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Construction of an *Escherichia coli-Rhodococcus* Shuttle Vector and Plasmid Transformation in *Rhodococcus* spp.

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A plasmid transformation system for *Rhodococcus* sp. strain H13-A was developed by using an *Escherichia coli-Rhodococcus* shuttle plasmid constructed in this study. *Rhodococcus* sp. strain H13-A contains three cryptic indigenous plasmids, designated pMVS100, pMVS200, and pMVS300, of 75, 19.5, and 13.4 kilobases (kb), respectively. A 3.8-kb restriction fragment of pMVS300 was cloned into pIJ30, a 6.3-kb pBR322 derivative, containing the *E. coli* origin of replication (*ori*) and ampicillin resistance determinant (*bla*), as well as a *Streptomyces* gene for thiostrepton resistance, *tsr*. The resulting 10.1-kb recombinant plasmid, designated pMVS301, was isolated from *E. coli* DH1(pMVS301) and transformed into *Rhodococcus* sp. strain AS-50, a derivative of strain H13-A, by polyethylene glycol-assisted transformation of *Rhodococcus* protoplasts and selection for thiostrepton-resistant transformants. Thiostrepton-resistant transformants were also ampicillin resistant and were shown to contain pMVS301, which was subsequently isolated and transformed back into *E. coli*. The cloned 3.8-kb fragment of *Rhodococcus* DNA in pMVS301 contains a *Rhodococcus* origin of replication, since the hybrid plasmid was capable of replication in both genera. The plasmid was identical in *E. coli* and *Rhodococcus* transformants as determined by restriction analysis and was maintained as a stable, independent replicon in both organisms. Optimization of the transformation procedure resulted in transformation frequencies in the range of 10^5 transformants per μg of pMVS301 DNA in *Rhodococcus* sp. strain H13-A and derivative strains. The plasmid host range extends to strains of *Rhodococcus erythropolis*, *R. globulerus*, and *R. equi*, whereas stable transformants were not obtained with *R. rhodochrous* or with several coryneform bacteria tested as recipients. A restriction map demonstrated 14 unique restriction sites in pMVS301, some of which are potentially useful for molecular cloning in *Rhodococcus* spp. and other actinomycetes. This is the first report of plasmid transformation and of heterologous gene expression in a *Rhodococcus* sp.

Members of the genus *Rhodococcus* are gram-positive, aerobic, nonsporulating, partially acid-fast actinomycetes, which were formerly classified as *Nocardia*, *Mycobacterium*, *Gordona*, or *Jensenia* spp. or as members of the "rhodochrous" complex (16, 17). *Nocardia* and *Mycobacterium* spp. are closely related to *Rhodococcus* spp., each exhibiting nocardioform morphology, having mycolic acids, *meso*-diaminopimelic acid, arabinose, and galactose in their cell walls, and having a high G+C content (>59 mol%) in their cellular DNA (17, 18, 29). Most members of the genus are saprophytic soil organisms, although several pathogenic species exist, including *Rhodococcus bronchialis*, a human pathogen, *R. equi*, an animal pathogen, and *R. fascians*, a plant pathogen (18).

Rhodococci exhibit a wide range of metabolic activities including antibiotic production (47, 48), amino acid production (49), degradation of alkanes and aromatic hydrocarbons, biotransformation of steroids and a number of xenobiotic compounds (34, 43), lignin degradation (13, 37), chemolithoautotrophic growth in the presence of hydrogen and carbon dioxide (1, 39), and production of biosurfactants (14, 26, 31, 35, 36, 40).

Genetic studies of *Rhodococcus* spp. have focused on mapping the *R. erythropolis* chromosome, with approximately 65 chromosomal markers established, by using a natural mating and recombination system (7). A lysogenic actinophage, φEC, a 47-kilobase (kb) double-stranded DNA phage, has been physically mapped by restriction analysis for potential use as a cloning vector in *Rhodococcus* spp. (8, 9). φEC can be transferred between fertile *Rhodococcus* strains either as a plasmid or as a prophage (6), and φEC DNA can be transfected into *R. erythropolis* protoplasts (9). A generalized transducing phage (Q4) for *R. erythropolis* has been described which may prove useful for fine-structural genetic mapping in *Rhodococcus* spp. (11).

Native plasmids in the hydrogen-oxidizing autotrophic strain *Rhodococcus* sp. (*Nocardia opaca* 1b) have been described (38, 41). The self-transmissible trait Aut^+, encoding genes for autotrophic growth in this strain, was previously thought to be plasmid localized, but is now considered to be a chromosomal trait (41). Thallium resistance is associated with large plasmids, (110 to 140 kb) in two Aut^+ strains (41).

In this study, we constructed an *Escherichia coli-Rhodococcus* shuttle vector and used this plasmid to develop a plasmid transformation system for the wild-type soil isolate, designated *Rhodococcus* sp. strain H13-A. This organism produces an extracellular glycolipid with surface-active properties (14).

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are shown in Table 1. *Rhodococcus* sp. strain H13-A, a wild-type soil organism previously isolated in this laboratory (14), was identified as a *Rhodococcus* sp. on the basis of cell wall chemotype, cellular lipid analyses, and biochemical tests as specified in *Bergey's Manual* (29). *Rhodococcus* sp. strain AS-7 is a pMVS200-cured derivative of *Rhodococcus* sp. strain H13-A

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TABLE 1. Bacterial strains and plasmids used in this study

Strain	Plasmid markers	Reference
Rhodococcus sp.		
H13-A(pMVS100, pMVS200, pMVS300)	Cryptic	This work
AS-7(pMVS100, pMVS300)	Cryptic	This work
AS-50(pMVS100, pMVS200)	Cryptic	This work
AS-50-1(pMVS100, pMVS200, pMVS301)	Ap^r $Thio^r$ (pMVS301)	This work
AS-50-2(pMVS100, pMVS200, pMVS302)	Ap^r $Thio^r$ (pMVS302)	This work
E. coli		
DH1		32
1830(pIJ30)	Ap^r $Thio^r$	45
DH1(pMVS301)	Ap^r $Thio^r$	This work
DH1(pMVS302)	Ap^r $Thio^r$	This work

and was used as the source of pMVS300 DNA (Fig. 1). *Rhodococcus* sp. strain AS-50 is a pMVS300-cured derivative of strain H13-A and was used as a source of pMVS200 DNA (Fig. 1).

E. coli DH1, which was used as the host for plasmid transformation, and *E. coli* 1830(pIJ30), which was used as a source of pIJ30 DNA, were obtained from Apcel Ltd., Slough, United Kingdom.

The coryneform bacteria *Arthrobacter globiformis* ATCC 8010, *Corynebacterium glutamicum* ATCC 13059, and *Brevibacterium linens* ATCC 9172 were obtained from the American Type Culture Collection, Rockville, Md.; *A. simplex* BRRL 35581 was obtained from Apcel, Ltd.

Rhodococcus strains, *R. equi* (*Nocardia restricta*) ATCC 14887-1, *R. globerulus* (*N. globerula*) ATCC 15903 and ATCC 19370, and *R. erythropolis* (*N. calcarea*) ATCC 19369 were obtained from George Brownell, Medical College of Georgia, Augusta. *R. erythropolis* ATCC 4277 and *R. rhodochrous* ATCC 13808 were type strains obtained from the American Type Culture Collection.

Growth conditions and culture media. *Rhodococcus* strains were grown at 30°C on NBYE medium, which contained 0.8% (wt/vol) nutrient broth (Difco Laboratories, Detroit, Mich.) and 0.5% (wt/vol) yeast extract (Difco). NBYE was supplemented with 1.5% (wt/vol) Bacto-Agar (Difco) for growth on solid medium. The hypertonic protoplast regeneration medium, R2YE, was described previously (22, 46). R2YE soft-agar overlays contained R2YE medium plus 0.6% Bacto-Agar. Protoplast buffer (P buffer), a hypertonic medium used for protoplast preparation and transformation, was described previously (33).

E. coli strains were grown at 37°C on Luria-Bertani (LB) medium (32) or LB medium supplemented with 1.5% Bacto-Agar for growth on solid medium. Liquid cultures were agitated at 250 rpm for *E. coli* strains or 300 rpm for *Rhodococcus* strains on a rotary shaker-incubator.

Thiostrepton, which was obtained from Salvadore Lucania, Squibb Institute of Medical Research, Princeton, N.J., was prepared in dimethyl sulfoxide.

Plasmid-curing methods. *Rhodococcus* sp. strain H13-A was grown in the presence of 50 μg of acridine orange per ml or 500 μg of sodium dodecyl sulfate per ml for 18 h. Surviving cells were plated onto NBYE agar and screened for plasmid loss by the small-scale plasmid isolation method developed for this strain.

Isolation of plasmid DNA. Plasmid DNA was isolated from *E. coli* by the alkaline lysis method (5) and was purified by centrifugation in cesium chloride-ethidium bromide density gradients (32). The boiling method of Holmes and Quigley (21) was used for rapid, small-scale isolation of *E. coli* plasmid DNA.

The following procedure was devised for large-scale isolation of plasmid DNA from *Rhodococcus* spp. Exponential-phase cells were harvested by centrifugation at 6,500 × *g* for 10 min and were washed once in 10 mM Tris–1 mM EDTA buffer (pH 8.0) (TE buffer). The cells were incubated at 37°C for 2 h in a buffer containing 0.05 M Tris, 0.01 M EDTA, 0.05 M NaCl, and 20% (wt/vol) sucrose (pH 8.0) plus 5 mg of lysozyme per ml. Cells were then lysed in 3.0% (wt/vol) sodium dodecyl sulfate in 0.05 M Tris hydrochloride buffer (pH 12.6) at 55°C for 2 h. Chromosomal DNA was precipitated with 5 M potassium acetate–acetate buffer (pH 4.8) (32) and centrifuged at 10,000 × *g* for 30 min. Plasmid DNA was precipitated from the resulting supernatant solution with isopropanol and was purified by centrifugation in cesium chloride-ethidium bromide density gradients (32).

The same procedure was scaled down for use with 1.5 ml of exponential-phase NBYE-grown cells for small-scale preparation of plasmid DNA from *Rhodococcus* spp., eliminating the final cesium chloride-ethidium bromide density gradient centrifugation.

Plasmid transformation. *E. coli* DH1 was transformed by the method of Hanahan (20). Transformants were selected on LB agar plates containing 100 μg of ampicillin per ml. The following method was developed for polyethylene glycol (PEG)-assisted transformation of *Rhodococcus* protoplasts.

Rhodococcus protoplast preparation. Mid-exponential-phase NBYE-grown cells were grown for 2 h in the presence of ampicillin (200 μg/ml). Cells were harvested in 5.0-ml portions by centrifugation at 1,600 × *g* for 5 min at 25°C, washed once with P buffer, centrifuged, suspended in 1.0 ml of P buffer containing 10 mg of lysozyme per ml, and incubated for 2 h at 35°C with intermittent agitation. This cell suspension was diluted with P buffer, centrifuged, washed once in P buffer, and centrifuged again. The resulting pellet containing protoplasts and intact cells was suspended in 150 μl of P buffer and used immediately for transformation. The percentage of protoplasts in the preparation was determined by differential plate counts on R2YE regeneration medium and on NBYE medium or by microscopic counts.

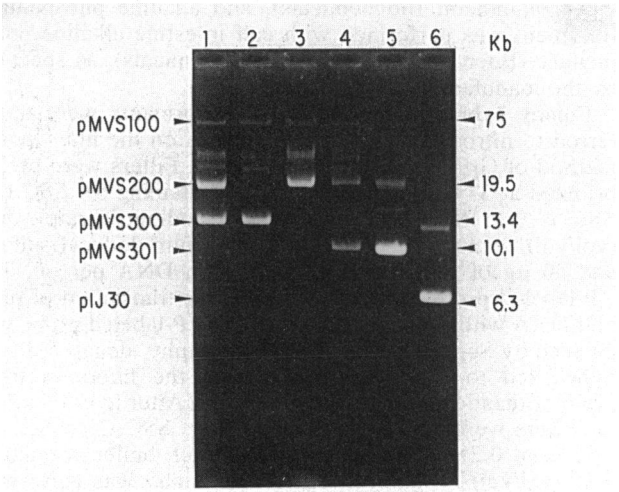

FIG. 1. Agarose gel electrophoresis of plasmid DNA isolated from *Rhodococcus* sp. strains H13-A (lane 1), AS-7 (lane 2), AS-50 (lane 3), and AS-50-1(pMVS301) (lane 4), *E. coli* DH1(pMVS301) (lane 5), and *E. coli* 1830(pIJ30) (lane 6).

Transformation of *Rhodococcus* protoplasts. Freshly prepared protoplasts were diluted in P buffer to a density of 2.0×10^7/ml. The protoplast suspension (100 μl) was mixed with 0.075 to 0.375 μg of plasmid DNA in 1 to 5 μl of TE buffer. PEG 8000 (25%, vol/vol; Sigma Chemical Co., St. Louis, Mo.) in P buffer (200 μl) was added, and the solution was gently mixed. After 10 min at 25°C, the protoplast suspension was diluted with P buffer and plated immediately onto freshly prepared and dehydrated R2YE agar plates. The plates were overlaid with R2YE soft agar containing 50 μg of thiostrepton per ml, after a 24-h regeneration period at 30°C, to select for thiostrepton-resistant transformants. Transformants were screened for ampicillin resistance by replica plating to NBYE medium containing ampicillin (30 μg/ml).

Determination of plasmid stability. *Rhodococcus* and *E. coli* plasmid-containing strains were grown under nonselective conditions on NBYE or LB medium, respectively, for 24 to 30 generations and were then plated onto the same medium. A total of 500 colonies were scored by replica plating for resistance to ampicillin (100 μg/ml for *E. coli*, 30 μg/ml for *Rhodococcus* spp.) and to thiostrepton (50 μg/ml for *Rhodococcus* spp.). The plasmid content was verified by small-scale plasmid preparation.

DNA biochemistry. Restriction endonuclease digestions were performed as specified by the manufacturers. Restriction enzymes were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind., New England BioLabs, Inc., Beverly, Mass., or Bethesda Research Laboratories, Inc., Gaithersburg, Md. Plasmid restriction mapping was performed by using a series of single and multiple restriction digestions of plasmid DNA. DNA fragments were separated by horizontal gel electrophoresis with gels prepared from 0.7% (wt/vol) agarose (International Biotechnologies, Inc., New Haven, Conn.) or 4.0% (wt/vol) Nu-Sieve agarose (FMC BioProducts, Rockland, Maine) to resolve small DNA fragments, with 0.04 M Tris-acetate–0.002 M EDTA electrophoresis buffer (pH 8.0) at 100 or 50 V, respectively. Gels were stained with ethidium bromide (0.5 μg/ml), and DNA was visualized with UV light. DNA fragment size was determined by comparison with *Hin*dIII-digested linear phage lambda DNA fragments and with a 1-kb ladder linear DNA standard (Bethesda Research Laboratories).

Ligations were performed with T4 DNA ligase (Boehringer Mannheim Biochemicals), and alkaline phosphatase treatment was performed with calf intestine alkaline phosphatase (Boehringer Mannheim Biochemicals), as specified by the manufacturer.

Colony hybridization. *E. coli* transformants were transferred to nitrocellulose filters and lysed on the filter by the method of Grunstein and Hogness (19). Filters were prehybridized at 37°C for 2 h in a buffer containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% (vol/vol) formamide, 0.1% (wt/vol) sodium dodecyl sulfate, and 50 μg of denatured salmon sperm DNA per ml. The ^{32}P-labeled probe was prepared by nick translation of plasmid DNA with [α-^{32}P]dATP (32). The ^{32}P-labeled probe was purified by Sephadex G-25 chromatography, denatured, and hybridized to DNA immobilized on the filters in fresh prehybridization buffer at 37°C for 18 h. After hybridization, the filters were washed at 37°C with 6× SSC, 2× SSC, 1× SSC, and 0.2× SSC in succession, each buffer containing 50% (vol/vol) formamide. Autoradiography was performed with Kodak XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) developed at −80°C for 18 h with an intensifying screen.

Southern hybridization. Transfer of DNA from agarose gels to nitrocellulose and Southern hybridization with the ^{32}P-labeled probe, prepared by nick translation of a purified plasmid restriction fragment, were performed by the method of Maniatis et al. (32).

Measurement of β-lactamase activity. β-Lactamase activity was measured with the chromogenic β-lactamase substrate PADAC [7-(thienyl-2-acetamide)-3-[-2-(4-*N*,*N*-dimethylaminophenylazo)pyridinium methyl]-3-cephem-4-carboxylic acid], described by Shindler and Huber (42). NBYE- or LB-grown cells were suspended in 20 mM Tris hydrochloride buffer (pH 8.0). The reaction was started by addition of 0.1 ml of cells or culture supernatant to 0.9 ml of 25 μM PADAC in 20 mM Tris hydrochloride buffer (pH 8.0). β-Lactamase activity was measured by monitoring the decrease in optical density at 570 nm at 30°C. Intact cells were treated prior to measurement of enzyme activity by addition of 10% toluene and 0.1% sodium deoxycholate.

Cell protein was measured by the method of Lowry et al. with bovine serum albumin as the standard (30).

RESULTS

Characterization of *Rhodococcus* plasmids. Three plasmids, pMVS100 (75.0 kb), pMVS200 (19.5 kb), and pMVS300 (13.4 kb), were found in the wild-type strain, *Rhodococcus* sp. strain H13-A (Fig. 1). Plasmid size was determined by restriction analysis of the purified plasmids. Each plasmid exhibited a unique restriction pattern with several different restriction enzymes. pMVS100, pMVS200, and pMVS300 are cryptic plasmids, having no detectable plasmid-linked antibiotic resistance or heavy-metal resistance markers.

Construction of an *E. coli-Rhodococcus* shuttle vector. The general strategy used for shuttle vector construction is shown in Fig. 2. The cloning vector, pIJ30, is an *E. coli* replicon derived from pBR322, containing an *E. coli* origin of replication (*ori*), an *E. coli* gene for ampicillin resistance

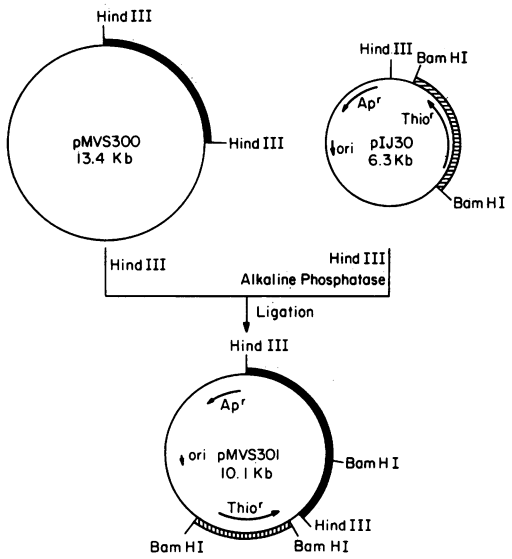

FIG. 2. Construction of the *E. coli-Rhodococcus* shuttle plasmid pMVS301. The 3.8-kb *Hin*dIII fragment (■) of pMVS300 was cloned into pIJ30, which is a pBR322 derivative containing the *E. coli* origin of replication (*ori*) and *bla* gene (ampicillin resistance) and *S. azureus* DNA (▨) with the *tsr* gene (thiostrepton resistance) (45). Arrows indicate the direction of transcription.

(*bla*), and a gene for thiostrepton resistance (*tsr*), derived from *Streptomyces azureus* (45). The native *Rhodococcus* plasmid, pMVS300, was digested with *Hin*dIII, yielding restriction fragments of 3.8 and 9.6 kb. The entire restriction digest was then ligated to the *Hin*dIII-digested, alkaline phosphatase-treated vector, pIJ30, which has a single *Hin*dIII restriction site. The ligation mixture was used to transform *E. coli* DH1, selecting for ampicillin resistance. Ampicillin-resistant transformants were analyzed by colony hybridization with ^{32}P-labeled pMVS300 DNA to detect recombinant plasmids. The plasmid content of presumptive transformants was verified by small-scale plasmid preparation, followed by digestion with the appropriate restriction enzyme. Two 10.1-kb recombinant plasmids were detected which contained the pMVS300-derived 3.8-kb *Hin*dIII restriction fragment cloned in two different orientations relative to the internal *Bam*HI cleavage site. These two plasmids were designated pMVS301 (Fig. 2) and pMVS302. Recombinant plasmids containing the 9.6-kb *Hin*dIII restriction fragment of pMVS300 were not detected among the transformants tested. Southern hybridization experiments confirmed that the 3.8-kb *Hin*dIII fragment cloned in pMVS301 and pMVS302 was derived from pMVS300 (data not shown).

Plasmid transformation in *Rhodococcus* spp. Transformation of *Rhodococcus* sp. strain AS-50 with pMVS301 initially yielded thiostrepton-resistant transformants at low transformation frequencies (<100 transformants per μg of DNA), with significantly higher frequencies resulting from optimization of the transformation procedure as described below. Thiostrepton-resistant transformants were not detected with pIJ30 as donor DNA. Likewise, spontaneous thiostrepton-resistant mutants of strain AS-50 were not detected when plasmid DNA was deleted in control transformation experiments.

Thiostrepton-resistant transformants of strain AS-50 were characterized to verify plasmid content and to examine the expression of the plasmid antibiotic resistance markers. The transformants were resistant to thiostrepton (>500 μg/ml) and to ampicillin (30 μg/ml), whereas the host strain, AS-50, was sensitive to 0.05 μg of thiostrepton per ml and 5 μg of ampicillin per ml. Each of the transformants contained the 10.1-kb plasmid, pMVS301. Strain AS-50-1(pMVS301), a representative transformant, contained pMVS301 as well as the native plasmids pMVS100 and pMVS200, which were present in the host strain, AS-50 (Fig. 1). *E. coli* DH1 was transformed with plasmid DNA prepared from *Rhodococcus* sp. strain AS-50-1(pMVS301), selecting for ampicillin-resistant transformants. The resulting transformants also contained pMVS301 (Fig. 1). The plasmid was again isolated from *E. coli* and transformed into *Rhodococcus* sp. strain AS-50, with isolation of thiostrepton-resistant transformants harboring pMVS301.

Restriction analysis showed that pMVS301 did not undergo rearrangement or deletion in either *E. coli* or *Rhodococcus*. An additional *Cla*I restriction site, located in the 3.8-kb *Rhodococcus* DNA fragment, was identified in the plasmid only when isolated from *Rhodococcus* transformants (Fig. 3). Since *Cla*I restriction sites are subject to methylation in *dam*$^+$ *E. coli* strains, this site was presumably methylated in *E. coli* DH1 and not in the *Rhodococcus* strain. With this exception, plasmid restriction patterns were identical regardless of plasmid source by using several different restriction enzymes.

Transformation of *Rhodococcus* sp. strain AS-50 with pMVS302 DNA also yielded thiostrepton-resistant transformants. pMVS302 differs from pMVS301 only in the orientation of the cloned 3.8-kb *Hin*dIII fragment of pMVS300 (Fig. 2). These transformants contained pMVS302 and were resistant to >500 μg of thiostrepton per ml and >750 μg of ampicillin per ml. Plasmid DNA isolated from *Rhodococcus* sp. strain AS-50-2(pMVS302), a thiostrepton-resistant transformant containing pMVS302, was used to transform *E. coli* DH1. The resulting ampicillin-resistant transformants contained pMVS302. Restriction analyses demonstrated that neither deletion nor rearrangement of pMVS302 occurred in *E. coli* or *Rhodococcus* spp.

Optimization of conditions for plasmid transformation in *Rhodococcus* spp. *Rhodococcus* transformants were not detected when PEG was omitted from the transformation mixture or when intact cells rather than protoplasts were used in the transformation procedure. PEG concentrations above 25% (wt/vol) were inhibitory, and higher transformation frequencies were obtained with PEG 8000 rather than PEG 3350 or PEG 1000. Treatment of cells with ampicillin (200 μg/ml) prior to lysozyme digestion of the cell wall was required for efficient protoplast formation. The protoplast regeneration efficiency was 75% for *Rhodococcus* sp. strains AS-50. The maximum transformation frequency obtained was 2×10^5 to 3×10^5 transformants per μg of DNA when *Rhodococcus*-derived pMVS301 or pMVS302 DNA was used to transform *Rhodococcus* sp. AS-50 (Table 2). Transformation frequencies were approximately 500-fold lower when *E. coli*-derived pMVS301 or pMVS302 DNA was used to transform *Rhodococcus* sp. strain AS-50 (Table 2). These results indicate the presence of a restriction-modification system in the *Rhodococcus* recipient, which can be overcome by using plasmid DNA isolated from *Rhodococcus* spp. for transformation.

Similar transformation frequencies were obtained when using the wild-type strain, *Rhodococcus* sp. strain H13-A, or strain AS-50 or AS-7 as recipient (Table 2). Transformation of *Rhodococcus* sp. strain H13-A or strain AS-7 with pMVS301 resulted in the loss of pMVS300, indicating incompatibility between pMVS300 and pMVS301. Homologous recombination of pMVS300 with pMVS301, which would result in the generation of a larger plasmid, was not detected in strain H13-A or AS-7 transformants. No homology was detected between the 3.8-kb *Hin*dIII fragment of pMVS301

TABLE 2. Plasmid transformation of *Rhodococcus* protoplasts

Recipient *Rhodococcus* strain	Donor DNA	Source of donor DNA	Transformation frequency (transformants/μg of DNA)
AS-50	pMVS301	*Rhodococcus* sp. strain AS-50-1(pMVS301)	1.9×10^5
AS-50	pMVS302	*Rhodococcus* sp. strain AS-50-2(pMVS302)	3.3×10^5
H13-A	pMVS301	*Rhodococcus* sp. strain AS-50-1(pMVS301)	1.0×10^5
AS-7	pMVS301	*Rhodococcus* sp. strain AS-50-1(pMVS301)	8.3×10^4
AS-50	pMVS301	*E. coli* DH1(pMVS301)	3.6×10^2
AS-50	pMVS302	*E. coli* DH1(pMVS302)	3.8×10^2

and pMVS100 or pMVS200 by Southern hybridization experiments (data not shown).

Host range of shuttle vector. Representatives of the genus *Rhodococcus* and several members of the coryneform group of bacteria were transformed with the shuttle plasmid pMVS301 by using the protoplast transformation method developed for *Rhodococcus* sp. strain H13-A. Thiostrepton-resistant transformants were obtained with *R. erythropolis* ATCC 4277, *R. globerulus* (*N. globerula*) ATCC 15903, and *R. equi* (*N. restricta*) ATCC 14887-1 at frequencies similar to those obtained with strain H13-A and derivatives (Table 3). Transformants were obtained at a 33-fold-lower frequency in *R. erythropolis* (*N. calcarea*) ATCC 19369. Thiostrepton-resistant transformants of these strains were stable upon repeated transfer to thiostrepton-containing medium. In addition, all exhibited resistance to ampicillin at levels ranging from 25 to 100 μg/ml, depending on the strain.

Thiostrepton-resistant transformants were not detected in *R. rhodochrous* ATCC 13808 or in the coryneform bacteria, *A. globiformis*, *C. glutamicum*, or *B. linens* (Table 3). Unstable transformants were obtained with *R. globerulus* (*N. globulera*) ATCC 19370 and *A. simplex* BRRL 35581, with loss of thiostrepton resistance following one or more transfers to thiostrepton-containing medium.

The conditions for the formation of protoplasts and the transformation of plasmid DNA were not optimized for these strains, and transformation frequencies may reflect nonoptimal conditions for any of several steps in the procedure. However, all strains tested formed viable protoplasts which regenerated on R2YE medium under the conditions established for *Rhodococcus* sp. strain H13-A. Low transformation frequencies in these strains could also reflect inability of the plasmid to replicate in the recipient, plasmid restriction in the recipient, or nonexpression of the *tsr* gene on the plasmid.

Shuttle vector stability. The shuttle plasmids pMVS301 and pMVS302 exhibited less than a 0.1% loss per generation in *E. coli* during growth under nonselective conditions. In *Rhodococcus* sp. strain AS-50-1(pMVS301), the plasmid exhibited a 1.0% loss per generation, whereas in *Rhodococcus* sp. strain AS-50-2(pMVS302), the plasmid was lost at a frequency of 7.3% per generation during growth under nonselective conditions. *Rhodococcus* transformants were routinely grown in medium containing 50 μg of thiostrepton per ml. Under these selective conditions, thiostrepton resistance was maintained as a stable, plasmid-borne trait.

TABLE 3. Transformation of nocardioform and coryneform bacteria with the *E. coli-Rhodococcus* shuttle plasmid pMVS301

Strain	Transformation frequency (transformants/μg of DNA)
Rhodococcus strains	
R. erythropolis ATCC 4277	6.4×10^5
R. erythropolis (*N. calcarea*) ATCC 19369	7.8×10^3
R. globerulus (*N. globerula*) ATCC 15903	2.4×10^5
R. globerulus (*N. globerula*) ATCC 19370	<1.0
R. equi (*N. restricta*) ATCC 14887-1	3.4×10^6
R. rhodochrous ATCC 13808	0
Coryneform bacteria	
A. simplex BRRL 35581	1×10^2
A. globiformis ATCC e8010	0
C. glutamicum ATCC 13059	0
B. linens ATCC 9172	0

TABLE 4. MIC of antibiotics for *E. coli* and *Rhodococcus* strains

Strain	MIC (μg/ml) of:	
	Ampicillin	Thiostrepton
Rhodococcus sp.		
H13-A	<5	0.05
AS-50	<5	0.05
AS-50-1(pMVS301)	30–50	>500
AS-50-2(pMVS302)	750–1,000	>500
E. coli		
DH1	<5	NS[a]
DH1(pMVS301)	>1,000	NS
DH1(pMVS302)	>1,000	NS

[a] NS, Not sensitive

β-Lactamase activity in transformants. The MICs of ampicillin for *Rhodococcus* transformants containing pMVS301 or pMVS302 were 6-fold and 150-fold higher, respectively, than that for the parent strain, AS-50 (Table 4). The MIC of ampicillin for *Rhodococcus* sp. strain AS-50-2(pMVS302) was 30-fold higher than that for strain AS-50-1(pMVS301) (Table 4). The MIC of thiostrepton, however, was similar for both strains (Table 4). The MIC of ampicillin was >1,000 μg/ml for *E. coli* DH1(pMVS301) and DH1(pMVS302) (Table 4). This MIC was 20- to 30-fold higher than that for *Rhodococcus* sp. strain AS-50-1(pMVS301), whereas the MIC for *Rhodococcus* sp. strain AS-50-2(pMVS302) was elevated, similar to those observed for the *E. coli* transformants (Table 4).

β-Lactamase activity in the cells and extracellular growth medium of *E. coli* and *Rhodococcus* transformants was measured to correlate levels of ampicillin resistance with β-lactamase activity. β-Lactamase activity was exclusively extracellular in *Rhodococcus* transformants, with no detectable cell-associated activity (Table 5). Cell extracts prepared by sonication of *Rhodococcus* cells showed no detectable β-lactamase activity (data not shown). In *E. coli* transformants, 70% of the β-lactamase activity was cell associated and 30% was present in the growth medium (Table 5). The specific activity of β-lactamase in pMVS301- and pMVS302-containing transformants was threefold higher than that of the host strain, *E. coli* DH1 (Table 5). Total β-lactamase activity was 7- and 47-fold higher in *E. coli* transformants containing pMVS301 and pMVS302, respectively, than in the corresponding *Rhodococcus* transformants, indicating lower levels of *bla* gene expression in *Rhodococcus* than in *E. coli* (Table 5). Total β-lactamase activity in *Rhodococcus* sp. strain AS-50-2(pMVS302) was 8-fold higher than that in *Rhodococcus* sp. strain AS-50-1(pMVS301) and 91-fold higher than in the parent strain, *Rhodococcus* sp. AS-50; however, β-lactamase activity was 11-fold higher in strain AS-50-1(pMVS301) than in strain AS-50 (Table 5). The relative β-lactamase activity in *Rhodococcus* strains correlated directly with the MIC of ampicillin for the respective transformants. The expression of the *E. coli bla* gene in *Rhodococcus* strains appears dependent on the orientation of the 3.8-kb *Hin*dIII *Rhodococcus* DNA fragment in the shuttle plasmid, suggesting that the *bla* gene is transcribed from a promoter in that segment of DNA.

Restriction analysis of pMVS301. A partial restriction endonuclease cleavage map of pMVS301 is shown in Fig. 3. The plasmid has 14 unique restriction sites, 8 of which are found in the cloned DNA fragment containing the *Rhodo-*

TABLE 5. β-Lactamase activity in *E. coli* and *Rhodococcus* strains

Strain	β-Lactamase activity (U/mg of cell protein)[a]	
	Cellular	Extracellular
Rhodococcus sp.		
AS-50	ND	0.03
AS-50-1(pMVS301)	ND	0.33
AS-50-2(pMVS302)	ND	2.73
E. coli		
DH1	5.30	0.04
DH1(pMVS301)	10.70	5.06
DH1(pMVS302)	12.50	6.42

[a] Defined as follows: 1 U of β-lactamase activity equals 1 μmol of PADAC hydrolyzed per min at 30°C.
[b] ND, Not detected.

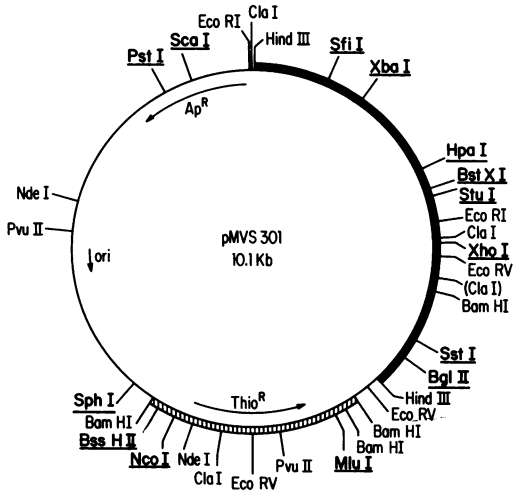

FIG. 3. Partial restriction map of pMVS301. Unique restriction endonuclease cleavage sites are underlined. Symbols: ■, *Rhodococcus* DNA with the *Rhodococcus* origin of replication; ▨, *Streptomyces* DNA carrying the *tsr* gene (thiostrepton resistance); —, DNA from pBR322 with the *E. coli* origin of replication (*ori*) and *bla* gene (ampicillin resistance). Arrows denote the direction of transcription. The *Cla*I restriction site in the *Rhodococcus* DNA, designated (ClaI), is not cleaved in DNA isolated from *E. coli* DH1(pMVS301).

coccus origin of replication. The essential regions in this segment of DNA, including the *Rhodococcus* plasmid replication functions, have not been physically mapped. The pBR322 portion of the plasmid has three unique restriction sites: *Pst*I, *Sca*I, and *Sph*I. The 1.8-kb *Bam*HI fragment which contains the thiostrepton resistance determinant has three unique restriction sites: *Mlu*I, previously determined from *tsr* sequence data (2, 22) and *Bss*HII and *Nco*I, determined by restriction mapping to lie upstream of the known *tsr* coding sequence. The known restriction endonuclease cleavage sites of pMVS301 are listed in Table 6.

DISCUSSION

We have developed a plasmid transformation system in *Rhodococcus* spp. which involves uptake of plasmid DNA by protoplasts in the presence of PEG. The transformation system is highly efficient, yielding more than 10^5 transformants per μg of *Rhodococcus*-derived plasmid DNA. Transformation was less efficient with *E. coli*-derived plasmid DNA, indicating the presence of a restriction-modification system in *Rhodococcus* spp. This transformation system was adapted from methods designed for the protoplast transformation of *Streptomyces* spp. (4) with identical protoplasting and regeneration media. Differences in this system include growth of cells in the presence of ampicillin rather than glycine, longer treatment times with lysozyme at a higher concentration, and transformation in the presence of PEG 8000 rather than PEG 1000.

The *E. coli-Rhodococcus* shuttle plasmids pMVS301 and pMVS302 were constructed as a prerequisite to the development of a plasmid transformation system in *Rhodococcus* spp. These bifunctional plasmids fulfilled the requirements for stable, independent replication in both *Rhodococcus* spp. and *E. coli* and had two selectable antibiotic resistance markers, encoding ampicillin resistance and thiostrepton resistance. The construction of these shuttle plasmids allowed us to perform initial cloning experiments and molecular characterization of the hybrid plasmids in *E. coli* and further genetic manipulation and molecular analyses in *E. coli* or *Rhodococcus* spp.

Both antibiotic resistance markers, the *Streptomyces* thiostrepton resistance gene *tsr* and the *E. coli* ampicillin resistance gene *bla* were expressed in *Rhodococcus* transformants. However, thiostrepton resistance is not a selectable marker in *E. coli* owing to outer membrane exclusion of thiostrepton by gram-negative bacteria (15). The level of *bla* gene expression differed in *E. coli* and *Rhodococcus* transformants, with lower β-lactamase activity detected in *Rhodococcus* transformants. This could be attributed to lower copy number of the plasmids, lower levels of *bla* gene transcription or translation, or increased rates of RNA or protein degradation in *Rhodococcus* spp. Interestingly, in *Rhodococcus* spp. β-lactamase activity was located exclusively in the extracellular medium, indicating that the organism can process and secrete heterologous proteins.

Rhodococcus transformants containing pMVS302 exhibited higher levels of ampicillin resistance and β-lactamase activity than those containing pMVS301. The two plasmids differed only in the orientation of the cloned fragment of *Rhodococcus* DNA located upstream of the *bla* gene. Expression of the *bla* gene in *E. coli* transformants was independent of the orientation of the cloned *Rhodococcus* DNA, with similar levels of ampicillin resistance and β-

TABLE 6. Restriction endonuclease cleavage sites in pMVS301

No. of sites	Restriction endonuclease
1	*Pst*I, *Sca*I, *Sph*I, *Mlu*I, *Bss*HII, *Bgl*II, *Hpa*I, *Stu*I, *Xho*I, *Sfi*I, *Nco*I, *Bst*XI, *Sst*I, *Xba*I
2	*Hin*dIII, *Eco*RI, *Nhe*I, *Nde*I, *Apa*I, *Pvu*II, *Bsm*I, *Bst*EII
3	*Ssp*I, *Sty*I, *Eco*RV, *Nru*I, *Sma*I
>3	*Bam*HI, *Cla*I, *Xma*III, *Pvu*I, *Ava*I, *Sal*I, *Sst*II, *Xma*I
0	*Sna*BI, *Spe*I, *Kpn*I, *Not*I, *Nsi*I, *Aat*I, *Asu*II, *Cvn*I

lactamase activity in transformants containing either plasmid. The orientation-dependent expression of the *E. coli bla* gene in *Rhodococcus* spp. suggests that *bla* gene transcription occurs from a *Rhodococcus* promoter in the cloned fragment rather than from its own promoter. Alternatively, pMVS302 may have a higher copy number than pMVS301 in *Rhodococcus* spp., although this seems unlikely since both plasmids contain the same origin of replication. In addition, estimates of plasmid copy number from gels of total DNA preparations indicate that pMVS301 and pMVS302 have similar copy numbers in *Rhodococcus* spp.

Several *Rhodococcus* species were transformed with the shuttle plasmid at high efficiencies, yielding stable plasmid transformants. The plasmid host range includes strains of *R. erythropolis*, *R. globerulus*, and *R. equi*. The only representative of the *R. rhodochrous* group tested in this study was not transformed with the vector. Representatives of other actinomycete genera, including *Streptomyces*, *Nocardia*, and *Mycobacterium* spp., were not tested for their ability to transform and maintain the vector in this study. The coryneform bacteria tested were not stably transformed with the plasmid, although protoplasts were readily formed and regenerated.

The *E. coli-Rhodococcus* shuttle vector constructed in this study has several potentially useful cloning sites. The unique *Pst*I site in the *bla* gene may be useful for self-cloning in *Rhodococcus* spp. by insertional inactivation of the ampicillin resistance determinant. However, inactivation of this marker would preclude further use of the vector in *E. coli*. Several other unique sites on the vector, such as the *Sph*I and the *Bgl*II sites, may be useful for nonselectional cloning in either *Rhodococcus* spp. or *E. coli*.

This report is the first description of a plasmid transformation system and of heterologous gene expression in *Rhodococcus* spp. The *E. coli-Rhodococcus* shuttle vectors constructed in this study are potentially useful for molecular cloning in this genus. In addition, this plasmid transformation and molecular cloning system may have broader applicability for cloning actinomycete genes of interest, including genes from the medically important pathogens *Nocardia* and *Mycobacterium* spp. *Mycobacterium* DNA has been cloned in both *E. coli* (10, 25, 28, 44, 50) and *Streptomyces* spp. (25). In general, mycobacterial genes are weakly expressed from their own promoters in *E. coli* (10, 25, 44). Cloned *Mycobacterium bovis* BCG DNA was expressed from its own transcriptional and translational signals more efficiently in *S. lividans* than in *E. coli* (25). Likewise, many *Streptomyces* genes are not transcribed efficiently in *E. coli* owing to the absence of promoter recognition and differences in promoter structure (2, 3), although recent studies have demonstrated the expression of certain *Streptomyces* promoters in *E. coli* (12, 24). Members of the genus *Streptomyces*, a sporeforming, mycelial actinomycete with a G+C content of 69 to 73 mol% (27), readily recognize heterologous promoters (3, 23), and substantial progress has been made in the development of molecular cloning and genetic transfer systems in this organism (22). The *Rhodococcus* host-vector system described in this study may provide an alternative for molecular cloning in the actinomycetes. *Rhodococcus* sp. strain H13-A is a nonpathogenic, nonmycelial, nonsporulating strain with a G+C content of 66 mol% (59 to 69 mol% G+C for the genus *Rhodococcus*), which falls within the range reported for members of the closely related genera *Mycobacterium* (62 to 70 mol% G+C) and *Nocardia* (60 to 69 mol% G+C) (29). This study has demonstrated the expression of genes in *Rhodococcus* spp. from both *E. coli* and *Streptomyces* spp. indicating that *Rhodococcus* spp. may be useful hosts for the expression of actinomycete DNA from its own regulatory signals.

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