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Characterization of the naphthalene-degrading bacterium, *Rhodococcus opacus* M213

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Abstract

Bacterial strain M213 was isolated from a fuel oil-contaminated soil in Idaho, USA, by growth on naphthalene as a sole source of carbon, and was identified as *Rhodococcus opacus* M213 by 16S rDNA sequence analysis and growth on substrates characteristic of this species. M213 was screened for growth on a variety of aromatic hydrocarbons, and growth was observed only on simple 1 and 2 ring compounds. No growth or poor growth was observed with chlorinated aromatic compounds such as 2,4-dichlorophenol and chlorobenzoates. No growth was observed by M213 on salicylate, and M213 resting cells grown on naphthalene did not attack salicylate. In addition, no salicylate hydroxylase activity was detected in cell free lysates, suggesting a pathway for naphthalene catabolism that does not pass through salicylate. Enzyme assays indicated induction of catechol 1,2-dioxygenase and catechol 2,3-dioxygenase on different substrates. Total DNA from M213 was screened for hybridization with a variety of genes encoding catechol dioxygenases, but hybridization was observed only with *catA* (encoding catechol 1,2-dioxygenase) from *R. opacus* 1CP and *edoD* (encoding catechol 2,3-dioxygenase) from *Rhodococcus* sp. I1. Plasmid analysis indicated the presence of two plasmids (pNUO1 and pNUO2). *edoD* hybridized to pNUO1, a very large (~750 kb) linear plasmid.

Keywords: Naphthalene metabolism; Biodegradation; Linear plasmids; Rhodococcus

1. Introduction

Virtually all that is known of the biochemistry and genetics of bacterial naphthalene metabolism was gained from analysis of pseudomonads such as *Pseudomonas putida* G7 and its resident plasmid, NAH7 [1,2]. Studies on naphthalene metabolism by pseudomonads were instrumental in developing an understanding of aromatic hydrocarbon metabolism and evolutionary relationships between diverse biodegradative strains. Now that the framework for studying naphthalene metabolism is well established, attention is turning toward diverse naphthalene-metabolizing bacteria and assessing the importance of non-pseudomonads, such as Gram-positive bacteria, in biodegradation of polyaromatic hydrocarbons in soils and other environments. A clear understanding of the fate of organic contaminants in the environment is dependent on an understanding of the pathways by which these compounds are degraded, and an understanding of the mechanisms by which genes encoding their metabolism are spread throughout a soil community.

Among the most metabolically versatile Gram-positive bacteria are *Rhodococcus* species. *Rhodococcus* strains are present in many soils and have been shown to metabolize a range of aromatic hydrocarbons and chlorinated aromatic compounds [3–13]. Little is known of the pathways or genetics of naphthalene metabolism by *Rhodococcus* strains, or how they may differ from those encoded by the archetypal *P. putida* NAH7 system [6]. Not surprisingly, genes from naphthalene metabolic pathways cloned from *Rhodococcus* strains are frequently not closely related to their counterparts from *Pseudomonas* strains, suggesting either different origins for these genes or early divergence from some common ancestor [14]. In addition, fundamental aspects of the plasmid biology of some *Rhodococcus* species, most notably *Rhodococcus opacus* strains, differ significantly from the plasmid biology of pseudomonads [13,15]. *R. opacus* strains appear to be characterized by

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linear plasmids and linear chromosomes, unlike the circular plasmids typical of naphthalene-metabolizing pseudomonads. While no linear plasmid has been previously implicated in the metabolism of naphthalene, it is likely that, should such a plasmid exist, it potentially may encode metabolic pathways different from those typical of Gram-negative strains.

We are interested in the genetics and biochemistry of naphthalene metabolism of strain M213, an *R. opacus* strain recently isolated from soil by virtue of its ability to utilize naphthalene as a sole source of carbon. This manuscript describes initial characterization of the biochemistry and genetics of *R. opacus* M213 as a prelude to detailed investigations of the pathway and genetic organization of aromatic hydrocarbon metabolism in this strain. We have partially characterized the range of aromatic substrates utilized for growth by M213, compared genes cloned from simple aromatic pathways of other *Rhodococcus* strains with M213, and investigated the potential role of plasmids in metabolism of aromatic hydrocarbons in this strain.

2. Materials and methods

2.1. Strain isolation

Strain M213 was isolated from gasoline-contaminated soil beneath a former gasoline station site in Moscow, ID, USA. Naphthalene-metabolizing bacteria were isolated by serial batch enrichments in mineral media containing naphthalene as sole source of carbon [16]. Strain M213 was selected for further study because of its suspected wide range of aromatic carbon substrate utilization.

2.2. Growth media

M213 was grown on mineral medium consisting of 4.8 g K_2HPO_4, 1.2 g KH_2PO_4, 1 g NH_4NO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.025 g $CaCl_2 \cdot 2H_2O$, 0.001 g $Fe_2(SO_4)_3$ per l deionized H_2O. For solid medium, 18 g agar was added. The substrates 2,4-dichlorophenol, 2,4-dichlorophenoxyacetic acid, 2-chlorobenzoic acid, 3-chlorobenzoic acid, 4-chlorobenzoic acid, benzoate, or *m*-toluate were added to the medium solution before autoclaving. For toluene, naphthalene, fluorene, phenol, phenanthrene and anthracene, the vapor plate method was used [17]. For growth on naphthalene in liquid medium, 2 g naphthalene was added per l mineral medium. Liquid cultures were grown at room temperature with shaking. *P. putida* G7 (NAH7) was used for comparison in this study.

To confirm the identity of M213 as *R. opacus*, M213 was screened for growth on mineral medium plates containing D-galactose, L-rhamnose, D-ribose, D-sucrose, D-turanose, D-arabitol, or *N*-acetyl-D-glucosamine for comparison with results obtained by Klatte et al. [5]. Controls consisting solely of mineral medium in the absence of carbon source were included in all experiments.

2.3. Fatty acid analysis

Cellular fatty acids were analyzed by the Microbial Identification Core Lab of the Interdisciplinary Center for Biotech Research, University of Florida as described by Sasser [18], and the results compared with other species in the available database.

2.4. 16S ribosomal RNA gene (rDNA) sequencing

Genomic DNA was isolated from M213 by a standard method [19], and 16S rDNA was amplified by PCR using the universal bacterial primers 27F (AGAGTTTGATCCMTGGCTCAG) and 1406R (ACGGGCGGTGTGTMC) [20]. The reaction mixture contained 2 μl 10× polymerase buffer, 1.5 mM $MgCl_2$, 1 pmole of each primer, 200 mM dNTP, 2.7 μl water, 10 μl DNA sample, and 2.5 units of Taq DNA polymerase (Promega, Madison, WI, USA). The reaction mixture was subjected to 35 cycles in a Perkin-Elmer DNA Model 2400 Thermal Cycler. Cycling parameters were 94°C and 30 s for denaturation, 58°C and 30 s for annealing, 72°C and 30 s for chain extension. At the end of the last cycle, 7 min were added as a chain extension step. The purified product (approximately 1.4 kb) was ligated into pCR@3.1 (Invitrogen, San Diego, CA, USA) and transformed to competent *Escherichia coli* cells (TOP10F′). Plasmid DNA from transformants was isolated using the protocol described by Nicoletti and Condorelli [21], and the insert was sequenced by the Interdisciplinary Center for Biotech Research core sequencing facility of the University of Florida. The 1373 bp partial rDNA sequence is registered with the National Center Biotechnology Information (NCBI) database (GenBank), under accession no. AF095715.

2.5. DNA isolation

2.5.1. Genomic DNA

The procedure described by Ausubel et al. [19] was used with minor modifications to improve cell lysis. For 250 ml cell cultures, cell pellets were resuspended in 21 ml TE (10 mM Tris–HCl, 1 mM EDTA, pH 8) and 2 ml lysozyme (100 mg ml^{-1} in 25 mM Tris, pH 8) and incubated at 37°C for 1 h. After incubation, 6.50 ml 20% sodium dodecyl sulfate (SDS) and 5 ml 5 M NaCl was added and mixed. DNA was then isolated from the lysate according to standard procedures [19].

2.5.2. Circular plasmid DNA

Three separate procedures designed for isolation of large circular plasmids were attempted, all with the following modification to improve cell lysis: To 250 ml centrifuged cell culture, 5 ml water and 500 μl lysozyme solution

(100 mg ml^{-1} in 25 mM Tris, pH 8) were added and incubated at 37°C for 1 h. To the mixture, 1.5 ml 20% SDS was added and incubated on ice for 10 min. The procedures described by Feng et al. [22], Anderson and McKay [23], and Schreiner et al. [24] were then followed.

2.5.3. Screening for linear megaplasmids

A modified version of the protocol described by Kalkus et al. [25] was used. M213 was grown in mineral naphthalene liquid medium to late log growth phase, 3 ml culture was harvested. The cells were resuspended in 100 μl EET buffer (10 mM Tris–HCl (pH 8), 0.1 M EDTA (pH 8), and 10 mM EGTA (pH 8)), and the cell solution mixed with an equal volume of low-melting point agarose (2% in water), allowed to solidify, and cut into plugs. The plugs were incubated in 0.5 mg ml^{-1} lysozyme, 0.6 mg ml^{-1} sodium *N*-lauroylsarcosine in EET for 1.5 h at 37°C, followed by an incubation in 0.1 mg ml^{-1} proteinase K, 10 mg ml^{-1} SDS in EET at 55°C overnight. The plugs were rinsed with TE buffer three times and stored in ES buffer (0.5 M EDTA (pH 8), 1% sodium *N*-lauroylsarcosine) at 4°C.

Pulsed field gel electrophoresis (PFGE) was performed with a Hoefer (San Francisco, CA, USA) PFGE electrophoresis system. The plugs were loaded into a 1% agarose gel (FisherBiotech, Fair Lawn, NJ, USA) in 0.5×TBE buffer and electrophoresed. Running voltage, run time, pulse time, and *F*/*R* ratio were 200 Volt DC, 24 h, 1 to 50, and 3:1, respectively. In order to determine whether the plasmids are linear or circular, pulse times of 1:40 and 1:80 were used [25], with lambda DNA concatemers (Promega) as linear standards and large circular plasmids [22] as circular DNA standards. Plasmids were also observed by clamped homogeneous electrical field (CHEF) electrophoresis (Bio-Rad, Hercules, CA, USA).

2.5. Enzyme assays

2.5.1. Spot test for catechol 2,3-dioxygenase (C23O) activity

The rapid spot test described by Pankhurst [26] was used to assess catechol 2,3-dioxygenase (C230) activity. Catechol dissolved in diethylether was sprayed onto 1 or 2 days old M213 and *P. putida* G7 colonies grown on naphthalene vapor plates. The color change to yellow indicates the presence of catechol 2,3-dioxygenase (meta-cleavage pathway) by the production of 2-hydroxymuconic semialdehyde.

2.5.2. Kinetic analysis

One liter of M213 and *P. putida* G7 grown in naphthalene-mineral liquid medium were harvested at late log growth phase and resuspended in phosphate buffer. Cells were lysed by sonication in the presence of 0.5 g glass beads, and cell debris removed by centrifugation at 8160×*g* for 10 min. To prevent enzymes from becoming inactivated, acetone was added to a final acetone concentration of 10% in the supernatant. The solution was filtered through 0.45 μm spin filter to remove any residual cell debris or glass bead fragments, and kept at 4°C. For the C230 assay, catechol solution (11 mg per 10 ml) and PBS buffer were added to a final volume of 980 μl. An aliquot of crude cell extract was added and the reaction was immediately monitored at 375 nm. Total protein concentrations were determined by a micro-protein determination kit and phenol reagent method for biologic fluids (procedure no. 690) (Sigma, St. Louis, MO, USA). Results were compared with C23O activities from a non-induced control (cells grown on glucose).

2.5.3. Salicylate hydroxylase assays

Cells grown on naphthalene as sole carbon source were lysed and crude extracts prepared as described above. Salicylate hydroxylase assays were conducted by analysis of consumption of NADH as described by Sze and Dagley [27].

2.6. Hybridization

For Southern blots, genomic DNA from M213 was digested with *Eco*RI, *Hin*dIII and *Bgl*II in separate reaction tubes, and the reactions were electrophoresed on 0.7% agarose gel. DNA was transferred to Hybond-N membrane (Amersham, Buckinghamshire, UK) using a vacuum blotter (Bio-Rad) according to the manufacturer's recommendations. For slot blots, denaturated DNA was applied directly onto Magna charge nylon membrane (MSI, Westborough, MA, USA) by vacuum slot blotter (Hoefer, San Francisco, CA, USA). DNA was fixed to the membrane with Fisherbiotech FB-UVXL-1000 model UV crosslinker (Pittsburg, PA, USA).

A previously described catechol 2,3-dioxygenase (C230) gene from *Rhodococcus rhodocrous* [28], *xylE* from the toluene degradation plasmid pWW0, a catechol 1,2-dioxygenase gene (*catA*) [29], and a chlorocatechol 1,2-dioxygenase gene (*clcA*) [30] were used as probes. Probes were labeled with ^{32}P-dCTP by random priming according to the vendor's recommendation (GibcoBRL, Gaithersburg, MD, USA). Membranes were washed with 2×SSC, 0.5% SDS for 5 min, and 2×SSC, 0.1% SDS for 15 min at room temperature. Final washes were with 0.1×SSC, 0.5% SDS for 2 h at 55°C for low stringency washing, 65°C for moderate stringency washing, and 69°C for high stringency. Hybridization was detected by exposure to X-ray film.

3. Results and discussion

3.1. Identification of strain M213

M213 is a large Gram-positive rod. Attempts to identify

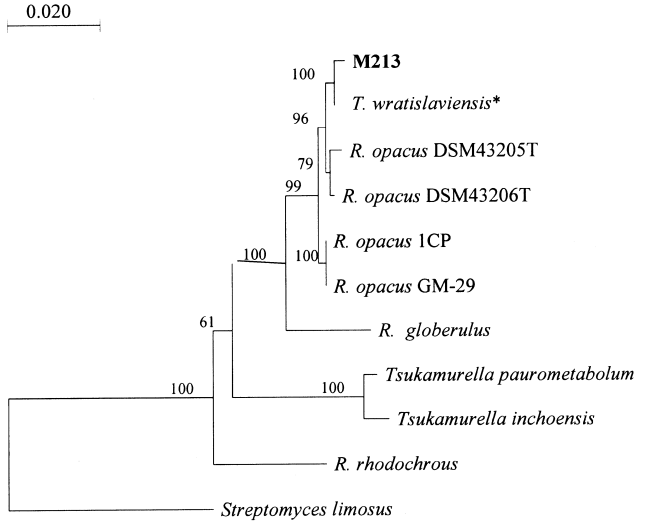

Fig. 1. Phylogenetic tree of M213 and related *Rhodococcus* species. *T. wratislaviensis* was originally misclassified and has not yet been officially classified (M. Goodfellow, personal communication). Tree was created by a Jukes and Cantor distance calculation with neighbor joining tree algorithm.

M213 by fatty acid analysis proved inconclusive; M213 shared only 38.3% similarity with *Nocardia* and *Rhodococcus* (highest matches with the local fatty acid database; data not shown). rDNA sequence analysis indicated that M213 is closely related to *R. opacus*, with more than 99% similarity to other *R. opacus* strains (Fig. 1).

To verify identification of M213 as *R. opacus*, the strain was grown with a variety of substrates as sole sources of carbon (Table 1) for comparison with reported characteristic strains of *Rhodococcus rhodochrous*, *Rhodococcus rhodnii*, *Rhodococcus erythropolis* and *R. opacus* [5]. M213 grew on all substrates tested, whereas *R. erythropolis* is not considered to use L-rhamnose and D-turanose, and most strains of *R. opacus* do not use L-rhamnose [5]. These data support classification of M213 as *R. opacus*.

3.2. Aromatic hydrocarbon utilization

The range of aromatic hydrocarbons and chlorinated aromatic compounds M213 was capable of using as growth substrates yielded some unexpected results (Table 2). The lack of growth on fluorene, phenanthrene, and anthracene suggested that the ability of M213 to metabolize polyaromatic hydrocarbons is limited to naphthalene, although M213 can metabolize a variety of single ring aromatic compounds, including some simple chlorinated aromatics.

Of greatest surprise was the apparent inability of M213 to utilize salicylate as a sole source of carbon. To our knowledge, salicylate is an intermediate in most naphthalene metabolic pathways described to date, and is the primary substrate for the lower pathway encoded by the well-studied *P. putida* G7 (NAH7). Possible explanations for the inability of M213 to grow on salicylate include: (1) M213 can metabolize salicylate but requires a metabolite higher in the pathway to induce the required enzymes; (2) salicylate is not transported into M213; and (3) M213 expresses a pathway for naphthalene metabolism that does not pass through salicylate.

To test the possibility that an additional inducer is required, resting cells of M213 grown on naphthalene were challenged with salicylate (data not shown). Salicylate was not lost from solution, whereas salicylate was completely lost from solution with *P. putida* G7 (NAH7). The possibility that M213 lacks the lower pathway for naphthalene metabolism, and hence is incapable of metabolizing salicylate, is unlikely because of the induction of a catechol 2,3-dioxygenase (a key lower pathway enzyme) during growth on naphthalene (discussed below). Finally, cell free lysates of M213 grown on naphthalene as a sole source of carbon failed to indicate salicylate hydroxylase activity, while such activity was present in *P. putida* G7 (NAH7) lysates (data not shown).

Another difference between M213 and *P. putida* G7 is the inability of M213 to utilize α-naphthol as a growth substrate. This may suggest that initial oxygenation of naphthalene may proceed differently from that observed in *P. putida* G7, or suggest different substrate specificities for the enzymes involved. Much more work is required to define the pathway for naphthalene catabolism by M213, including studies to elucidate the inability of the strain to metabolize salicylate.

The inability of M213 to utilize 2,4-dichlorophenol was somewhat unexpected since another *R. opacus* strain (*R.*

Table 1
Growth of M213 on substrates characteristic of *Rhodococcus*

Substrate	M213[a]	*R. opacus* (%)[b]	*R. erythropolis* (%)[b]	*R. rhodochrous* (%)[b]	*R. rhodnii* (%)[b]
D-galactose	+++	100	85	85	100
L-rhamnose	++	0	0	23	0
D-ribose	++	100	92	15	33
D-sucrose	+++	100	92	31	0
D-turanose	+++	100	0	0	0
D-arabitol	+++	100	92	100	100
N-acetyl-D-glucosamine	+++	100	77	0	0

[a]+++, good growth; ++, relatively slow growth.
[b]% strains tested showing growth [5].

opacus 1CP) was isolated by virtue of its ability to grow on 2,4-dichlorophenol [31].

3.3. Enzyme assays

To further investigate the observed differences between M213 and *P. putida* G7 (NAH7), enzyme activities involved in naphthalene catabolism were assayed. The spot test for detection of catechol 2,3-dioxygenase (C23O) activity [26] revealed bright yellow M213 colonies grown on naphthalene vapors, indicative of C230 activity. The specific activity of C23O in cell free lysates of M213 was somewhat lower (6.9 units mg^{-1} protein) than was observed for G7 (7.8 units mg^{-1} protein). No C23O activity was observed when M213 was grown on glucose as a sole source of carbon (i.e. non-inducing conditions) (data not shown). Induction of C23O during growth of M213 on naphthalene was expected as this is an important enzyme in most naphthalene lower pathways previously described. It was also suspected from growth of M213 on *m*-toluate, a compound that is metabolized through a meta-cleavage pathway in many cases. Induction of C23O during growth on naphthalene indicates that the lower pathway for naphthalene degradation is not completely missing (if at all), regardless of the inability of the strain to metabolize salicylate.

Catechol 1,2-dioxygenase activities were observed for M213 during growth on benzoate, as is typical of *P. putida* G7 (NAH7). Catechol 1,2-dioxygenase activity has also been reported for *R. opacus* 1CP.

3.4. Similarity with cloned dioxygenase genes

To determine the relative similarity between catechol dioxygenases encoded by M213 with other *Rhodococcus* strains, a series of hybridizations were conducted.

Table 2
Growth of M213 on aromatic hydrocarbon and chlorinated aromatic compounds

Substrate	Growth[a]
Naphthalene	+++
Toluene	+++
Fluorene	−
Phenanthrene	−
Anthracene	−
m-Toluate	+++
2,4-Dichlorophenol	−
2,4-Dichlorophenoxyacetic acid	−
2-Chlorobenzoic acid	+
3-Chlorobenzoic acid	+
4-Chlorobenzoic acid	−
Benzoate	++
p-Hydroxybenzoate	+++
α-Naphthol	−
Phenol	++

[a]+++, good growth; ++, relatively slow growth; +, very slow growth; −, no growth.

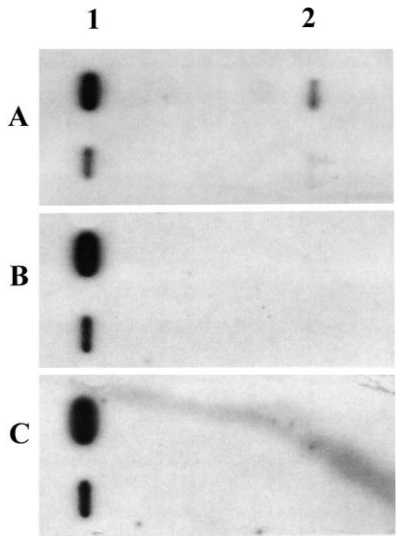

Fig. 2. Slot-blot hybridization of *clcA* from *R. opacus* 1CP to M213 DNA. A: Low stringency wash. Lanes: 1, positive control DNA; 2, M213 DNA. B: Moderate stringency wash. C: High stringency wash. The top slot in each of these panels represents 10 times the mass of target DNA relative to the mass of DNA in the lower slot.

Chlorocatechol 1,2-dioxygenase gene (*clcA*) from *R. opacus* 1CP hybridized with genomic DNA from M213, although only at low stringencies of washing. Hybridization was observed for *clcA* after low stringency washing (Fig. 2A); however, hybridization disappeared after washing at moderate stringency (Fig. 2B and C). The observation that M213 does not grow on 2,4-dichlorophenol and only poorly on 2- and 3-chlorobenzoate (Table 2) agrees with the poor hybridization observed with *clcA*. It is likely that the hybridization observed following the low stringency wash was due to heterologous hybridization with another oxygenase (perhaps *catA*; *catA* shares 55.4% sequence similarity with *clcA*), and that M213 does not encode a chlorocatechol dioxygenase. Absence of significant hybridization with *clcA* does not indicate the absence of any chlorocatechol dioxygenase, but the poor growth or lack of growth on most chlorinated aromatics tested is strongly suggestive that the strain does not harbor a chlorocatechol dioxygenase.

The catechol 1,2-dioxygenase (C12O) gene (*catA*) from *R. opacus* 1CP [29] remained hybridized at the moderate and higher stringency washings (Fig. 3B and C); however, hybridization intensely decreased more than 50% relative to the lower washing temperature (Fig. 3A and C), indicating that the C12O gene harbored by M213 diverges significantly from *catA* of 1CP. This indicates divergence between the C12O gene encoded by the two *R. opacus* strains, although they are closely related.

To our knowledge, no C23O genes have been cloned from *R. opacus* strains, although some were recently cloned from various *Rhodococcus* species that included species similar to *R. opacus*. To explore possible similarities between M213 and C23O genes cloned from other

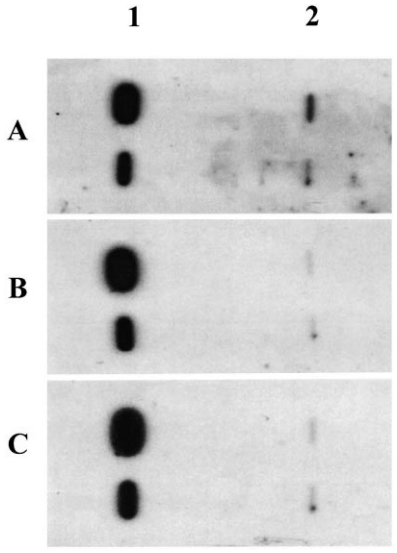

Fig. 3. Slot-blot hybridization of *catA* from *R. opacus* 1CP to M213 DNA. A: Low stringency wash. Lanes: 1, positive control DNA; 2, M213 DNA. B: Moderate stringency wash. C: High stringency wash. The top slot in each of these panels represents 10 times the mass of target DNA relative to the mass of DNA in the lower slot.

Rhodococcus species, we tested those cloned from *R. rhodocrous* CTM [28] and various *Rhodococcus* strains of various species (*edoA*, *edoB*, *edoC*, and *edoD*) [32]. No hybridization was observed between blots of M213 genomic DNA and catechol 2,3-dioxygenase (C230) gene from *R. rhodochrous* CTM or with *edoA*, *edoB*, or *edoC* (data not shown). Significant hybridization was observed, however, with *edoD* (data not shown).

Kulakov et al. [32] originally cloned *edoD* from *Rhodococcus* sp. I1, which was isolated from a creosote-contaminated soil and was tentatively characterized by analysis of partial 16S rDNA sequences as being closely related to *R. opacus* and *Rhodococcus marinonancens*. They reported that *edoD* cloned from I1 also hybridized strongly to DNA from *R. rhodochrous* P200, a strain isolated from the same site. Both I1 and P200 are capable of growing on naphthalene and toluene as sole sources of carbon. rDNA sequences for I1 and P200 were not available from GenBank for comparison with that of M213 at the time of this writing. Interestingly, *Rhodococcus* sp. I1 hybridized with *edoA*, *edoB*, and *edoC*; and *R. rhodochrous* P200 hybridized with *edoA*, and *edoB*. Our observation that M213 hybridized only to *edoD* and not to *edoA*, *edoB* or *edoC* indicates that M213 is significantly different with respect to the diversity of C23O genes, and possibly with respect to the diversity of pathways for aromatic hydrocarbon catabolism that may be present in these strains.

Kulakov et al. [32] reported that sequence analysis of *edoD* indicated similarity with Type II C23Os. C23Os may be divided into two subgroups (I and II), with the majority described to date belonging to Type I. Type II C23Os differ significantly from Type I dioxygenases at the nucleotide and amino acid levels, and are related to the C23O from *Ralstonia eutropha* JMP222 [33,34]. The observed sequence similarity of *edoD* with C23O genes from Gram-negatives such as *R. eutropha* and *S. paucimobilis* [32] strongly suggests that these genes were transferred between Gram-positive bacteria and Gram-negative bacteria via horizontal genetic exchange that was likely mediated by plasmids.

3.5. Plasmids

Genes encoding metabolism of naphthalene are typically carried on plasmids, although to our knowledge, no naphthalene-degrading plasmids have yet been described for *Rhodococcus*. Many *Rhodococcus* strains harbor plasmids, and some of these encode biodegradation of environmentally relevant compounds [4,13,28,35,36]. In order to screen M213 for the presence of plasmids, four different plasmid isolation protocols were applied [22–25]. Three of these were developed for detection of large, circular plasmids in both Gram-positive and Gram-negative strains, but all three failed to detect plasmid DNA in M213 (data not shown). A possible reason for the failure of these methods to detect plasmid DNA is that they were developed for detection of circular, not linear, DNA. Most *R. opacus* plasmids described to date are linear, rather than the circular form common among other bacterial species. Linear chromosomal DNA appears also to be characteristic of this species [25].

It is likely that screening M213 by pulsed field gel electrophoresis of cells lysed in agarose plugs would reveal linear and circular plasmids, if present in M213. Three bands were observed by this method (Fig. 4). The top band corresponded with the agarose well, indicating that a significant amount of nucleic acid did not migrate out of the well. This band hybridized strongly with the 16S rDNA gene cloned from M213 (Fig. 4B), indicating that much of this band consists of chromosomal DNA. The

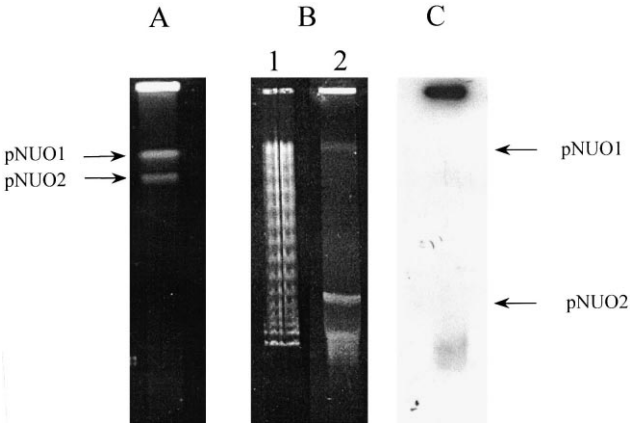

Fig. 4. *R. opacus* M213 plasmids. A: CHEF gel electrophoresis of total DNA from M213. B: Pulsed field electrophoresis gel of total DNA from M213. Lanes: 1, 1 kb linear size marker; 2, M213 DNA. C: Southern blot of gel in panel B with 16S rDNA.

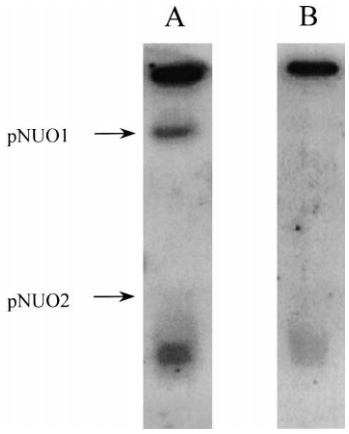

Fig. 5. Southern blots of M213 DNA with *edoD* and *catA*. A: Hybridization with *edoD*. B: Hybridization with *catA*.

second band, labeled pNUO1, exhibits an electrophoretic mobility corresponding with that of a 750 kb linear size marker. Linear DNA may be distinguished from circular DNA by observing different mobilities as a function of pulse time on PFGE [25]. The mobility of pNUO1 varied with pulse time similarly to standard linear DNAs, unlike standard circular DNAs (data not shown), indicating that pNUO1 is linear. In addition, pNUO1 does not hybridize with 16S rDNA, and indicating it is not a chromosome, but is a linear megaplasmid. A second plasmid (pNUO2) is much smaller and exhibits an electrophoretic mobility between the 200 and 300 kb bands of the linear size marker. Data were inclusive as to whether pNUO2 is linear or circular (data not shown). Fig. 4A is presented as indication that the lower, diffuse band observed in Fig. 4B is composed of degradation products of chromosomal and plasmid DNA and not truly a distinct band. DNA presented in Fig. 4A, B, and C are from the same preparation, and this lower smear disappears with CHEF electrophoresis.

3.6. Genetic location (plasmid versus chromosome) of catA and edoD

In order to determine whether the sequences hybridizing to *catA* and *edoD* are chromosomally or plasmid encoded, Southern blots of M213 DNA were hybridized with the known genes (Fig. 5). *catA* clearly hybridized to DNA remaining in the well (assumed to be a mixture of chromosomal and plasmid DNA) and to the smear of DNA assumed to be degradation products of chromosome and plasmid (Fig. 5B). Since no hybridization was observed between *catA* and either pNUO1 or pNUO2, *catA* is likely to be chromosomally encoded. It is possible, however, that a very large plasmid may have been retained in the well along with the chromosome, and that *catA* hybridized with this unseen plasmid.

Hybridization was observed between *edoD* and pNUO1 (Fig. 5A) and with DNA retained in the well. This indicates that *edoD* is part of pNUO1, and it is likely that a significant amount of pNUO1 was retained in the well along with the chromosome and cell debris. No hybridization was observed between either *catA* or *edoD* and pNUO2, and its function is cryptic at this time.

Hybridization between *edoD* and pNUO1 is not proof that naphthalene catabolism is encoded by pNUO1. It is, however, strongly suggestive that genes involved in naphthalene catabolism are carried by this very large plasmid, and could explain the distribution of sequences similar to *edoD* between different *Rhodococcus* species and Gram-negative species.

4. Conclusions

Our data suggest that at least part of the naphthalene catabolic pathway is encoded by a very large linear plasmid (pNUO1). The naphthalene pathway encoded by M213 may differ significantly from other systems in that salicylate does not appear to be an intermediate. Much work remains to elucidate the genetics and biochemistry of naphthalene catabolism in *R. opacus* M213.

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