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Conservation of Plasmid-Encoded Dibenzothiophene Desulfurization Genes in Several Rhodococci†

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The cloned sulfur oxidation (desulfurization) genes (*sox*) for dibenzothiophene (DBT) from the prototype *Rhodococcus* sp. strain IGTS8 were used in Southern hybridization and PCR experiments to establish the DNA relatedness in six new rhodococcal isolates which are capable of utilizing DBT as a sole sulfur source for growth. The ability of these strains to desulfurize appears to be an exclusive property of a 4-kb gene locus on a large plasmid of ca. 150 kb in IGTS8 and ca. 100 kb in the other strains. Besides a difference in plasmid profile, IGTS8 is distinguishable from the other strains in at least the copy number of the insertion sequence IS*1166*, which is associated with the *sox* genes.

There is considerable interest in developing a biocatalytic system as precombustion technology for the specific removal of organic sulfur from coal and petroleum products, since combustion of these compounds emits noxious oxides of sulfur which contribute to acid rain. Specific breakage of carbon-sulfur bonds in organosulfur compounds has the added advantage of conserving the calorific value of the fuels, since the carbon skeleton in these molecules is left intact (for recent reviews, see references 7, 8, 14, 19).

Dibenzothiophene (DBT) has been used as a model *S*-heterocycle for studying desulfurization in a number of microorganisms (3, 5, 10, 13, 20, 23, 28). *Rhodococcus* sp. strain IGTS8 is a prototype sulfur-specific desulfurization bacterium for which the first molecular cloning and characterization experiments on the genes responsible for sulfur oxidation have been described previously (4, 22). In this plasmid-encoded pathway, three genes (*soxABC* [we have adopted the *sox* nomenclature {5} because of its priority over *dsz* {22}]) arranged in an operonic manner and spanning a 4-kb region (Fig. 1) are responsible for the metabolism of DBT to 2-hydroxybiphenyl (2-HBP) and sulfate. SoxC, a 45-kDa protein bearing sequence relatedness to members of the acyl coenzyme A dehydrogenase family, was found to mediate the initial oxidation of DBT to DBT sulfone. This enzyme was recently characterized and named as a sulfide/sulfoxide monooxygenase which requires reduced flavin mononucleotide for activity (18). SoxA (a 50-kDa protein) and SoxB (a 40-kDa protein) are believed to act concertedly in transforming DBT sulfone to 2-HBP and sulfate (4, 22). The question of whether this is a direct conversion or one that proceeds via the formation of a sulfonate intermediate (2′-hydroxybiphenyl-2-sulfonate) is unresolved (9).

In this study, we carried out a comparative molecular analysis of six rhodococcal strains with probes derived from the IGTS8 *sox* genes and the insertion sequence elements (IS*1166* and IS*1295*) that are associated with the desulfurization pathway (Fig. 1) (6). The results provide a first indication of the conservative nature of the *sox* genotype and establish differences and similarities among desulfurization strains isolated from different geographic locations.

Bacterial strains, nondesulfurizing mutants, and plasmids. Table 1 lists the bacterial strains and plasmids used in this study. The basic physiological and desulfurization properties of *Rhodococcus* sp. strains X309 and X310 (formerly the nonmucoid ECRD-1 and mucoid isolates of *Arthrobacter* spp., respectively) have been described previously (17). The isolation and desulfurization properties of strains B1, If, Ig, and Ih will be described separately (11). Desulfurization was evaluated by the DBT spray plate assay and analysis of 2-HBP (4, 5).

Heat treatment (15) was used to isolate mutants of X309. Bacterial cells were subcultured twice a week in 10 ml of Luria-Bertani broth (0.5-ml overnight culture in 9.5 ml of fresh medium) at 36.5°C, the highest temperature allowing growth. After 10 and 11 subculturings, 50% of the colonies plated on minimal salts medium plus DBT (0.52 mM; Aldrich Chemical Co., Milwaukee, Wis.) were found to be DBT desulfurization negative (DBT^-). Both DBT^+ and DBT^- isolates were screened for the presence of plasmids. Isolates, designated X309-10-1, X309-10-2, X309-11-15, and X309-11-20 (Table 1) were used for further study.

Sequencing of 16S rDNAs. Sequencing of the gene coding for the small ribosomal subunit (16S rRNA) is a well-established method for identifying bacteria (21). Correct strain identification is both a prerequisite for genetic manipulations and an essential piece of pretest information required by the stringent regulations governing the use of microorganisms for biotechnology applications. By using conventional PCR methods and the eubacteria primers 27f and 1492r (16), near-full-length 16S ribosomal DNAs (rDNAs) were amplified from the genomic DNAs of the various isolates. The products were purified with a QIAEX gel extraction kit (Qiagen) and sequenced with an Applied Biosystems model 373A automated fluorescent sequencer and the Taq DyeDeoxy terminator cycle system. The X309 and B1 DNAs were sequenced on both strands with the 16S primers and additional primers derived from the emerging se-

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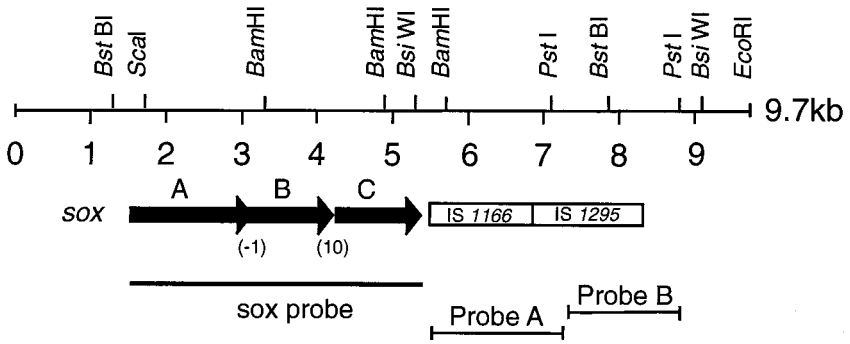

FIG. 1. Location of desulfurization (*sox*) genes and insertion sequence elements (IS*1166* and IS*1295*) in *Rhodococcus* sp. strain IGTS8 (adapted from reference 6 and sequence from GenBank accession no. U08850). Probes shown are as follows: a 4-kb *sox* probe released from pSAD231-4 (Table 1) after double digestion of the vector sequence with *Hin*dIII and *Xba*I; probe A, a 1.2-kb PCR-amplified fragment with pSAD68-3 (Table 1) used as a template and primers 5′-CGACAGCGGTG TTGGTCGGTCGTTGC and 5′-CGATGGGTCGTTCGAGCAGCTTGCC, which correspond to nucleotides 1784 to 1809 and a complementary sequence of nucleotides 2974 to 2998 shown in reference 6; and probe B, a 1.7-kb *Pst*I fragment from pSAD68-3. Seven additional *Pst*I sites in the pSAD68-3 cloned insert are not shown. Numbers in parentheses: −1, overlapping stop/start codon; 10, *soxBC* intergenic space.

quences. For the other strains (X310, If, Ig, and Ih), only the ends of the 16S rDNAs were sequenced (data not shown).

For analysis, the sequence of X309 was first compared with others in a nonredundant sequence database at the National Center for Biotechnology Information by using the BLASTN program (1). The highest score was obtained with *Rhodococcus* sp. strain P6 (EMBL accession no. X77780) which has 95% positional identity with the X309 sequence. This match provided the basis for further sequence comparison in an updated *Rhodococcus* 16S rDNA database which contains the sequences of 13 species of *Rhodococcus* (24). Results of the various binary comparisons showed that the three closest candidates to X309 are *Rhodococcus erythropolis* (DSM 43066), *Rhodococcus globerulus* (DSM 43954), and *Rhodococcus* sp. strain DSM 43943, which exhibit 99.7, 98.1, and 97.8% identity, respectively. In the X309-*R. erythropolis* comparison, no gap was needed for an optimal alignment. Interestingly, *Mycobacterium chlorophenolicum* (DSM 43826), *Gordona aichiensis* (DSM 43978), and *Gordona sputi* (DSM 44019), which were previously identified as *Rhodococcus* spp. (reference 24 and references therein) all gave lower scores (92.6 to 93.4%) than those obtained with the rhodococci.

The B1 sequence is 99% identical to X309. Partial 16S rDNAs of the other isolates (X310, If, Ig, and Ih) also had their highest scores with *R. erythropolis* (data not shown). In this study, the various strains are simply referred to as *Rhodococcus* spp. For a phylogenetic dendrogram of *Rhodococcus* species and a discussion of this taxon, see reference 24.

Plasmid profile and Southern hybridization. The procedure for isolating large plasmids from *Rhodococcus* spp., as modified from that of Tardif et al. (26), is as follows (provided in detail since we found it useful also in the isolation of large and small plasmids from gram-negative sphingomonads and *Escherichia coli*).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid carrier	Genotype or description	Source and/or reference
Rhodococcus sp. strain IGTS8 (ATCC 53968)	DBT^+; pSOX (150 kb)	K. Young (4)
Rhodococcus sp. strain UV1	DBT^- (UV mutant; loss of pSOX)	K. Young (4)
Rhodococcus sp. strain X309[a] (ATCC 55309)	DBT^+; pSOX (100 kb)	M. Grossman (reference 17 and this study)
Rhodococcus sp. strain X310[b] (ATCC 55310)	DBT^+; pSOX (100 kb)	M. Grossman (reference 17 and this study)
Rhodococcus sp. strain X309-10-1	DBT^- (heat mutant; loss of pSOX)	This study
Rhodococcus sp. strain X309-10-2	DBT^- (heat mutant; plasmidless)	This study
Rhodococcus sp. strain X309-11-15[c]	DBT^+ (heat mutant; pSOX)	This study
Rhodococcus sp. strain B1	DBT^+	Emulsion of bitumen (11); this study
Rhodococcus sp. strain If	DBT^+	Calgary, Canada, soil (11); this study
Rhodococcus sp. strain Ig	DBT^+	Calgary, Canada, soil (11); this study
Rhodococcus sp. strain Ih	DBT^+	Calgary, Canada, soil (11); this study
Sphingomonas yanoikuyae B1	PAH^+[d]; 32- and 223-kb plasmids	D. Gibson (University of Iowa); 29
Escherichia coli XL1-blue(pSAD231-4)	4-kb *Bsi*WI-*Bst*BI (*soxABC*) fragment	K. Young (6)
E. coli XL1-blue(pSAD68-3)	8.0-kb *Eco*RI-*Sca*I fragment of IGTS8 DNA containing IS*1166*/IS*1295* cloned in *Eco*RI/*Hin*cII sites of pBluescript SK^-	K. Young (6)
E. coli HB101 (RP4)	60-kb plasmid	26
Agrobacterium tumefaciens C58	200- and 410-kb plasmids	S. Farrand (University of Illinois)
Pseudomonas putida G1 (ATCC 17453)	>200-kb CAM[e] plasmid	ATCC[f]

[a] Formerly *Arthrobacter* sp. strain ECRD-1.
[b] Formerly *Arthrobacter* sp. mucoid strain.
[c] A related isolate is X309-11-20.
[d] Polycyclic aromatic hydrocarbon.
[e] Camphor.
[f] American Type Culture Collection.

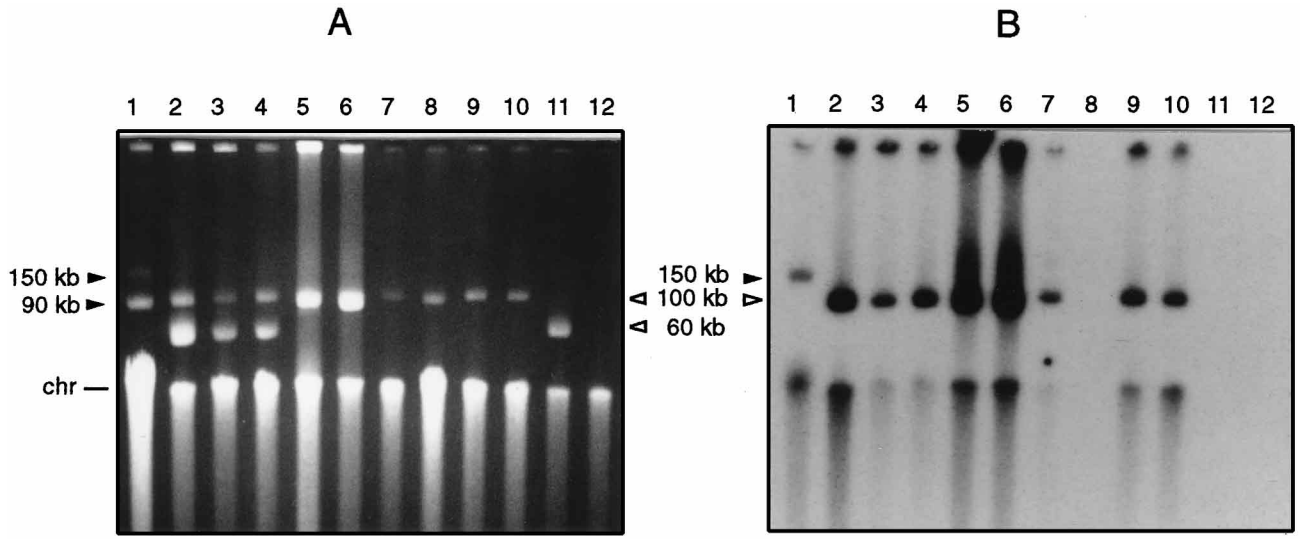

FIG. 2. (A) Plasmid profile in seven desulfurizing rhodococci and heat-treated mutants. Electrophoresis was carried out in a 0.55% agarose gel and run overnight at 50 V in 40 mM Tris-acetate–1 mM EDTA buffer. Lanes: 1, IGTS8; 2, X309; 3, X310; 4, B1; 5, If; 6, Ig; 7, Ih; 8, UV1; 9, X309-11-15; 10, X309-11-20; 11, X309-10-1; 12, X309-10-2. Plasmid markers used in separate experiments to provide the size estimates indicated alongside the gels are listed in Table 1. Closed arrowheads indicate migration positions of IGTS8 plasmids; open arrowheads indicate migration positions of X309 plasmids and related plasmids. chr, chromosomal DNA. (B) Southern blot of the gel shown in panel A. The *sox* probe (Fig. 1) was labelled with [α-^{32}P]dATP by the random priming method (25). Hybridization was carried out under stringent conditions, 6× SSC at 65°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The nylon membrane was washed in 0.1 × SSC–0.1% SDS at 65°C (25). Hybridization signals at the origin of the gel or in the chromosomal DNA region are apparently due to sheared plasmid DNA associated with protein or with the chromosomal DNA. Notice that samples from UV1, X309-10-1, and X309-10-2 (lanes 8, 11, and 12, respectively), which are devoid of the *sox* plasmid but display an abundant chromosomal DNA band, gave a clean background.

(i) Grow 10 ml of bacterial culture in Luria-Bertani broth to an $A_{600\ nm}$ of ~1.

(ii) Resuspend pelleted cells in 0.5 ml of solution I (50 mM glucose–25 mM Tris-HCl [pH 8.0]–10 mM EDTA containing 10 mg of lysozyme per ml). Incubate at 37°C for at least 30 min.

(iii) Place a 100-μl aliquot of cell mix in four Eppendorf tubes. To each tube add 200 μl of a freshly made solution of 0.2 N NaOH–4% sodium dodecyl sulfate (SDS). Incubate on ice for 10 min.

(iv) Add 150 μl of 5 M potassium acetate (pH 5) and incubate on ice for an additional 10 min.

(v) Spin samples in an Eppendorf centrifuge (5 min at room temperature and maximum speed). Remove 400 μl of supernatant from two tubes and pool.

(vi) Add 400 μl of buffer-saturated phenol (Gibco/BRL). Mix solution gently and add 400 μl of chloroform. Mix and centrifuge as in step v.

(vii) Transfer 750 μl of the aqueous-phase solution to a clean tube. Precipitate DNA by adding an equal volume of isopropanol. Mix gently and incubate at room temperature (30 min).

(viii) Collect plasmid DNAs by a 15 min centrifugation in an Eppendorf microcentrifuge.

(ix) Wash DNA pellets in 80% ethanol. Dry briefly under vacuum. Resuspend each pellet in 25 μl of 10 mM Tris-HCl–1 mM EDTA buffer (pH 7.5).

(x) Apply 5 to 10 μl of DNA solution to a 0.55% agarose gel. Stain DNA with ethidium bromide (1 μg/ml) after electrophoresis (0.2 V/cm) in Tris-borate-EDTA (8.9 mM borate) as the running buffer (26). Alternatively, 40 mM Tris-acetate–1 mM EDTA buffer may be used for electrophoresis.

A common plasmid pattern consisting of two large plasmids was found in isolates X309, X310, and B1 (Fig. 2A). Strains If, Ig, and Ih each contained a single plasmid which was similar in size to the larger plasmid found in X309, X310, and B1. This common plasmid is henceforth referred to pSOX since it hybridized to the *sox* gene probe derived from IGTS8 (Fig. 2B). Loss of pSOX in mutants X309-10-1 and X309-10-2, which led to no hybridization signals (Fig. 2B, lanes 11 and 12), provided definitive evidence that the desulfurization phenotype was plasmid coded. Two other classes of mutants are exemplified by isolate X309-11-15, which lost the small plasmid, and isolate X309-10-2, which lost both plasmids.

The molecular size of pSOX(309) and related counterparts is evidently smaller than that of pSOX(IGTS8). Using several closed circular supercoiled plasmid markers (Table 1), our estimates for pSOX(309) and pSOX(IGTS8) were 100 kb and 150 kb, respectively. The smaller plasmid in IGTS8 was estimated to be 90 kb, and that of X309 and others was estimated to be 60 kb. In arriving at these sizes, we isolated the two plasmids from X309 individually and obtained summation of the restriction fragments derived from *Eco*RI and *Eco*RV digests (data not shown). Neither the 90-kb plasmid in IGTS8 nor the 60-kb plasmid in X309 and others hybridized to the *sox* probe (Fig. 2B). UV1 is a *sox*-negative mutant of IGTS8 that had retained the 90-kb plasmid (4).

Our size estimates for the IGTS8 plasmids do not agree with the values of 50 and 120 kb reported by Denome et al. (4). Those values were, however, extrapolated from linear DNA markers and under pulsed-field gel electrophoresis (PFGE) configuration, which normally does not resolve closed circular plasmid DNAs according to their molecular weights (2). We examined the possibility of plasmid pSOX being a linear molecule and carried out PFGE on genomic DNAs from IGTS8, UV1, X309, and selected mutants. Figure 3 indicates that X309, its mutants, and B1 all contain a DNA species which migrates at ca. 250 kb. In contrast, IGTS8 harbors a ca. 400-kb species which is lost in the UV1 mutant. This result is, however, consistent with the finding of Denome et al. (4) that an extra DNA species (400 kb) is present in IGTS8 but was lost in UV1 along with the *sox* plasmid. Under different electrical-pulse conditions, both the 250- and 400-kb DNA species were found to be linear (data not shown), since these DNAs mi-

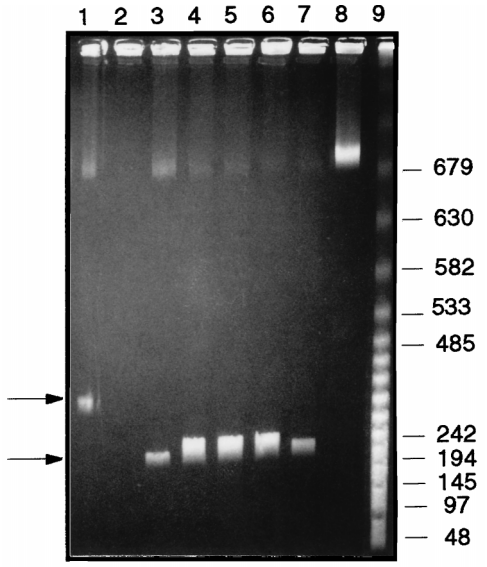

FIG. 3. PFGE of *Rhodococcus* sp. strain IGTS8 DNA in comparison with its UV1 mutant and other rhodococci. Agarose plugs of the bacterial cultures were prepared according to the procedure of Kauc et al. (12). Lanes: 1, IGTS8; 2, UV1; 3, X309; 4, X309-10-1; 5, X309-11-15; 6, X309-10-2; 7, B1; 8, *Pseudomonas putida* G1 (control); 9, molecular size markers. Separation was carried out in an AutoBase apparatus purchased from Mandel Scientific Ltd. The electrophoresis principle is that described by Turmel et al. (27). The duration of electrophoresis was 65 h. ROM card 4, with a separation range of 100 to 700 kb, was used. Numbers (in kb) alongside the gel are derived from the Lambda Ladder PFG Marker (New England Biolabs). The positions of linear DNA species are indicated by arrows.

grated to the same positions relative to the size markers (2). Southern blotting with the *sox* probe indicated that pSOX in IGTS8, X309, and X309-11-15 did not penetrate into the gel, as the signals were located in the well (data not shown). This characteristic is typical of closed circular supercoiled DNAs when they are subjected to PFGE (2).

Hence, besides plasmid size, the presence of a larger linear DNA species in X309 and related isolates distinguishes the prototype IGTS8 from the other strains.

Amplification of common PCR fragments. It was of interest to establish by PCR the extent of *sox* sequence relatedness among the various desulfurization strains in addition to the positive hybridization data shown in Fig. 2. The available *sox* DNA sequence from IGTS8 (GenBank accession no. U08850) was used to design primers for *soxAB* gene amplification (Fig. 4). PCR primers were 5′-CGCGATGACTCAACAACGAC (underlined is the presumptive *soxA* start codon) and 5′-CTA TCGGTGGCGATTGAGGC (underlined is the stop codon of *soxB*) of *Rhodococcus* sp. strain IGTS8. Amplification conditions were 94°C for 1 min, 65°C for 1 min, and 72°C for 4 min, for 25 cycles. As a result, the new isolates all yielded the same 2.5-kb fragment as those found in the positive control DNAs, IGTS8, and pSAD231-4 (Fig. 4). Subsequent *Pvu*II endonuclease digestion of these amplified fragments produced an identical restriction pattern consisting of three fragments. The 530-, 693-, and 1,236-bp fragments were expected from the IGTS8 *sox* DNA sequence. We sequenced approximately 360 bp of *soxA* from X309 and found this region to be identical to that of IGTS8. In a separate PCR experiment, we amplified a 610-bp fragment corresponding to codons 161 to 363 of *soxC* in all desulfurization isolates (data not shown). These findings led us to conclude that the *sox* locus is extremely conserved among the rhodococcal strains used in this study.

Strain differentiation by using insertion sequence elements. At the 3′ end of the *soxC* gene in IGTS8, Denome and Young (6) found two putative insertion sequence elements, IS*1166* and IS*1295* (Fig. 1); the former was also detected in at least two other *Rhodococcus* species. Since insertion elements are potentially useful in strain characterization (30), specific probes of IS*1166* and IS*1295* (probes A and B, respectively [Fig. 1]) were generated and used in Southern hybridization experiments. In Fig. 5, probe A was used to compare *Bam*HI-restricted total DNAs prepared from the new rhodococcal isolates and some of their derivatives to IGTS8 and its UV1 mutant.

As previously noted (6), IGTS8 contains four copies of IS*1166*, as there are eight hybridizing fragments (Fig. 5) and a *Bam*HI site within this element (Fig. 1). The UV1 strain is short one copy (two bands of 6.5 and 0.8-kb) due to the absence of plasmid pSOX. Isolates X309, X309-11-15, If, and Ig all yielded two hybridizing bands, indicating that the one copy of IS*1166*-like sequence in these strains is associated with plasmid pSOX. On the other hand, both B1 and Ih yielded four hybridizing bands, indicating either the presence of two plasmid-borne copies or that one copy is on the chromosome. To distinguish between these possibilities, a cured strain of these isolates will be needed. As expected, no hybridizing band was observed in isolates X309-10-1 and X309-10-2, which had been cured of the desulfurization plasmid (Fig. 5).

Southern blots of total DNA from IGTS8, UV1, X309, and mutants X309-10-1, X309-10-2, and X309-11-15, each of which was digested with *Eco*RI, were also hybridized with probe B, which is specific for IS*1295* (Fig. 1). Consistent with previous findings (6), only one hybridizing band (ca. 23 kb) was found in IGTS8; X309 and isolate X309-11-15 (cured of the small plasmid only) yielded results similar to that of IGTS8, except that the bands appeared to be slightly larger (data not shown). As expected, a negative result was obtained with the cured X309-10-1, X309-10-2, and UV1 strains.

Conclusions. By the criteria of plasmid content, size, and distribution of IS*1166*-like elements, it is clear that *Rhodococcus* sp. strain X309 and related isolates are genetically distinct from the prototype IGTS8 desulfurization strain. This study provides additional evidence that insertion elements (30) as well as PFGE techniques are useful molecular tools for strain differentiation.

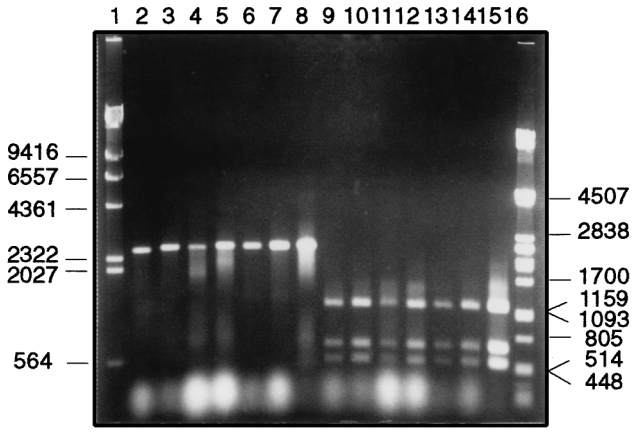

FIG. 4. Agarose gel electrophoresis of PCR-amplified fragments from various desulfurization strains and generation of a common *Pvu*II restriction profile. A 10-μl aliquot of the PCR reaction mixture was electrophoresed in a 0.75% agarose gel. Lanes: 1 and 16, *Hin*dIII- and *Pst*I-digested lambda DNA markers, respectively, with some sizes (in bp) indicated alongside; 2 and 9, IGTS8; 3 and 10, X309; 4 and 11, If; 5 and 12, Ig; 6 and 13, Ih; 7 and 14, B1; 8 and 15, pSAD231-4. Lanes 9 to 15 show *Pvu*II-digested DNAs. A slight "smile" effect in this gel is apparent. The broad spot at the gel front is dye and degraded RNA.

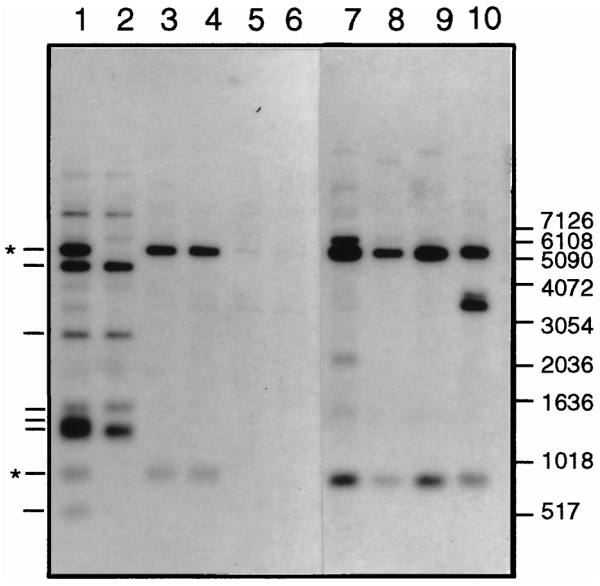

FIG. 5. Distribution of an IS*1166*-like element in the newly isolated *Rhodococcus* spp., in contrast to those present in IGTS8 and mutant UV1. Total DNA from bacterial isolates was isolated by the Marmur method (25). *Bam*HI-digested DNA from each sample was separated by agarose gel electrophoresis and transferred to a nylon membrane. Hybridization was carried out with probe A (Fig. 1), which was labelled with [α-^{32}P]dATP by the random primer method. Hybridizations were performed under stringent conditions at 65°C; washing was carried out in 0.1× SSC and 0.1% SDS at 65°C. Lanes: 1, IGTS8; 2, UV1; 3, X309; 4, X309-11-15; 5, X309-10-1; 6, X309-10-2; 7, B1; 8, If; 9, Ig; 10, Ih. Numbers alongside are sizes (in bp) from the 1-kb DNA marker (Gibco/BRL). The eight reference bands in IGTS8 are marked on the left. *, bands originating from pSOX. Bands above ~10 kb represent partially digested DNA.

For the first time, we established the conserved nature of the *sox* locus, which is hitherto exclusive to IGTS8. It will be interesting to see whether the *sox* genes in other sulfur-specific desulfurization strains (e.g., *Rhodococcus* sp. strain SY1 [previously *Corynebacterium* {20}], *R. erythropolis* D-1 [10], *R. erythropolis* UM9 [23], and *Agrobacterium* spp. [3]) are also conserved and plasmid encoded. It is tempting to speculate on a *Rhodococcus*-associated sulfur-specific pathway, since most of the desulfurization strains isolated to date belong to this genus. The possibility of sulfur-specific removal from DBT by sulfate-reducing bacteria has been examined (7). However, these bacteria metabolize DBT to hydrogen sulfide and biphenyl. Our negative results with DNA of *Desulfovibrio desulfuricans* G20 (genomic DNA supplied by J. Wall, University of Missouri) by PCR and *sox* hybridization (data not shown) provide circumstantial evidence that a different pathway is operational in these anaerobic organisms.

Nucleotide sequence accession numbers. The 1,439-bp rDNA sequence of Rhodococcus strain X309 and the 1,413-bp rDNA sequence of Rhodococcus strain B1 have been submitted to GenBank and assigned accession no. U87968 and U87969, respectively.

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