, 5′-GAACNGTTC-3′, was located between −35 and −10 bases of the promoter region. The palindromic sequence, 5′-GAAC NGTTC-3′, has been identified as the consensus sequence within the putative promoter region of the *B. subtilis* DNA damage-inducible genes (5).

ORF2, which was designated *uvrB*, encoded a 60-amino-acid protein with a molecular mass of 7.5 kDa. The ATG start codon was preceded by a putative potential ribosome binding site (GGAGA) located 5 bp upstream. There was an apparent promoter sequence (−10 and −35 region) upstream of the start codon. Comparison of the deduced amino acid sequence of *uvrB* did not show significant homology with any other reported protein.

ORF3 encoded a 74-amino-acid sequence with a molecular mass of 8.7 kDa. Comparison of the deduced amino acid sequence of ORF3 did not show any significant homology with any other reported protein.

Generation of deletion mutants and their UV resistance. The deletion mutants were generated from pMG503-1 with a nested deletion kit (Nippon Gene, Inc.). The deletion mutants pMG505-1, pMG506-1, and pMG507-1 possessed fragments between 30.5 and 27.7, between 29.5 kb and 27.7, and between 28.5 and 27.7 kb of the pAD1 map, respectively (Fig. 2). The deletion mutant pMG505-1 possessed ORF1, ORF2, and a part of ORF3. pMG506-1 possessed ORF1, and pMG507-1 possessed a part of ORF1.

UV resistance was not expressed by any clone in *E. coli* DH1(*recA*), *E. coli* ME8412(*umuC*), or *E. coli* ME8386(*umuC*) (data not shown). Plasmids carrying each deletion mutant and the *E. faecalis-E. coli* shuttle vector pAM401 were constructed. Each clone was digested with *Eco*RI to cleave the *Eco*RI site of pMW119 and was then cloned into the *Eco*RI site of pAM401. Recombinants between pAM401 and each of the plasmids pMG505-1, pMG506-1, and pMG507-1 were designated pMG505, pMG506, and pMG507, respectively (Fig. 2). The cloned DNA was introduced into *E. faecalis* UV202 by electrotransformation, and the transformants were examined for UV resistance. As shown in Fig. 1B, deletion mutants encoding ORF1 conferred UV resistance.

UV resistance of wild-type FA2-2 strain harboring pAD1 or pMG506. To examine whether pAD1 or the cloned UV resistance determinant enhances the UV resistance of wild-type *E. faecalis* FA2-2, the survival curves of *E. faecalis* FA2-2 carrying pAD1 or pMG506, which encodes the cloned UV resistance determinant, were plotted. The results are shown in Fig. 1C. The number of cells which survived decreased in a logarithmic fashion starting 30 s after UV irradiation (Fig. 1C). The num-

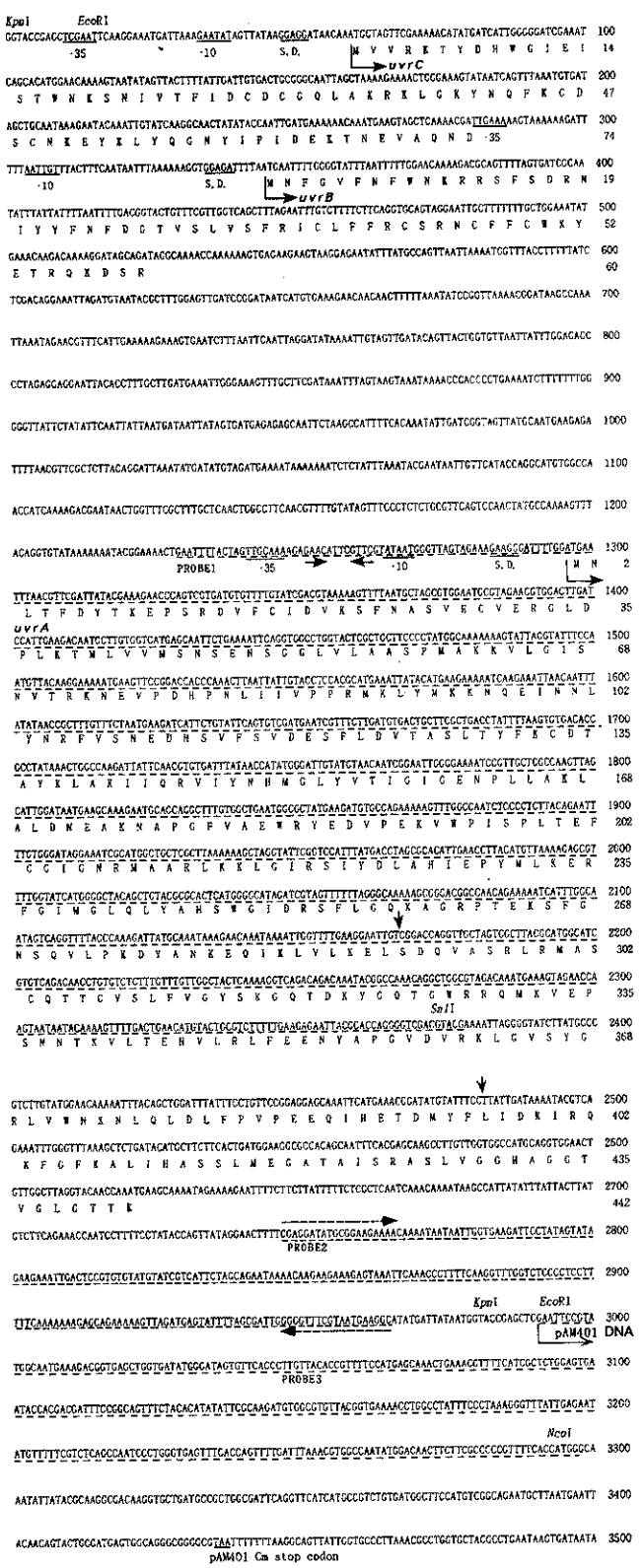


FIG. 3. Nucleotide sequence of the *uvr* determinant of plasmid pAD1 and the deduced amino acid sequence. The potential promoter (−10 and −35) and Shine-Dalgarno (S.D.) ribosome binding sequences are underlined. The palindromic consensus sequences (GAACNGTTC) in the *uvrA* promoter region are indicated by horizontal arrows and are described in the text. The vertical arrows mark the locations of the Tn*917* insertions that gave rise to altered UV resistance, which are described in the text. The sequences of synthetic oligonucleotides used for Northern blot analyses are underlined with dashed lines (probe 1, probe 2, and probe 3). Dashed horizontal arrows show the PCR primer used for synthesis of probe 2.

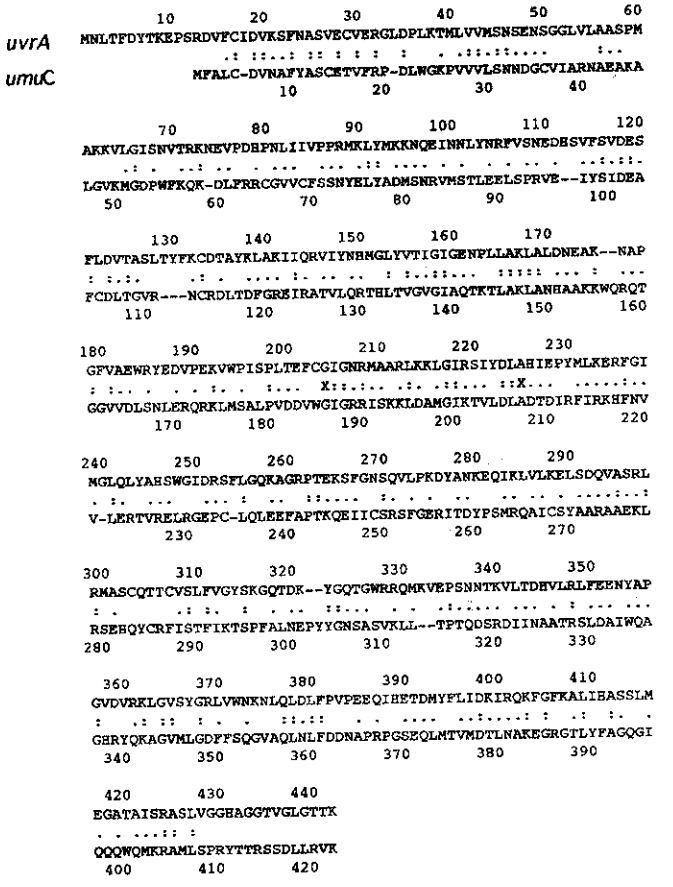


FIG. 4. Comparison of the amino acid sequence of the predicted *uvrA*-encoded protein of the *uvr* determinant of pAD1 with the amino acid sequence of the *umuC*-encoded protein of *E. coli*. Identical residues are shown by two dots, and similar residues are shown by a single dot.

ber of FA2-2 cells carrying pAD1 or pMG506 which survived was about 1.5 orders of magnitude greater than that of the plasmid-free FA2-2. The results indicated that the UV resistance determinant also enhances the UV resistance of wild-type *E. faecalis* FA2-2.

Transcript analysis of the *uvrA* gene. DNA sequence analysis revealed that the potential promoter sequences were located upstream of the start codons of *uvrA*, *uvrB*, and *uvrC*. The *uvrA* transcript was analyzed in the deletion mutants by Northern hybridization. Probe 1, corresponding to a 1,144-bp sequence of *uvrA*, was used (Fig. 3). The results of Northern analysis are shown in Fig. 5. Probe 1 did not detect any significant amount of transcript from RNA isolated from UV202 carrying pMG504, which contained *uvrA*, *uvrB*, and *uvrC* (Fig. 5, lane 1). One transcript with a length of 1.54 kb was detected in RNA isolated from UV202 carrying pMG505, which contained *uvrA* and *uvrB* (Fig. 5, lane 2). Two transcripts of 1.54 and 2.14 kb in length were detected in RNA isolated from UV202 carrying pMG506, which contained *uvrA* (Fig. 5, lane 3).

Inducible DNA repair systems are induced by DNA-damaging agents such as UV radiation, mitomycin C, and other

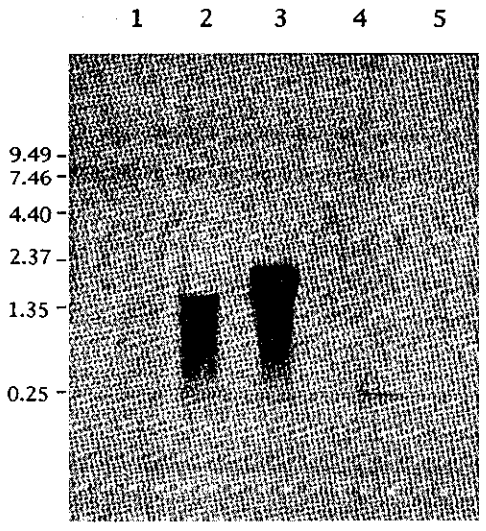


FIG. 5. Northern blot analyses of the *uvrA* gene. Probe 1 is shown in Fig. 3. Total RNA prepared from *E. faecalis* UV202 cells carrying the cloned *uvr* determinant and the deletion mutants was tested with probe 1. Lane 1, cells harboring pMG504; lane 2, cells harboring pMG505; lane 3, cells harboring pMG506; lane 4, cells harboring pMW119::pAM401; lane 5, plasmid-free *E. faecalis* UV202. The position of the RNA markers is noted on the left in kilobases.

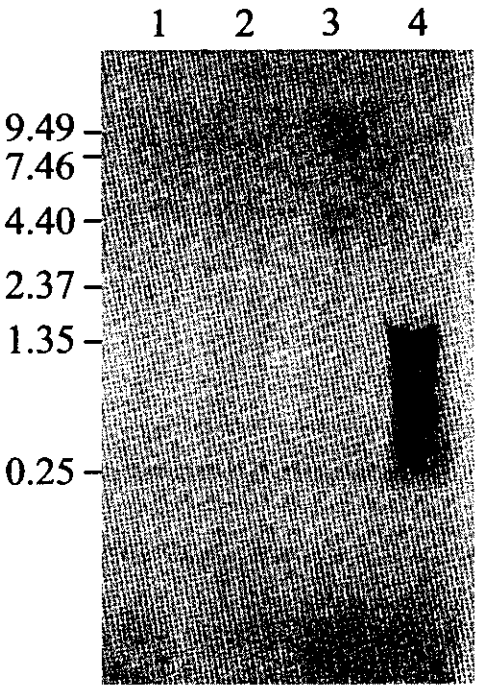


FIG. 6. Induction of transcription of the *uvrA* gene by mitomycin C. *E. faecalis* UV202 carrying pMG504, which contained *uvrA*, *uvrB*, and *uvrC*, was exposed to mitomycin C as described in Materials and Methods. Transcript of the strain was examined at 1, 2, and 3 h after mitomycin C exposure. The experimental procedure for Northern blot analysis was similar to that described for Fig. 5. Lane 1, without exposure to mitomycin C; lanes 2, 3, and 4, 1, 2, and 3 h after mitomycin C exposure, respectively. Numbers at left show positions in kilobases.

chemical agents. We examined whether the pMG504 *uvrA* determinant, which contains *uvrA*, *uvrB*, and *uvrC*, is expressed in response to mitomycin C. The results of Northern analysis after exposure to mitomycin C are shown in Fig. 6. A transcript which hybridized to probe 1 and corresponded to the shorter RNA (1.54 kb) was detected 3 h after mitomycin C induction.

Two transcripts of different lengths were detected in UV202 carrying pMG506. These implied that the transcripts were initiated from the same promoter of *uvrA* and terminated at different regions downstream of *uvrA*. To determine whether the transcripts terminated at different points, two different probes were prepared for Northern blot analysis. One probe (probe 2) corresponded to a 219-bp fragment constructed from a 2,747- to 2,965-bp region of the downstream C-terminal region of *uvrA*. This fragment is included in pAD1 DNA (Fig. 3). The other probe (probe 3) corresponded to a 306-bp fragment constructed from 2,991 to 3,297 bp of the downstream C-terminal region of *uvrA*, and this fragment is included in the vector DNA (Fig. 3). The UV202 transcript carrying pMG506 was analyzed with the two probes by Northern hybridization. As shown in Fig. 7, probe 2 hybridized to both the 1.54- and 2.14-kb transcripts (Fig. 7A), but probe 3 did not detect the 1.54-kb transcript (Fig. 7B). These indicated that the two transcripts terminated at different points.

DISCUSSION

Tn*917* insertion mutants which had altered UV resistance were mapped to two different locations at 28.2 and 28.6 kb of the pAD1 map by DNA sequence analysis. The data were not consistent with a previous report that the Tn*917* inserts of pAD1 UV-sensitive mutants are mapped approximately from 27.8 to 29 kb of the pAD1 map (50). On the basis of the insertion mutagenesis, the 2.9-kb fragment which lies between 27.7 and 30.6 kb of the pAD1 map was cloned, and the cloned fragment conferred UV resistance activity. The *uvr* determinant consisted of three ORFs designated *uvrA*, *uvrB*, and *uvrC*, which were located between 28.0 and 29.3 kb, 30.1 and 30.3 kb, and 30.4 and 30.6 kb of the pAD1 map, respectively. *uvrA*, *uvrB*, and *uvrC* encoded proteins of 442, 60, and 74 amino acids, respectively. The deletion mutant pMG506, which contained only *uvrA*, expressed *uvr* in *E. faecalis* UV202. The Tn*917* inserts of pAD1 UV-sensitive mutants were mapped to *uvrA*. These indicated that *uvrA* was a structural gene for *uvr* encoded on the pAD1. pAD1 and the cloned *uvrA* also enhanced the UV resistance of wild-type *E. faecalis* FA2-2, indicating that the *uvr* determinant of pAD1 generally confers enhanced UV resistance on *E. faecalis*.

Two *uvrA* transcripts of different sizes, which hybridized to a probe corresponding to an 1,144-bp sequence in the *uvrA* gene, were detected in UV202 carrying pMG506. The molecular size of one transcript was 1.54 kb, and that of the other was 2.14 kb. One potential promoter sequence was identified upstream of the start codon of the *uvrA* gene. However, the reported terminator was not identified downstream of the *uvrA* gene. The *uvrA* gene transcript could be initiated at a transcription start site upstream of *uvrA* and could be terminated at two different points downstream of *uvrA*. Northern analysis with two probes corresponding to two different regions downstream of *uvrA* indicated that the transcripts terminated at two different points downstream of *uvrA*.

The 1.54-kb *uvrA* transcript was detected in UV202 carrying pMG505, which contains *uvrA* and *uvrB*; however, the 2.14-kb transcript was not detected in these cells. The result suggests that the *uvrB* product terminates transcription at 1.54 kb from the *uvrA* promoter region. The *uvrA* transcript was not detected in any significant amount in UV202 carrying pMG504, which contains *uvrA*, *uvrB*, and *uvrC*; however, the 1.54-kb transcript was detected in the strain exposed to mitomycin C.

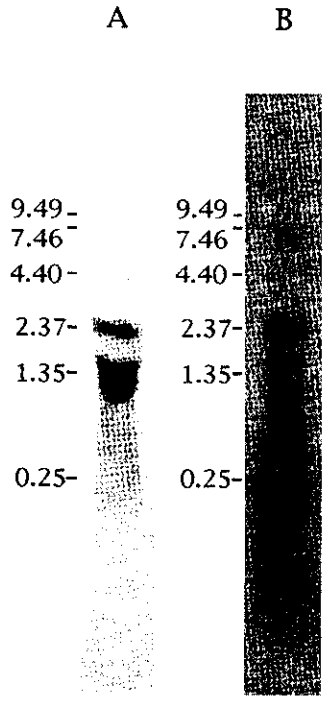


FIG. 7. Northern blot analyses of downstream of the *uvrA* gene. Total RNA prepared from *E. faecalis* UV202 carrying pMG506 was tested with probe 2 (A) and probe 3 (B). The position of the RNA markers is noted on the left of each panel in kilobases.

These results indicate that the *uvrC* product represses the expression of the *uvrA* gene and that this negative regulation is derepressed by DNA-damaging agents.

The regulatory mechanism of the inducible DNA repair systems has been extensively characterized in the SOS regulatory system of *E. coli* (34). The SOS system comprises about 20 unlinked genes. *umuDC* is a dicistronic operon which encodes the structural genes for mutagenic repair. The *mucAB* operon is a plasmid-borne analog of the *umuDC* operon (15, 38, 39). *lexA* and *recA* are regulatory genes which encode the repressor and activator of the SOS gene transcripts, respectively (4, 36, 46). The genes are coordinately induced by a variety of DNA-damaging agents (55, 56). The SOS response is highly conserved in *B. subtilis* (5, 32, 33). However, in contrast to *E. coli*, little is known about the regulation of the SOS-like system in gram-positive bacteria. The deduced amino acid sequence of the *uvrA*-encoded protein has 20% homology with that of the *umuC*-encoded protein of *E. coli* (33, 39, 60). A palindromic sequence, GAACNGTTC, was identified within the *uvrA* promoter region. This palindromic sequence, which is located between −35 and −10 bases of the promoter region, may function as an operator site to regulate expression of the *uvrA* gene. The palindromic sequence GAACNGTTC has been identified as a consensus sequence in the promoter regions of the *din* genes, which are *B. subtilis* DNA damage-inducible genes (32), and functions as an essential operator site for the regulation of the SOS system in *B. subtilis* (5).

E. faecalis UV202 is a derivative of *E. faecalis* JH2-2 (61) and is UV sensitive and deficient in recombination (61). The gene, which determines the UV sensitivity of *E. faecalis* UV202, is not yet understood. The UV resistance determinant of pAD1 enhances the UV resistance of UV202. This suggests that UV202 is a mutant deficient in one of the genes involved in the SOS-like system of *E. faecalis*. *uvrA* is a plasmid-borne, inducible structural gene which enhances the resistance of its *E. faecalis* host to the DNA-damaging agent UV light. As indicated above, the *uvrA* gene was regulated by the plasmid-borne *uvrC* product. The expression of *uvrA* was induced by DNA-damaging chemical agents, such as mitomycin C, and lack of *uvrC* gave rise to the derepression of *uvrA*. This indicated that *uvrA* was not regulated by an *E. faecalis recA* and *lexA*-like system similar to the SOS system but was regulated by *uvrC*. However, the palindromic sequence found in the promoter region was identical to the consensus sequence found in the promoter regions of the *din* genes. This implied that *uvrA* was originally a member of the SOS system and had developed a specific regulatory gene, *uvrC*, in the course of evolution.

The UV resistance determinants examined to date are associated with the hemolysin-bacteriocin determinant on pAD1-related plasmids (6, 10, 23, 28). The hemolysin-bacteriocin protein is associated with virulence in animal models (6, 25, 28). A significant number of *E. faecalis* clinical isolates produce hemolysin-bacteriocin (22, 26). More than 50% of the *E. faecalis* clinical isolates studied carry transferable hemolysin-bacteriocin genes (22, 26). More than 90% of these hemolysin-bacteriocin plasmids are identical to pAD1 (23). Thus, pAD1 is a typical *E. faecalis* hemolysin-bacteriocin plasmid. The UV resistance determinant contributes to the protection of *E. faecalis* strains carrying pAD1-like plasmids against UV light or the damage of DNA. Together with hemolysin-bacteriocin and the aggregation substance, the UV resistance determinant may contribute to pathogenicity.

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High-Level Plasmid-Mediated Gentamicin Resistance and Pheromone Response of Plasmids Present in Clinical Isolates of *Enterococcus faecalis*

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Eleven pheromone-responding plasmids encoding erythromycin or gentamicin resistance were isolated from multiresistant clinical *Enterococcus faecalis* isolates. The plasmids were classified into six types with respect to their pheromone responses. The three erythromycin resistance plasmids responded to different pheromones. Of the eight gentamicin resistance plasmids, four plasmids responded to same pheromone. Southern hybridization studies showed that the genes involved in regulation of the pheromone response were conserved in the drug resistance plasmids.

Certain *Enterococcus faecalis* conjugative plasmids confer a mating response to the small sex pheromones secreted by potential recipient cells (1–4, 12, 13). This mating signal induces the synthesis of a surface aggregation substance that facilitates the formation of mating aggregates and plasmid transfer (3, 40). Other genes encoded by these plasmids include the β-hemolysin/bacteriocin (Hly/Bac), bacteriocin, and antibiotic and UV light resistance genes (9, 20–24, 26, 30–32).

High-level gentamicin-resistant or β-lactamase-producing *E. faecalis* strains cause serious nosocomial infections (28–30, 38). Murray and colleagues first isolated the β-lactamase-producing strain *E. faecalis* HH22 (32) and showed that a single strain of a β-lactamase-producing HH22-like strain was disseminated throughout a broad geographical area in the United States (33). *E. faecalis* HH22 harbors the drug resistance pheromone-responsive plasmid pBEM10, which encodes penicillin and gentamicin resistance, and pAM323, which encodes erythromycin resistance (31).

In this report, we describe the drug resistance of clinical *E. faecalis* isolates obtained in Japan, the drug resistance plasmids, and the pheromone responses of these plasmids.

Drug resistance of clinical *E. faecalis* isolates and isolation of a conjugative drug resistance plasmid. One hundred clinical *E. faecalis* isolates were examined for drug resistance as described previously (22, 26). Approximately half of the strains were resistant to two or more drugs. Most (76%) of the strains

TABLE 1. Transferable drug resistance plasmid and the pheromone response

Strain[a]	Drug resistance pattern of donor cells[b]	Plasmid (molecular size [kb])[c]	Drug resistance encoded on pheromone-responding plasmid[b]	Transfer frequency (transconjugants/donor cells)[d]	Related pheromone[e]
GF101	Em Gm Tc	pSM101 (40.0)	Em	4.2×10^{-2}	cSM1
GF102	Em Gm Tc	pSM102 (52.7)	Em	8.0×10^{-1}	cSM2
GF103	Cm Em Gm Sm Tc	pSM103 (59.0)	Em	3.9×10^{-1}	Self-clumper
GF104	Em Gm Tc	pSM104 (80.0)	Gm	3.6×10^{-1}	cSM3
GF105	Em Gm Sm Tc	pSM105 (80.0)	Gm	6.9×10^{-1}	cSM3
GF106	Cm Em Gm Tc	pSM106 (98.5)	Gm	1.2×10^{-1}	cSM3
GF107	Cm Em Gm Tc	pSM107 (94.6)	Gm	4.6×10^{-1}	cSM3
GF108	Em Gm Tc	pSM108 (64.5)	Gm	1.3×10^{-1}	cSM4
GF109	Cm Gm Tc	pSM109 (64.5)	Gm	1.2×10^{-2}	cSM4
GF110	Gm Sm Tc	pSM110 (71.0)	Gm	1.0×10^{-2}	cSM5
GF111	Em Gm Sm Tc	pSM111 (80.8)	Gm	2.4×10^{-2}	cSM6

[a] Strains GF103, GF108, GF109, and GF111 were isolated from a hospital in Maebashi City, Japan, and strains GF106 and GF107 were isolated from Isesaki City Hospital. The other strains were isolated from Gunma University Hospital.
[b] Em, erythromycin; Gm, gentamicin; Tc, tetracycline; Cm, chloramphenicol; Sm, streptomycin.
[c] Plasmid DNA was isolated from the transconjugant by the alkaline lysis method (37).
[d] Transfer frequency was tested by conjugative transfer from *E. faecalis* JH2SS (Str^r Spc^r) (42) to FA2-2 (Rif^r Fus^r) (8) in broth mating experiments as described previously (13).
[e] The pheromone responses (aggregation) of the plasmids were examined as described previously (13, 23).

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were resistant to tetracycline. Between 30 and 40% of the strains were resistant to gentamicin or erythromycin. Ampicillin- or vancomycin-resistant strains were not isolated. Most (60%) of the drug-resistant strains exhibited a clumping response with a culture filtrate of plasmid-free *E. faecalis* FA2-2 (Rif^r Fus^r) (8), suggesting that the strains harbor a pheromone-responding plasmid. Of the drug-resistant and pheromone-responding strains, 48 strains were selected at random, and the transferability of drug resistance to *E. faecalis* FA2-2 was examined by broth mating as described previously (13). About 60% of the gentamicin or erythromycin resistance determinants were transferred. Only 1 of the 44 tetracycline resistance determinants was transferred.

Eleven nonhemolytic, multidrug-resistant strains were selected for use in experiments performed to identify drug-resistant conjugative plasmids (Table 1). Erythromycin- or gentamicin-resistant transconjugants that harbored a single plasmid were isolated by repeated transfer experiments (Table 1).

The *Eco*RI restriction profile of each plasmid is presented in Fig. 1A. The gentamicin resistance plasmids pSM104 and pSM105 and plasmids pSM108 and pSM109 exhibited the same *Eco*RI restriction profiles, respectively. Several *Eco*RI fragments were found to be of identical size in pSM104, pSM105, pSM106, and pSM107.

E. faecalis isolates carrying the gentamicin resistance plasmid were tested for their resistance to various aminoglycoside antibiotics as described by Courvalin et al. (10, 11). The strains had an aminoglycoside antibiotic resistance spectrum identical to that of the strain carrying the gentamicin resistance plasmid pIP800 (data not shown) (10, 11). The results indicated that the gentamicin resistance phenotype is due to the presence of 2′-APH and 6′-AAC, the same aminoglycoside-modifying enzymes encoded by pIP800 (10, 14).

Pheromone response of the drug resistance plasmids. Donor cells of strain JH2SS (Str^r Spc^r) (42) carrying a drug resistance plasmid were exposed (120 min) to a strain FA2-2 culture filtrate (pheromone) to induce aggregation-mating functions before a short (10-min) mating period as described previously (21). The transfer frequency of the drug resistance plasmids from the induced JH2SS donor cells to the FA2-2 recipient cells was between 10^{-2} and 10^{-4} per donor cell for each mating experiment. The transfer frequency of the plasmid from the uninduced donor cells to the recipient cells was less than 10^{-7} per donor cell. These results indicate that transfer of the drug resistance plasmids was pheromone dependent.

The pheromone response of the drug resistance plasmids was classified by determining as described previously (13, 23) the pheromone responses (aggregation) of *E. faecalis* FA2-2 or OG1-10 (12) carrying the plasmids. Strains containing each of the drug resistance plasmids were examined for their response to the culture filtrate prepared from each drug-resistant strain (23). Strains or culture filtrates of strains carrying the Hly/Bac plasmid pAM714 (pAD1::Tn*917*) (21), pOB1 (35), pYI2 (23), or the bacteriocin plasmid pPIT7022 (pPD1::Tn917) (17), which respond to the pheromones cAD1, cOB1, cYI2, and cPD1, respectively, were included in these experiments. The drug resistance plasmids were classified into six types with regard to their pheromone responses (Table 1). The gentamicin resistance plasmids pSM104, pSM105, pSM106, and pSM107 respond to the same pheromone. pSM108 and pSM109 respond to another type of pheromone.

Pheromone-related conjugation systems have been well studied in pAD1 (3–5, 7, 21, 25, 39, 40, 43, 44), pCF10 (27, 36), and pPD1 (17, 18, 34, 41). Genes involved in the regulation of the pheromone response are clustered in a 7-kb region on each plasmid (3, 4, 17), and there is homology among the plasmids.

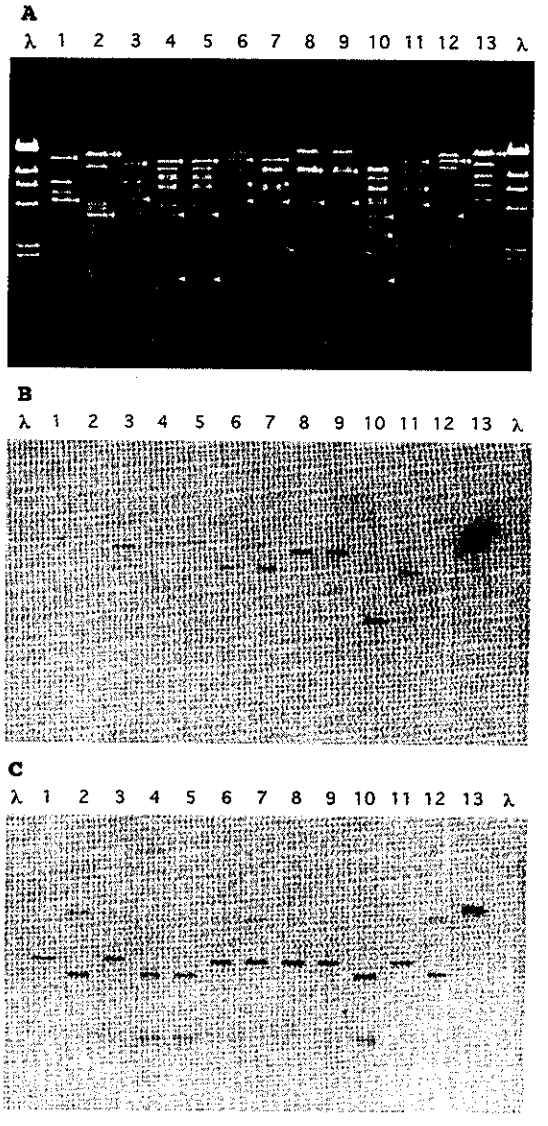

FIG. 1. Agarose gel electrophoresis of *Eco*RI-digested drug resistance plasmid DNAs and hybridization of cloned pPD1 fragments to the *Eco*RI fragments. Plasmid DNA was isolated by the alkaline lysis method (36). (A) Agarose gel electrophoresis of *Eco*RI-digested drug resistance plasmid DNAs. Lane 1, pSM101 (erythromycin resistance); 2, pSM102 (erythromycin resistance); 3, pSM103 (erythromycin resistance); 4, pSM104 (gentamicin resistance); 5, pSM105 (gentamicin resistance); 6, pSM106 (gentamicin resistance); 7, pSM107 (gentamicin resistance); 8, pSM108 (gentamicin resistance); 9, pSM109 (gentamicin resistance); 10, pSM110 (gentamicin resistance); 11, pSM111 (gentamicin resistance). Bacteriophage λ DNA digested with *Hin*dIII was used as a molecular size marker. Marker DNA was run on the first and last lanes of the gel. The white solid circles to the right of each lane indicate the fragments which hybridized with probe 1 containing the regulatory region genes of pPD1. The white arrowheads to the right of each lane mark the fragment which hybridized with probe 2 containing the genes for the possible surface exclusion protein and the N-terminal region of the aggregation substance gene of pPD1. Duplicate gels were Southern blotted onto a Biodyne A nylon membrane (Pall Co., Glen Cove, N.Y.) and hybridized (37) to probe 1 (B) or probe 2 (C). The probes were derived as described in the text and in the legend to Fig. 2.

There is also homology between the genes of the possible surface exclusion protein and aggregation substance, which are located downstream of the regulatory region (17–19). The drug resistance plasmids were studied for homology with DNA probes derived from plasmid pPD1 (17). An *Eco*RI-*Sal*I frag-

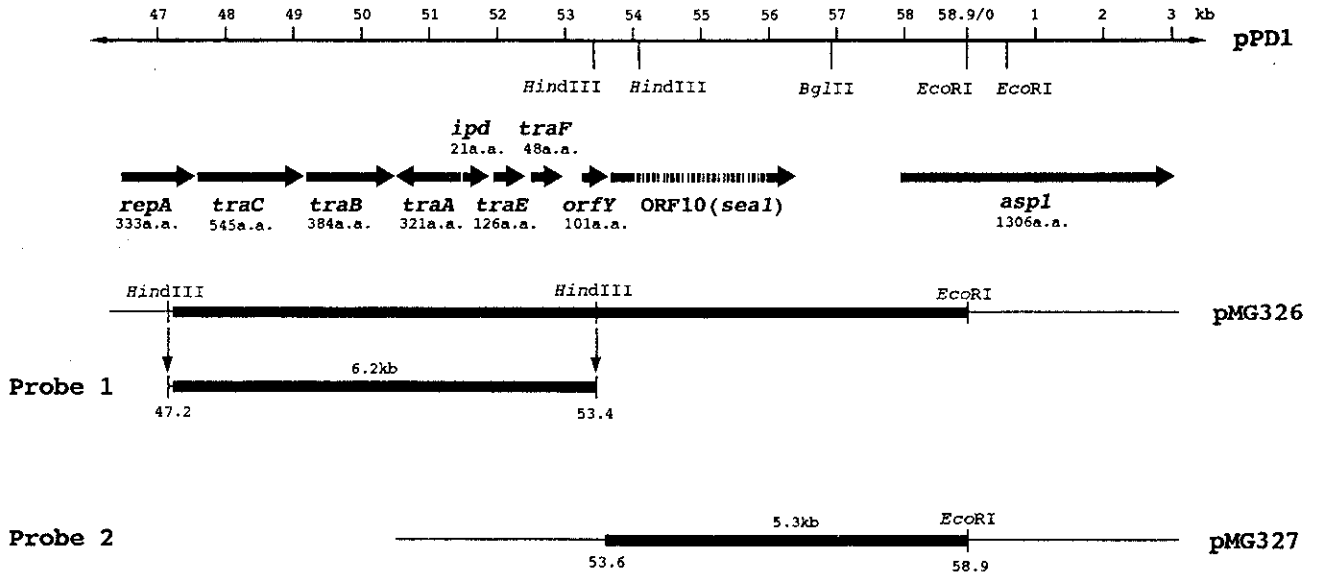

FIG. 2. Physical map of the regulatory region of the pheromone response of pPD1 and its subclones. Thick horizontal arrows indicate the open reading frames on the pPD1 physical map and the direction of transcription of the open reading frame (ORF). The dotted line of ORF10 (*seal*) was the gene of the possible surface exclusion protein (17, 18). Thick lines respresent the cloned pPD1 DNA fragment. Thin lines represent pMW119 (Nippon Gene Co., Ltd., Toyama, Japan) vector DNA. Deletion mutant pMG326 possessed a fragment located between 47.2 and 58.9 kb on the pPD1 map and contained regulatory region genes, a possible surface exclusion gene (ORF10 [*seal*]), and the N-terminal region of the aggregation substance gene (*aspl*). Probe 1 (6.2-kb fragment), which was obtained from pMG326 as described in the text, was used for Southern blot analysis. The results of the Southern blot analysis are presented in Fig. 1B. Deletion mutant pMG327 possessed a fragment located between 53.6 and 58.9 kb of the pPD1 map and was used as probe 2 for Southern blot analysis. The results of that Southern blot analysis are presented in Fig. 1C. a.a., amino acid.

ment (16.7 kb) lying between 42.2 and 58.9 kb of the pPD1 map was cloned into vector pMW119. The deletion mutants of the cloned fragment were produced as described previously (16), and the deletion mutants pMG326 and pMG327 were obtained (Fig. 2). Plasmid pMG326 was digested with *Hin*dIII, and the digested DNA was submitted to agarose gel electrophoresis. A 6.2-kb DNA fragment which contains the regulatory region genes was eluted from the agarose gel and was used as probe 1 (Fig. 2). pMG327 was used as probe 2 and contains a possible surface exclusion protein gene and the N-terminal region of the aggregation substance gene (Fig. 2). Each probe hybridized to specific *Eco*RI fragments from each of the drug resistance plasmids (Fig. 1). These results indicate that the genes of the regulatory region and the pheromone-related downstream genes of pPD1 were homologous with genes carried on the pheromone-responsive drug resistance plasmids of the clinical isolates.

Concluding comments. In our strains, the gentamicin or erythromycin resistance determinants frequently resided on a pheromone-responding plasmid. In some cases, a kanamycin or erythromycin resistance determinant was frequently transferred (unselected) with the pheromone-responding erythromycin or gentamicin plasmid, suggesting that a nontransferable kanamycin or erythromycin plasmid was mobilized by a transferable plasmid (data not shown).

Most *E. faecalis* strains were tetracycline and minocycline resistant (data not shown); however, the tetracycline resistance trait did not transfer in broth mating experiments and transferred by filter mating only at a relatively low frequency (38a), suggesting that the tetracycline resistance determinant resides on a conjugative transposon (6, 15). These results raise the possibility that the pheromone-responding plasmids and the conjugative transposon appear to play a major role in the spread of multiple drug resistance in clinical *E. faecalis* isolates.

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Role of Aminoglycoside 6′-Acetyltransferase in a Novel Multiple Aminoglycoside Resistance of an Actinomycete Strain #8: Inactivation of Aminoglycosides with 6′-Amino Group Except Arbekacin and Neomycin

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Role of Aminoglycoside 6′-Acetyltransferase in a Novel Multiple Aminoglycoside Resistance of an Actinomycete Strain #8: Inactivation of Aminoglycosides with 6′-Amino Group Except Arbekacin and Neomycin

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From a rare actinomycete strain #8 isolated from soil as arbekacin (ABK) resistant, we cloned a gene segment (0.9 kb) conferring multiple resistance to aminoglycoside (AG) antibiotics with 6′-NH_2 including semisynthetic ones except ABK and neomycin (NM). Enzymatic modification using cell free extracts from *Streptomyces lividans* TK21/pANT-S2 carrying the cloned gene revealed that the gene coded for an AG 6′-acetyltransferase [AAC(6′)] capable of acetylating all of the tested AGs with 6′-NH_2 including semisynthetic ones and astromicin. The substrate specificty of the enzyme was thus similar to that of AAC(6′)-Ie of *Enterococcus faecalis*. Antibiotic assay revealed a weak but clear antibiotic activity of 6′-*N*-acetylABK (8% of ABK activity) in contrast with substantial inactivation by the AAC(6′) of the other AGs including amikacin and isepamicin. The NM acetylation by the AAC(6′) also did not result in NM inactivation. It seems thus likely that AAC(6′)-dependent resistance to ABK and NM, if it emerges, will remain at low level.

Aminoglycoside (AG) antibiotics have been and still are playing important roles in curing various infectious diseases caused by Gram-positive as well as Gram-negative bacteria. Especially, semisynthetic AGs such as amikacin (AMK) and isepamicin (ISP) have been most widely used in the last decade[1,2]. Furthermore, arbekacin (ABK)[3], an anti-MRSA (methicillin-resistant *Staphylococcus aureus*) agent in Japan, has also been used widely since its approval in 1990. The most serious problem to the activty of these AGs is resistant bacteria carrying AG-modifying or inactivating enzymes[1~7]; *i.e.* AG phosphotransferases (APHs), adenylyltransferases (AADs), acetyltransferases (AACs) and a bifuntional enzyme AAC(6′)/APH(2″). According to recent reports[8~11], AAC(6′) and AAC(6′)/APH(2″) have been increasing their importance in clinically-occurring AG resistant bacteria such as *Pseudomonas aeruginosa*, MRSA and *Enterococcus faecalis*.

As to ABK resistance in MRSA, AAC(6′)/APH(2″)-dependent resistance[1,12] has been exclusively reported although modification sites for AAC(3), AAC(2′) and AAC(6′) exist in ABK molecule. Therefore, HOTTA *et al.* have been interested in the possible emergence of AAC-dependent ABK resistance and to check this possibility they employed AACs of actinomycete origin. Actually AAC(3) and AAC(2′) of *Streptomyces* origin were examined for capability of modification as well as inactivation of ABK[13,14]. Consequently, it turned out that ABK was relatively readily converted to 3″-*N*-acetylABK and 2′-*N*-acetylABK by the AAC(3) and AAC(2′),

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respectively. However, these two acetylated ABK products were found to retain substantial antibiotic activity so that the cloned AAC(3) or AAC(2′) did not confer ABK resistance.

HOTTA *et al.* have been studying the phenotypic diversity in multiple AG resistance of actinomycetes and the underlying biochemical and genetic basis[15,16]. Recently they isolated a soil actinomycete strain designated #8 with resistance to ABK to which actinomycetes are generally sensitive. Since the strain showed a novel AG resistance profile, they started to characterize the underlying genetic and biochemical basis for the resistance. This paper deals with the modification and inactivation of AGs by an AAC(6′) cloned from the strain #8. We believe this is the first characterization report on the AAC(6′) of actinomycete origin.

Materials and Methods

Strains

Strain #8 is an actinomycete that was isolated as an ABK resistant from a soil collected at a water fall (Johren-no-taki) in Izu peninsula in Japan. *Streptomyces lividans* TK21 was used as the host for gene manipulation.

Chemicals

AG antibiotics were available from the antibiotic collection at National Institute of Infectious Diseases. The other chemicals used for enzymatic modification and DNA manipulation were commercially available.

Taxonomic Characterization

According to the methods[17] used regularly for the characterization of actinomycetes, strain #8 was characterized in terms of morphology and physiology. Diaminopimelic acid type of cell wall and the G+C content of DNA were also analyzed according to the methods[18,19] reported, respectively.

Cloning of AG Resistance Gene

Preparation of total DNA and protoplasts, and transformation were carried out according to a genetic manipulation method of *Streptomyces*[20]. Total DNA prepared from mycelia grown in Tryptic Soy Broth (Difco) was cut partially with *Sau* 3AI and run on an agarose gel to separate DNA fragments. The resulting fragments ranging 4~10 kb were extracted with GeneClean II kit (BIO101) and ligated with plasmid vector pIJ702 cut with *Bgl* II. Subsequently, a protoplast suspension (40 μl) prepared from *S. lividans* TK21 was mixed with the ligation mixture (12 μl) in order to get transformed. Then the protoplasts were spread and incubated at 27°C for 2 weeks on soft R2YE medium supplemented with thiostrepton (10 μg/ml). The resulting colonies were replica plated on ISP No. 2 agar medium containing 10 μg/ml of an AG.

Enzymatic Modification of AGs

Acetylation reaction was carried out under the following conditions; 250 μg/ml AG, 0.1 M phosphate buffer (pH 7.0), 10% (v/v) cell free extract (S30) and 4 mM acetylCoA in a 50 μl reaction mixture. After incubation at 37°C, the acetylation of AGs and the remaining antibiotic activity of the reaction mixtures were monitored by TLC and paper disk assay, respectively. TLC was carried out on a silica gel plate (E. Merck Art. 5712) by developing with 5% KH_2PO_4. AGs and their acetylation products were detected by spraying ninhydrin reagent.

Antibiotic Resistance

Aerial mycelium of strain #8 grown on ISP No. 4 agar medium (Difco) for 2 weeks at 27°C was streaked on ISP No. 2 agar medium plates supplemented with AGs. The growth was scored after 7 day incubation at 27°C.

Isolation and Structure Determination of Acetylated Products

AGs [ABK (20 mg; 36.2 μmol), AMK (20 mg; 34.2 μmol) and ASTM (10 mg; 24.7 μmol)] were incubated with 5 mM acetylCoA and 3~10% (v/v) cell free extracts (72.6 mg protein/ml) in 20 ml (10 ml for ASTM) of the reaction mixture containing 0.1 M phosphate buffer (pH 7.0). After incubated at 37°C for 6~8 hours, the reaction mixtures were loaded on columns of Amberlite CG50 (NH_4^+, 20 ml). Subsequently, the columns were washed with 40 ml of water, and eluted with aqueous ammonia (1%, 0.4% and 0.2% for acetylated products of ABK, AMK and ASTM, respectively). The eluates were collected as approximately 2 ml fractions and the fractions with positive reactions to ninhydrin and Rydon-Smith reagents were pooled and concentrated to yield a colorless solids. As results, 15.1 mg, 14.4 mg and 7.9 mg of the purified acetylation products of ABK, AMK and ASTM, respectively, were obtained.

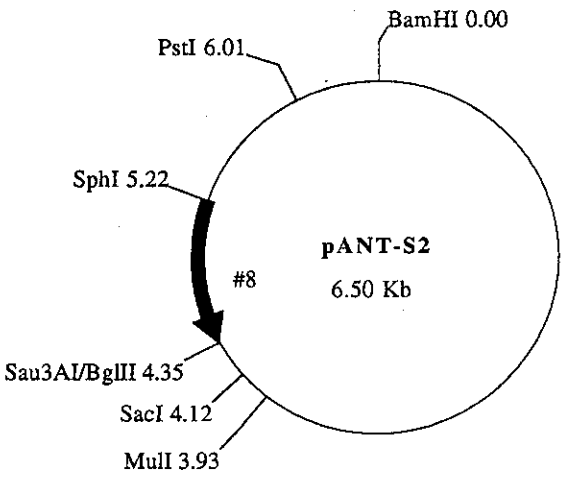
Results

Taxonomic Properties of Strain #8

Taxonomically, strain #8 showed the following

properties. On ISP media (Nos. 2, 3, 4 and 5), the strain showed good growth with white surface color and developed flexuous aerial mycelia and spores with smooth surface. The strain is capable of utilizing the following sugars; *i.e.* glucose, arabinose, xylose, galactose, sucrose, mannitol, inositol, rhamnose and raffinose as sole carbon sources for growth. The following physiological tests gave negative results; melanine production, gelatin liquifaction, milk peptonization, milk coagulation and starch hydrolysis. Nitrate reduction was positive. The G+C content of DNA was estimated at 73.2%, provided that 71.7% was obtained from the nucleotide sequence of the cloned DNA segment containing an AAC(6′) gene (unpublished). Cell wall analysis using whole mycelia indicated that cell wall contained *meso*-diaminopimelic acid (data not shown) so that the strain was regarded to be categorized into so called rare actinomycetes. No antibiotic productivity has been detected so far.

Fig. 1. Plasmid pANT-S2.



Cloning of AG Resistance Gene

The transformation treatment of *S. lividans* TK21 protoplasts with the ligation mixture of *Bgl* II-cut pIJ702 (a high copy number vector plasmid) and *Sau* 3AI-cut DNA fragments from strain #8 resulted in the formation of a number of colonies on ISP No. 2 medium containing ribostamycin (RSM). One of the colonies was then subcultured and designated as strain #8-2. This RSM-resistant strain was confirmed to contain a recombinant plasmid designated pANT8-2 with a 6.9 kb *Sau* 3AI fragment derived from the strain #8. Subsequent subcloning provided pANT-S2 (Fig. 1) containing a 0.9 kb *Sph* I-*Sau* 3AI fragment derived from the strain #8.

AG Resistance

As shown in Table 1, strain #8 showed multiply resistance (25 μg/ml or higher) to kanamycin (KM)-, gentamicin (GM)-, astromicin (ASTM)- and neomycin (NM)-group AGs, but specific sensitivity (<2.5 μg/ml) to paromomycin (PRM) and GM when examined on ISP No. 2 agar medium supplemented with AG. The AGs to which the strain #8 was resistant turned out to possess 6′- NH_2 commonly.

On the other hand, *S. lividans* TK21/pANT-S2 showed clear resistance ranging 10~200 μg/ml to RSM and other AGs including semisynthetic AGs such as AMK, dibekacin (DKB), ISP and netilmicin (NTL). These AGs commonly possess 6′-NH_2, suggesting pANT-S2 contains an AAC(6′) gene. However, the strain did not show clear resistance to ABK and NM both of which also possess 6′-NH_2, although the resistance levels to these two AGs were a little bit higher than those of *S. lividans* TK21.

AG Acetylation by the Cell Free Extract from *S. lividans* TK21/pANTS-2

As shown in Fig. 2, all of the examined AGs but PRM were converted in the presence of acetylCoA. The acetylated AGs commonly possess 6′-NH_2 including ones (ABK and NM) to which *S. lividans* TK21/pANT-S2 did not show clear resistance. Thus all of the semisynthetic AGs such as ABK[1] and ISP[5] known to be refractory to AAC(6′) of clinical origin were acetylated by the AAC(6′) of strain #8. The acetylation rate was relatively fast with AMK and ISP and relatively slow with ABK and DKB. Exceptionally, no PRM acetylation and incomplete gentamicin (GM) acetylation were observed. These results should be due to that PRM lacks 6′-NH_2 and GM contains a component (GM-C_1 with 6′-C- as well as 6′-*N*-methyl groups) known to be refractory to AAC(6′)[5].

When the reaction mixtures with complete substrate conversion were examined for antibiotic activity, those of ABK and NM showed a clear antibiotic activity. By contrast, the other reaction mixtures showed no significant antibiotic activity.

On the other hand, neither phosphotransferase activity nor adenylyltransferase activity was detectable (data not shown).