JOURNAL OF BACTERIOLOGY, Aug. 2004, p. 4894–4902 Vol. 186, No. 15
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.15.4894–4902.2004

A Novel *p*-Nitrophenol Degradation Gene Cluster from a Gram-Positive Bacterium, *Rhodococcus opacus* SAO101

Wataru Kitagawa, Nobutada Kimura,* and Yoichi Kamagata

Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan

Received 7 March 2004/Accepted 29 April 2004

***p*-Nitrophenol (4-NP) is recognized as an environmental contaminant; it is used primarily for manufacturing medicines and pesticides. To date, several 4-NP-degrading bacteria have been isolated; however, the genetic information remains very limited. In this study, a novel 4-NP degradation gene cluster from a gram-positive bacterium, *Rhodococcus opacus* SAO101, was identified and characterized. The deduced amino acid sequences of *npcB*, *npcA*, and *npcC* showed identity with phenol 2-hydroxylase component B (reductase, PheA2) of *Geobacillus thermoglucosidasius* A7 (32%), with 2,4,6-trichlorophenol monooxygenase (TcpA) of *Ralstonia eutropha* JMP134 (44%), and with hydroxyquinol 1,2-dioxygenase (ORF2) of *Arthrobacter* sp. strain BA-5-17 (76%), respectively. The *npcB*, *npcA*, and *npcC* genes were cloned into pET-17b to construct the respective expression vectors pETnpcB, pETnpcA, and pETnpcC. Conversion of 4-NP was observed when a mixture of crude cell extracts of *Escherichia coli* containing pETnpcB and pETnpcA was used in the experiment. The mixture converted 4-NP to hydroxyquinol and also converted 4-nitrocatechol (4-NCA) to hydroxyquinol. Furthermore, the crude cell extract of *E. coli* containing pETnpcC converted hydroxyquinol to maleylacetate. These results suggested that *npcB* and *npcA* encode the two-component 4-NP/4-NCA monooxygenase and that *npcC* encodes hydroxyquinol 1,2-dioxygenase. The *npcA* and *npcC* mutant strains, SDA1 and SDC1, completely lost the ability to grow on 4-NP as the sole carbon source. These results clearly indicated that the cloned *npc* genes play an essential role in 4-NP mineralization in *R. opacus* SAO101.**

Nitroaromatic compounds have been used in a number of ways, including in medicines, explosives, and pesticides (17, 32). Wide use of these nitroaromatic compounds and their subsequent release leads to environmental pollution. Due to this potential toxicity and persistence in the environment, rapid removal and detoxification of these compounds are necessary. *p*-Nitrophenol (4-NP) is among such compounds found in many different environments. This compound is used on a large scale in the synthesis of the aspirin substitute acetaminophen and in the manufacture of pesticides such as parathion and methylparathion (25, 32). In the environment, such pesticides are hydrolyzed and transformed to 4-NP; these pesticides have been considered to be the main source of 4-NP that has been detected in the environment (18, 19, 23). The toxicology and carcinogenicity of 4-NP have been studied and reviewed by the Agency for Toxic Substances and Disease Registry (1).

Several 4-NP-degrading bacteria have been isolated, and their degradation pathways have been studied. As shown in Fig. 1, the two major initial degradation pathways of 4-NP have been characterized. The degradation pathway in which 4-NP is converted to maleylacetate via hydroquinone (hydroquinone pathway) (Fig. 1, top) was preferentially found in gram-negative bacteria such as *Burkholderia* spp. and *Moraxella* spp. (21, 25). The degradation pathway in which 4-NP is converted via 4-nitrocathechol (4-NCA) and hydroxyquinol (hydroxyquinol pathway) (Fig. 1, bottom), was preferentially found in gram-positive bacteria such as *Bacillus* spp. and *Arthrobacter* spp. (10, 11). Although many studies of 4-NP degradation have been reported, genetic information related to 4-NP degradation remains limited. In association with the hydroquinone pathway, the 4-NP degradation genes were cloned from *Pseudomonas* sp. strain ENV2030 and *Pseudomonas putida* JS444 (32). In the case of the hydroxyquinol pathway, no 4-NP catabolic gene has been reported. The only nucleotide sequence of 4-NP degradation genes (*nphA1A2*) thus far reported was from *Rhodococcus* sp. strain PN1. The gene products NphA1 and NphA2 are reported to convert 4-NP to 4-NCA, but the genes involved in the further degradation of 4-NCA and its metabolites from strain PN1 have not been reported to date (26). Therefore, more information about the catabolic genes involved in 4-NP metabolism is still needed, and in particular, information about those genes that act on the hydroxyquinol pathway is considered to be highly desirable.

A gram-positive 4-NP degrader, *Rhodococcus opacus* SAO101, was originally isolated as a bacterium able to degrade dibenzo-*p*-dioxin (12). This strain also degrades biphenyl, naphthalene, phenol, benzene, dibenzofuran, dibenzo-*p*-dioxin, and 4-NP. In this study, a novel 4-NP catabolic gene cluster that acts on the hydroxyquinol pathway from strain SAO101 was isolated and characterized. First, we cloned the 4-NP degradation genes with the aid of PCR and by screening a genomic library. Second, the enzyme activities expressed in *Escherichia coli* were examined. We then isolated mutants with insertionally inactivated 4-NP catabolic genes and analyzed the mutants to address the functional importance of the 4-NP genes in strain SAO101. This is the first report of 4-NP degradation genes that are responsible for converting 4-NP to maleylacetate.

* Corresponding author. Mailing address: Institute for Biological Resources and Functions, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki 305-8566, Japan. Phone: 81-29-861-6591. Fax: 81-29-861-6587. E-mail: n-kimura@aist.go.jp.

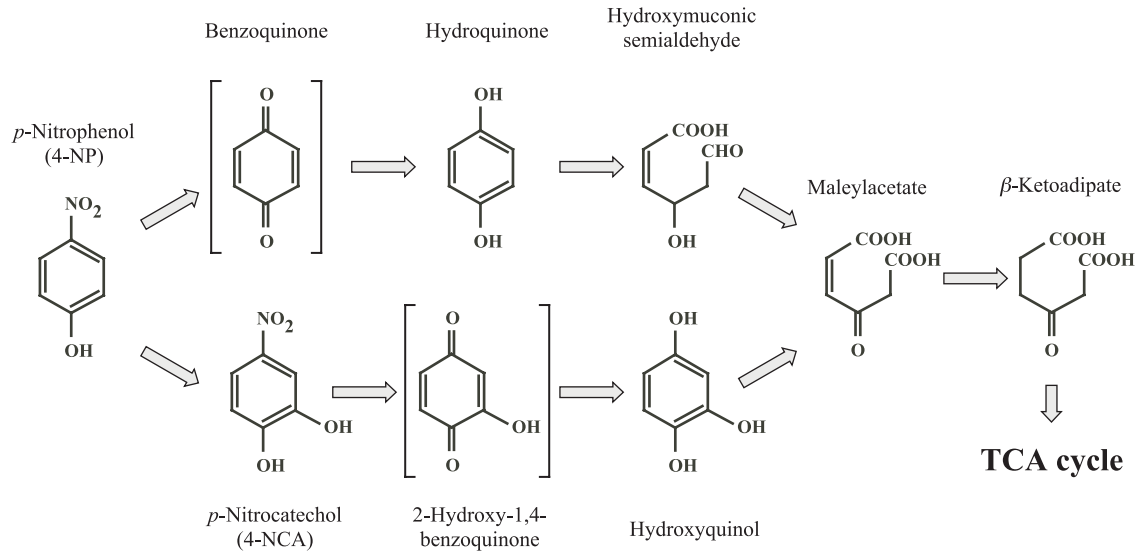

FIG. 1. Proposed degradation pathway for 4-NP (11, 25, 32). TCA, trichloroacetic acid.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The plasmids and bacterial strains used are listed in Table 1. Strain SAO101 was grown in Luria-Bertani (LB) medium and minimal salt medium (MM) with 4-NP or 4-NCA at 30°C (12). A resting-cell assay of *Rhodococcus* strains was also performed in MM supplemented with 500 μM substrate at 30°C. In addition, *E. coli* strains were grown in LB medium at 30 or 37°C; 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml, 100 μg of ampicillin per ml, 50 μg of kanamycin per ml, and 30 μg of chloramphenicol per ml were added to the medium when needed.

DNA manipulations and analysis. Standard DNA techniques were performed basically as described elsewhere (13, 14, 24). Nucleotide sequence analyses were performed with a CEQ2000 DNA analysis system (Beckman Coulter). For the sequence analyses, GeneWorks (Intelligenetics, Mountain View, CA) and GENETYX (Software Development, Tokyo, Japan), as well as the online BLAST homology search (http://www.ncbi.nlm.nih.gov/) and ClustalW multiple-sequence alignment (http://www.ddbj.nig.ac.jp/) software packages were used.

RT-PCR analyses. The total RNA of strain SAO101 cells grown in LB medium or 4-NP-supplemented MM were extracted with the RNeasy minikit (Qiagen). Reverse transcriptase PCR (RT-PCR) analyses were performed with the ReverTra Ace-α kit (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. Five primer sets were used for RT-PCR. Primer set A (forward, 5′-TCG GACACTTCGCAAGTGG-3′; reverse, 5′-CGGTAGTACGACAGTGGATTG C-3′) amplified the internal 409 bp of *npcB*. Primer set B (forward, 5′-CGCCT ACCACGAATTCTGG-3′; reverse, 5′-ATTGCCGATGTGCTGAACG-3′) amplified the internal 583 bp of *npcA*. Primer set C (forward, 5′-CAGGAGTT CATCCTGCTCTCC-3′; reverse, 5′-CACGAAGTCCTTGATCAGCG-3′) amplified the internal 567 bp of *npcC*. Primer set D (forward, 5′-GCAATCCACT GTCGTACTACCG-3′; reverse, 5′-CCAGAATTCGTGGTAGGCG-3′) amplified 388 bp of the *npcB-npcA* spanning region. Primer set E (forward, 5′-TTCAGC

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or origin
Strains		
R. opacus		
SAO101	4-NP degrader, wild type, 4-NP$^+$	12
SDC1	*npcC* gene-disrupted mutant of SAO101, 4-NP$^-$ (Km^r)	This study
SDA1	*npcA* gene-disrupted mutant of SAO101, 4-NP$^-$ (Km^r)	This study
E. coli		
JM109	*recA1 endA1 gyrA96 thi hsdR*17 (r_K^- m_K^+) *e*14$^-$ (*mcrA*) *supE44 relA1* Δ(*lac-proAB*)/F′[*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Takara Shuzo
Rosetta(DE3)pLysS	F$^-$ *ompT hsdS*$_β$ (r_B^- m_B^-) *gal dcm*, *lacY1* (DE3) pLysSRARE (Cm^r)	Novagen
Plasmids		
pBluescriptII KS, SK	Cloning vector, Ap^r	Stratagene
pBS-aphII	Cloning vector, Ap^r Km^r	This study
pHQ157	Strain SAO101 cosmid library clone containing *npc* genes, Ap^r	This study
pET17b	Expression vector, Ap^r	Novagen
pETnpcBA	pET17b derivative carrying the *npcBA* genes	This study
pETnpcB	pET17b derivative carrying the *npcB* gene	This study
pETnpcA	pET17b derivative carrying the *npcA* gene	This study
pETnpcC	pET17b derivative carrying the *npcC* gene	This study

[a] 4-NP$^+$, growth on 4-NP; 4-NP$^-$, no growth on 4-NP; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance.

ACATCGGCAATCC-3′; reverse, 5′-GAGAGCAGGATGAACTCCTGG-3′) amplified 1,112 bp of the *npcA-npcC* spanning region.

Construction of high-expression vectors of *npc* genes. Each *npc* gene was PCR amplified and ligated to an expression vector, pET17b (Novagen). All of these genes were amplified with a forward primer designed to contain the NdeI restriction site at its own ATG start codon in order to achieve ligation at the NdeI ATG site of the expression vector. The resultant plasmids pETnpcBA, pETnpcB, pETnpcA, and pETnpcC contained *npcBA*, *npcB*, *npcA*, and *npcC*, respectively. All four expression plasmids were introduced into the expression host strain *E. coli* Rosetta(DE3)pLysS (Novagen).

Crude cell extract enzyme assay. The crude cell extract enzyme assay was performed basically like the 2,4,6-trichlorophenol (2,4,6-TCP) catabolic enzyme assay previously reported by Hatta et al. and Takizawa et al. (9, 27). NpcBA was demonstrated by detecting the depletion of the substrate. To detect NpcB and NpcA (4-NP/4-NCA monooxygenase) enzyme activity, the cell extracts were diluted with the same volume of modified GPME buffer (27), and final concentrations of 200 μM substrate, 5 μM flavin adenine dinuleotide, and 2 mM NADH were added to the reaction mixture, which was then incubated at 30°C with gentle shaking. A 0.1-ml aliquot of the reaction mixture was removed at appropriate times and subjected to high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) analyses. To detect the NpcC enzyme (hydroxyquinol 1,2-dioxygenase), enzymatic activity was measured in 25 mM phosphate buffer (pH 7.2) containing 200 μM hydroxyquinol, 0.5 mM $FeSO_4$, and 5% (vol/vol) cell extract at 30°C with gentle shaking. A 0.1-ml aliquot was withdrawn and analyzed as described above. Protein concentrations were determined with a Bio-Rad Laboratories protein assay kit with bovine serum albumin as the standard.

Analytical methods. (i) HPLC analysis. Each 0.1-ml sample from the crude cell extract enzyme assay was mixed with an equal volume of methanol immediately after sampling. The samples were then centrifuged at 15,000 × *g* for 20 min, and the supernatants were subjected to HPLC analysis. HPLC analysis was carried out at room temperature with an Alliance 2690 system (Waters, Milford, Mass.) equipped with a μBondasphere column (3.9 [inner diameter] by 150 mm; Waters). The mobile phase was a mixture of water (69.93%), acetonitrile (30.0%), and acetate (0.07%), and the flow rate was 2.0 ml/min. In this analysis, 4-NP and 4-NCA were detected at 320 nm and hydroxyquinol was detected at 254 nm, using a UV spectrophotometric detector.

(ii) GC-MS analysis. Each 0.1-ml sample from the crude cell extract enzyme assay and from the resting-cell assay was mixed with 5 μl of concentrated HCl, and each sample was saturated with NaCl; 0.2 ml of ethyl acetate was then added and mixed vigorously for 5 min. The samples were centrifuged at 15,000 × *g* for 5 min, and the upper ethyl acetate phase was recovered. Samples extracted with ethyl acetate were evaporated and treated with an *N,O*-bis(trimethylsilyl) acetamide kit (B0911; Tokyo Kasei, Tokyo, Japan) to generate a trimethylsilyl derivative. GC-MS analysis was performed on a GC-17A, QP5050 GC-MS system (Shimadzu, Kyoto, Japan) equipped with a DB-5 capillary column (30 m by 0.25 mm) (Agilent Technologies, Palo Alto, Calif.). Helium was used as the carrier gas at a flow rate of 0.8 ml/min. The oven parameters were 50°C for 2 min and then a 30°C/min increase to a final temperature of 250°C for 3 min.

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been deposited in the DDBJ, EMBL, and GenBank databases under accession no. AB154422.

RESULTS

PCR cloning of the hydroxyquinol 1,2-dioxygenase gene. To obtain 4-NP degradation genes, we first attempted to isolate the hydroxyquinol 1,2-dioxygenase gene, whose gene product (enzyme) converts hydroxyquinol to maleylacetate. This gene is presumed to be involved in the 4-NP degradation pathway (Fig. 1). Three published hydroxyquinol dioxygenase gene sequences, i.e., *hadC* of *Burkholderia pickettii* (9), *tftH* of *Burkholderia cepacia* AC1100 (5), and *dxnF* of *Sphingomonas wittichi* RW1 (2), were aligned, and a primer set was designed. With this primer set and the total DNA of strain SAO101 as a template, PCR was carried out. A DNA fragment of approximately 620 bp was amplified, and its nucleotide sequence showed similarity with those of some known hydroxyquinol 1,2-dioxygenase genes. Therefore, we used it in further experiments.

Isolation of 4-NP degradation genes. To obtain the flanking regions of the PCR-cloned gene, colony hybridization against *E. coli* cells containing a strain SAO101 cosmid gene library was performed with the PCR-cloned gene probe. A total of 1,000 *E. coli* colonies were screened, and four clones gave a positive hybridization signal. Among the positive clones, a cosmid designated pHQ157 was extracted, and the flanking nucleotide sequences of the PCR-cloned gene in pHQ157 were analyzed by the gene walking method. A 7.5-kb nucleotide sequence was determined, and seven open reading frames (ORFs) (ORF1 to -7) and one incomplete ORF (ORF8) were found in the sequenced region (Fig. 2A). The deduced amino acid sequences for the ORFs were used for a BLAST homology search (Table 2). The ORF1 product showed 33% identity with NodD (a LysR-type transcriptional regulator for *nod* genes) of *Rhizobium* sp. strain HW17b (28). The ORF2 and ORF3 products showed 32 and 44% identity with PheA2 (phenol 2-hydroxylase component B [reductase component]) of *Geobacillus thermoglucosidasius* A7 (7) and TcpA (2,4,6-TCP monooxygenase) of *Ralstonia eutropha* JMP134 (16), respectively. Moreover, the ORF3 product showed 42% identity with HadA (2,4,6-TCP monooxygenase) of *Ralstonia pickettii* DTP0602 (27). These two ORFs (ORF2 and ORF3) appeared to encode a reductase component and an oxygenase component, respectively, of a monooxygenase system. ORF4, which contained the complete sequence of the gene fragment cloned by PCR, seemed to encode a hydroxyquinol 1,2-dioxygenase. This ORF showed the highest identity (76%) with ORF2 (hydroxyquinol 1,2-dioxygenase) of *Arthrobacter* sp. strain BA-5-17 (20) and also showed 43 to 52% identity with hydroxyquinol 1,2-dioxygenases from gram-negative bacteria, such as HadC of *R. pickettii* DTP0602 (9) and TcpC of *R. eutropha* JMP134 (16). Of the other ORFs found in the sequenced region, the products of ORF5 to -8 showed similarity with a putative secreted protein, a putative YjeB/Rrf2-type transcriptional regulator, a putative SoxR/MerR-type transcriptional regulator, and a putative drug transporter, respectively. Structural similarities between the ORF3 product and two known monooxygenase systems, TcpA and HadA 2,4,6-TCP monooxygenase, were observed. Therefore, ORF2 and ORF3 appeared to encode an oxygenase system especially for phenolic compounds. In addition, the ORF4 product is known to share similarities with TcpC and HadC hydroxyquinol 1,2-dioxygenase of 2,4,6-TCP degradation pathways. Therefore, based on these sequence similarities as well as the analogy between the 2,4,6-TCP degradation pathways (9, 16) and the proposed 4-NP degradation pathway (Fig. 1), we postulated that the products of ORF2 to -4 were involved in 4-NP catabolism as 4-NP monooxygenase and hydroxyquinol 1,2-dioxygenase.

Transcription of ORF2, ORF3, and ORF4 in 4-NP-grown cells of strain SAO101. To determine whether the genes were transcribed in 4-NP-grown cells of strain SAO101, RT-PCR analysis was performed with total RNA extracted from strain SAO101 cells grown on 4-NP. Total RNA extracted from SAO101 cells grown on LB medium was used as a control. With the use of primer set C (see Materials and Methods), a fragment of approximately 570 bp was amplified from the total RNA extracted from 4-NP-grown cells, but this fragment was

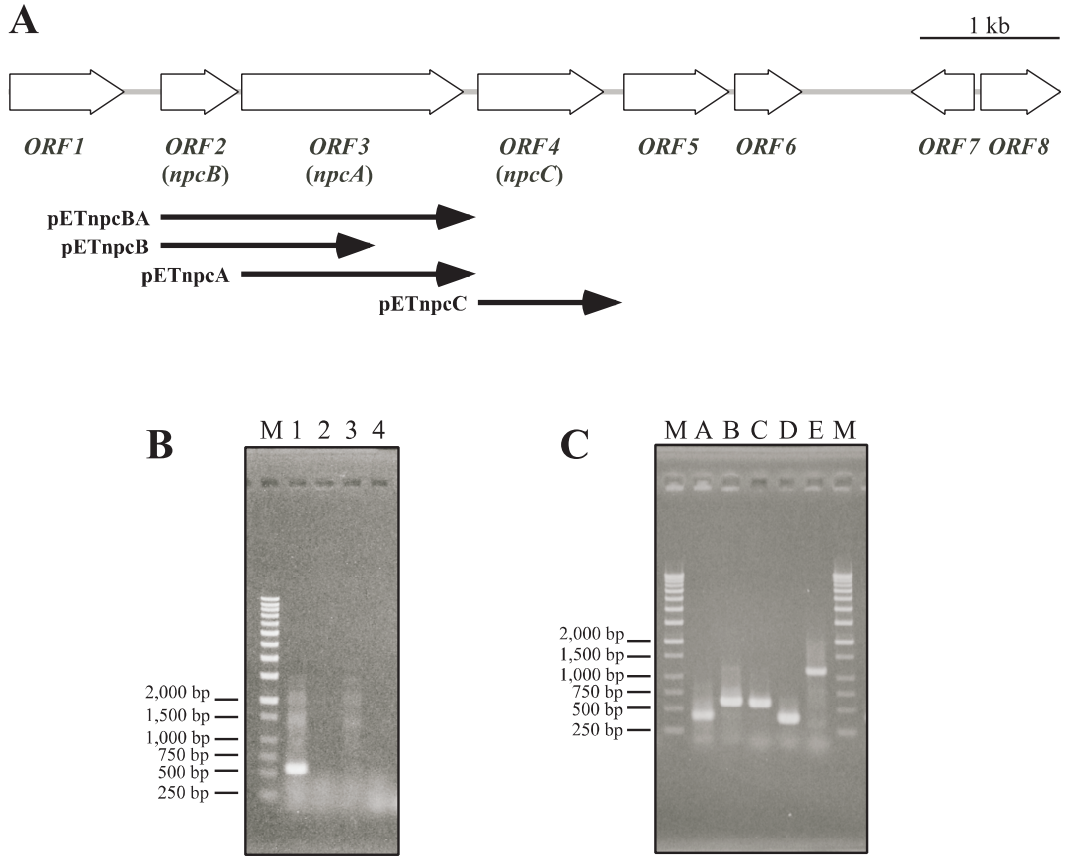

FIG. 2. (A) Organization of the ORFs found in *R. opacus* SAO101 and the nucleotide sequence region used for expression in *E. coli*. The large arrows indicate the size and the direction of each ORF. The small arrows indicate the region cloned into pET17b for gene expression. The direction of the cloned genes was identical to that of the T7 promoter of pET17b. (B) RT-PCR analysis of PCR-amplified gene fragment *npcC*. Total RNAs isolated from 4-NP-grown SAO101 cells (lanes 1 and 2) and from LB-grown cells (lanes 3 and 4) were used as the templates. Lanes 2 and 4, reactions performed without reverse transcriptase as a control. Lane M, molecular size markers. (C) RT-PCR analysis of ORF2 to -4 (*npcBAC*). Total RNA isolated from 4-NP-grown SAO101 cells was used as the template. Primer sets A to E for amplification of an internal fragment of ORF1, an internal fragment of ORF2, an internal fragment of ORF3, a spanning region of ORF1 and ORF2, and a spanning region of ORF1 and ORF2, respectively, were used (lanes A to E, respectively). Lanes M, molecular size markers.

not amplified from LB-grown cells (Fig. 2B). These results suggested that the identified genes were involved in the 4-NP catabolic pathway. RT-PCR analysis of ORF2 to -4 was also performed in order to clarify the transcriptional features of the genes. Total RNA extracted from 4-NP-grown cells of strain SAO101 was used as the template, and five oligonucleotide sets (A to E) were used as the PCR primers (see Materials and Methods). As shown in Fig. 2C, RT-PCR with each of the primer sets amplified DNA fragments of the expected sizes in this experiment. These results indicated that ORF2 to -4 are transcribed as a single operon and that the transcription is induced in the presence of 4-NP or its metabolite(s). We then designated ORF2 to -4 the nitrophenol catabolic genes *npcB*, *npcA*, and *npcC*, respectively.

Expression of *npc* genes in *E. coli*. To confirm the expression of the *npc* genes isolated from strain SAO101 in *E. coli* cells, plasmid pETnpcB containing *npcB*, pETnpcA containing *npcA*, pETnpcC containing *npcC*, and pETnpcBA containing *npcBA* were constructed by using expression vector pET17b (Table 1 and Fig. 2A), and these plasmids were introduced into *E. coli* Rosetta(DF3)pLysS. Each clone was IPTG induced, and the crude cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Distinctive overexpressed proteins with molecular masses of approximately 23.4, 60.0, and 37.8 kDa were observed in *E. coli* harboring pETnpcB, pETnpcA, and pETnpcC, respectively (data not shown). The sizes of these overexpressed proteins were consistent with the expected sizes of the gene products of *npcB* (20.1 kDa), *npcA* (59.7 kDa), and *npcC* (33.2 kDa), respectively. Unexpectedly, only one overexpressed protein, thought to be NpcB, was observed in *E. coli* carrying pETnpcBA.

Crude cell extract enzyme assay of *E. coli* harboring *npcB*, *npcA*, and *npcC*. In order to characterize the function of the *npc* gene products, crude cell extract enzyme assays were performed as described in Materials and Methods. As described above, *npcB* and *npcA* were presumed to encode 4-NP monooxygenase and *npcC* was presumed to encode a hydroxyquinol 1,2-dioxygenase. The enzyme assay of NpcBA with crude cell extract was done by measuring the depletion of the substrate (Table 3). Neither the crude cell extract of *E. coli* harboring *npcB* nor that of *E. coli* harboring *npcA* transformed 4-NP. Transformation of 4-NP was achieved only when the reaction

TABLE 2. BLAST homology search results for deduced amino acid sequences

Protein	Homolog (% identity, score)	Function	Origin	Accession no.
ORF1	NodD (33, 131)	Transcriptional regulator for *nod* genes (LysR type)	*Rhizobium* sp. strain HW17b	AAA85282
	NodD3 (33, 131)	Transcriptional regulator for *nod* genes (LysR type)	*Rhizobium leguminosarum*	P23720
ORF2 (NpcB)	PheA2 (32, 98.2)	Phenol 2-hydroxylase component B (reductase)	*Geobacillus thermoglucosidasius* A7	AAF66547
	NtaB (37, 97.8)	Nitrilotriacetate monooxygenase component B (reductase)	*Chelatobacter heintzii* ATCC 29600	P54990
ORF3 (NpcA)	TcpA (44, 421)	2,4,6-TCP monooxygenase	*Ralstonia eutropha* JMP134	AAM55214
	HadA (42, 411)	Chlorophenol 4-hydroxylase	*Ralstonia pickettii* DTP0602	BAA13105
	TftD (44, 406)	Chlorophenol 4-monooxygenase component 2	*Burkholderio cepacia* AC1100	AAC23548
ORF4 (NpcC)	ORF2 (76, 450)	Hydroxyquinol 1,2-dioxygenase	*Arthrobacter* sp. strain BA-5-17	BAA82713
	HadC (52, 265)	Hydroxyquinol 1,2-dioxygenase	*Ralstonia pickettii* DTP0602	BAA13107
	TcpC (50, 262)	6-Chlorohydroxyquinol-1,2-dioxygenase	*Rolstonia euiropha* JMP134	AAM55216
	DxnF (46, 249)	Hydroxyquinol 1,2-dioxygenase	*Sphingomonas wittichi* RW1	CAA51371
	TftH (43, 227)	Chlorohydroxyquinol 1,2-dioxygenase	*Burkholderia cepacia* AC1100	I40182
ORF5	SCO7191 (47, 202)	Putative secreted protein	*Streptomyces coelicolor* A3(2)	NP_631249
	mlr0977 (48, 214)	Putative protein	*Mesorhizobium loti*	NP_102662
ORF6	SAV1362 (65, 166)	Putative transcriptional regulator (YjeB/Rrf2 type)	*Streptomyces avermitilis* MA-4680	NP_822537
	mll1642 (46, 107)	Putative transcriptional regulator (YjeB/Rrf2 type)	*Mesorhizobium loti*	NP_103182
ORF7	SMc00182 (55, 154)	Putative transcriptional regulator (SoxR/MerR type)	*Sinorhizobium meliloti*	NP_385930
	PA2273 (55, 153)	Putative transcriptional regulator (SoxR/MerR type)	*Pseudomonas aeruginosa* PA01	NP_250963
ORF8	Rv1634 (47, 129)	Putative drug transporter	*Mycobacterium tuberculosis* H37Rv	NP_216150
	RSc2324 (37, 110)	Putative transmembrane protein	*Ralstonia solanacearum* GMI1000	NP_520445

was performed with a mixture of the crude cell extracts of *E. coli* harboring *npcB* and *npcA*. The results clearly indicated that both *npcB* and *npcA* are indispensable for 4-NP conversion. A small amount of hydroxyquinol, which appeared to be an intermediate of the 4-NP degradation pathway, was identified in the reaction mixture by GC-MS analysis. No other metabolite was detected in the reaction mixture. Transformation activity was also observed when 4-NCA and 2,4,6-TCP, as well as 4-NP, were used as substrates. On the other hand, 2,4,6-trinitrophenol was not transformed (Table 3). In this experiment, 4-NP and 4-NCA were transformed at almost same efficiency, and hydroxyquinol was also detected when 4-NCA was used as a substrate. Similar observations were reported for components A and B of the two-component monooxygenase system from *Bacillus sphaericus* JS905 (11). In strain JS905, both components A and B of 4-NP monooxygenase were essential for the transformation activity, and 4-NP was transformed to 2-hydroxy-1,4-benzoquinone via 4-NCA by same monooxygenase system. The 2-hydroxy-1,4-benzoquinone was presumed to then be converted to hydroxyquinol by an unidentified (nonspecific) quinone reductase. On the basis of previously reported information and our results, although 2-hydroxy-1,4-benzoquinone was not detected, 4-NP degradation in strain SAO101 is likely to be catalyzed by an *npcB*- and *npcA*-encoding monooxygenase to form 4-NCA, and then 4-NCA would be converted into 2-hydroxy-1,4-benzoquinone by the same monooxygenase. The generated 2-hydroxy-1,4-benzoquinone might also be converted to hydroxyquinol by an unidentified quinone reductase in both strain SAO101 and crude cell extract of *E. coli*.

TABLE 3. Conversion of phenolic compounds by *npcB* and *npcA* gene products in crude cell extract of *E. coli*

Substrate	Sp act (pmol/μg of protein/min)[a]	Relative activity[b]
4-NP	26.4 ± 2.2	100
4-NCA	25.0 ± 2.3	95
2,4,6-TNP[c]	<0.1	
2,4,6-TCP	4.6 ± 0.7	17

[a] Results are the mean of three independent experiments with standard deviations noted.
[b] Relative activity was standardized by setting the activity for 4-NP as, 100.
[c] 2,4,6-TNP, 2,4,6-trinitrophenol.

The crude cell extract of *E. coli* harboring *npcC* converted

hydroxyquinol, and the metabolite maleylacetate was identified in the reaction mixture by GC-MS analysis. These results suggested that *npcB* encodes the reductase component of 4-NP/4-NCA monooxygenase, *npcA* encodes the oxygenase component of 4-NP/4-NCA monooxygenase, and *npcC* encodes hydroxyquinol 1,2-dioxygenase.

Gene inactivation of *npcA* and *npcC* in strain SAO101. To address the functional significance of *npc* genes in strain SAO101, *npc* gene-disrupted mutants were constructed and analyzed. Insertional gene disruption was performed by a single-crossover method, as described previously (13). Since illegitimate recombination frequently occurs in *Rhodococcus* strains, the candidates for the mutants were screened by colony PCR. One genuine *npcA* mutant and one genuine *npcC* mutant were obtained from 12 and 28 candidate kanamycin-resistant mutants, respectively, in this study.

An *npcC* mutant designated strain SDC1 and an *npcA* mutant designated strain SDA1 were isolated, and the transformation and assimilation of 4-NP, 4-NCA, and hydroxyquinol were examined. Both mutant strains SDC1 and SDA1 completely lost the ability to grow on 4-NP, 4-NCA, and hydroxyquinol. In the resting-cell assay with wild-type strain SAO101, the yellow color of 4-NP disappeared within 3 to 8 h (approximate conversion rate, 125 μM/unit of optical density at 600 nm [OD_{600}] = 1 cell/h), and no intermediate had accumulated in the medium (data not shown). Moreover, the yellow-orange color of 4-NCA disappeared within 3 to 8 h, without the accumulation of any intermediate. In contrast, a dark-brown pigment gradually accumulated in the medium after the disappearance of the yellow color of 4-NP and the yellow-orange color of 4-NCA in the resting-cell assays with strain SDC1. This dark-brown pigment was identified as hydroxyquinol by GC-MS analysis. In the resting-cell assay with strain SDA1, 4-NP gradually changed into a yellow-orange compound after 2 days of incubation (approximate conversion rate, 250 μM/OD_{600} unit = 1 cell/day); further conversion was not observed, even after longer incubation periods. The yellow-orange compound was identified as 4-NCA by GC-MS analysis. In the resting-cell assay of strain SDA1, 4-NCA was not transformed. These results revealed that the *npc* genes were functionally active and absolutely essential for 4-NP mineralization in strain SAO101.

Even in the resting-cell assay of the *npcA*-disrupted stain SDA1, 4-NP was converted slowly to 4-NCA. These observations suggest the existence of another 4-NP degradation gene(s) in strain SAO101. The unknown gene product might transform 4-NP to 4-NCA but might not further convert 4-NCA.

Localization of *npc* genes. Many aromatic degradation genes are known to be encoded on plasmid DNAs (3, 6, 21, 22, 29–31). In particular, several *Rhodococcus* strains harbor aromatic degradation genes on large linear plasmids (4, 15, 24). Three strains of 4-NP-degrading bacteria, i.e., *Arthrobacter protophomiae* RJK100 (3), *B. cepacia* RJK200 (21), and *Arthrobacter aurescens* TW17 (8), have been reported to harbor plasmids involved in 4-NP degradation. In order to confirm the localization of the *npc* genes on replicons of strain SAO101, pulsed-field gel electrophoresis (PFGE) and Southern hybridization against the PFGE-separated DNAs with an *npcC* probe were performed. The PFGE analysis revealed that strain SAO101 had three large linear plasmids, designated pWK301 (1,100 kb), pWK302 (1,000 kb), and pWK303 (700 kb) (N. Kimura, W. Kitagawa, and Y. Kamagata, submitted for publication). By Southern hybridization analysis, a unique positive hybridization signal was observed at the position of the origin of electrophoresis (data not shown). This result indicated that the *npc* genes were encoded on chromosomal DNA.

DISCUSSION

We successfully isolated the 4-NP metabolic genes, i.e., *npcBAC*, from a gram-positive bacterium, *R. opacus* SAO101. In the present study, a mixture of NpcB and NpcA converted 4-NP to hydroxyquinol and also converted 4-NCA to hydroxyquinol; moreover, NpcC converted hydroxyquinol to malelylacetate in a crude cell extract of *E. coli*. Based on these results and the structural information regarding the enzymes involved, the *npcB* gene encodes the reductase component of 4-NP/4-NCA monooxygenase, the *npcA* gene encodes the oxygenase component of 4-NP/4-NCA monooxygenase, and the *npcC* gene encodes hydroxyquinol 1,2-dioxygenase. This is the first study to isolate and characterize the 4-NP degradation genes that convert 4-NP to maleylacetate via the hydroxyquinol pathway.

The deduced amino acid sequences and gene organization of the *npcBAC* genes from strain SAO101 indicate an evolutionary relationship with the 2,4,6-TCP degradation genes *tcpABC* from *R. eutropha* JMP134 (16) and *hadABC* from *R. pickettii* DTP0602 (9, 27). However, some differences were noted. The reductase component *npcB* of strain SAO101 was located upstream of the oxygenase component *npcA*, in contrast to the finding that putative reductase component genes *tcpB* and *hadB* were located downstream of the oxygenase component genes *tcpA* and *hadA* of *R. eutropha* JMP134 and *R. pickettii* DTP0602, respectively. The deduced amino acid sequences of *npcA* and *npcC* showed 42 to 52% identity with the corresponding *tcpA* and *tcpC* genes and *hadA* and *hadC* genes; however, the deduced amino acid sequence of *npcB* showed much lower identity, i.e., 30 and 27%, to the corresponding *tcpB* and *hadB* amino acid sequences. The phylogenetic trees of the NpcA, NpcB, and NpcC proteins are shown in Fig. 3. As shown in these trees, NpcA, TcpA, and HadA are in the same branch, and they seem to comprise a nitro- or chloro-substituted phenol oxygenase family (Fig. 3A). Moreover, NpcC, TcpC, and HadC are in the same branch as members of the hydroxyquinol 1,2-dioxygenase family (Fig. 3C). The present results indicate that these proteins are closely related. On the other hand, NpcB is apparently distant from TcpB and HadB (Fig. 3B). From an evolutionary perspective, these findings indicate that the reductase gene *npcB* has a relationship to the *tcp* and *had* genes that is more distant than the evolutionary relationship of the corresponding monooxygenase genes (*npcA*, *tcpA*, and *hadA*) and the corresponding hydroxyquinol 1,2-dioxygenase genes (*npcC*, *tcpC*, and *hadC*). It should also be noted that different characteristics of the reductase were also observed in our enzyme activity experiments. Purified TcpA protein did not show monooxygenase activity by itself; however, in the presence of *E. coli* flavin reductase, it showed monooxygenase activity. In the case of HadA, the crude cell extract of HadA-expressing *E. coli* did not require HadB for

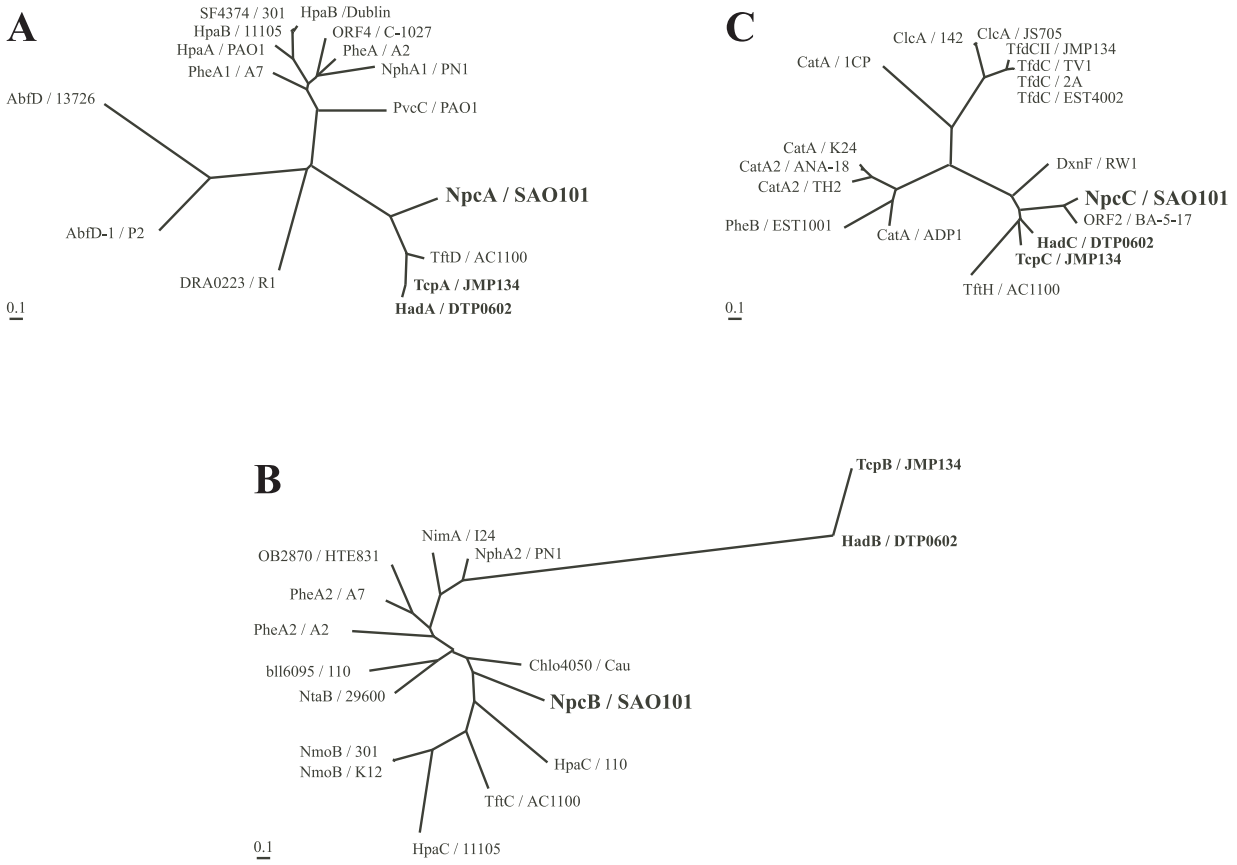

FIG. 3. Phylogenetic trees of NpcA (A), NpcB (B), and NpcC (C) proteins. Protein and strain abbreviations and accession numbers: NpcA/SAO101, nitrophenol monooxygenase oxygenase component of *R. opacus* SAO101 (AB154422); TcpA/JMP134, chlorophenol monooxygenase of *R. eutropha* JMP134 (AF498371); HadA/DTP0602, chlorophenol monooxygenase of *R. pickettii* DTP0602 (D86544); TftD/AC1100, chlorophenol monooxygenase component 2 of *B. cepacia* AC1100 (U83405); NphA1/PN1, 4-nitrophenol hydroxylase component A of *Rhodococcus* sp. strain PN1 (AB081773); PheA1/A7, phenol 2-hydroxylase component A of *G. thermoglucosidasius* A7 (AF140605); PheA/A2, phenol 2-hydroxylase component A of *Geobacillus thermoleovorans* A2 (AAC38324); PvcC/PAO1, pyoverdine biosynthesis protein of *Pseudomonas aeruginosa* PAO1 (AAC21673); ORF4/C-1027, chlorophenol-4-monooxygenase of *Streptomyces globisporus* C-1027 (AAL06674); HpaB/Dublin, 4-hydroxyphenylacetate 3-monooxygenase large chain of *Salmonella enterica* subsp. *enterica* serovar Dublin (AAD53503); SF4374/301, 4-hydroxyphenylacetate 3-monooxygenase large chain of *Shigella flexneri* 2a strain 301 (NP_710083); HpaB/11105, 4-hydroxyphenylacetic hydroxylase of *E. coli* ATCC 11105 (Z29081), HpaA/PAO1, 4-hydroxyphenylacetate 3-monooxygenase large chain of *P. aeruginosa* PAO1 (NP_252780); AbfD/13726, 4-hydroxybutyryl coenzyme A dehydratase of *Clostridium aminobutyricum* ATCC 13726 (P55792); AbfD-1/P2, 4-hydroxybutyryl coenzyme A dehydratase of *Sulfolobus solfataricus* P2 (NP_343847); DRA0223/R1, 4-hydroxyphenylacetate-3-hydroxylase of *Deinococcus radiodurans* R1 (NP_285546); NpcB/SAO101, nitrophenol monooxygenase reductase component of *R. opacus* SAO101 (AB154422); TcpB/JMP134, putative reductase of *R. eutropha* JMP134 (AF498371); HadB/DTP0602, putative reductase of *R. pickettii* DTP0602 (D86544); TftC/AC1100, chlorophenol monooxygenase component 1 of *B. cepacia* AC1100 (U83405); NphA2/PN1, 4-nitrophenol hydroxylase component B of *Rhodococcus* sp. strain PN1 (AB081773); PheA2/A7, phenol 2-hydroxylase component B of *G. thermoglucosidasius* A7 (AF140605); PheA2/A2, phenol 2-hydroxylase component B of *G. thermoleovorans* A2 (AAQ04677); NtaB/29600, nitrilotriacetate monooxygenase component B of *Chelatobacter heintzii* ATCC 29600 (U39411); Chlo4050/Cau, putative reductase of *Chloroflexus aurantiacus* (ZP_00020998); bll6095/110, putative nitrilotriacetate monooxygenase of *Bradyrhizobium japonicum* USDA 110 (NP_772735); OB2870/HTE831, phenol 2-hydroxylase component B of *Oceanobacillus iheyensis* HTE831 (NP_693792); NmoB/K12, 4-hydroxyphenylacetate 3-monooxygenase small chain of *E. coli* K-12 (BAA35774); NmoB/301, 4-hydroxyphenylacetate 3-monooxygenase small chain of *S. flexneri* 2a strain 301 (NP_706929); HpaC/110, putative monooxygenase of *B. japonicum* USDA 110 (NP_773930); NimA/I24, putative reductase of *Rhodococcus* sp. strain I24 (AAL61657); HpaC/11105, 4-hydroxyphenylacetate 3-monooxygenase oxidoreductase component of *E. coli* ATCC 11105 (Z29081); NpcC/SAO101, hydroxyquinol 1,2-dioxygenase of *R. opacus* SAO101 (AB154422); TcpC/JMP134, hydroxyquinol 1,2-dioxygenase of *R. eutropha* JMP134 (AF498371); HadC/DTP0602, hydroxyquinol 1,2-dioxygenase of *R. pickettii* DTP0602 (D86544); TftH/AC1100, hydroxyquinol 1,2-dioxygenase of *B. cepacia* AC1100 (U19883); DxnF/RW1, hydroxyquinol 1,2-dioxygenase of *S. wittichi* RW1 (CAA51371); ORF2/BA-5-17, hydroxyquinol 1,2-dioxygenase of *Arthrobacter* sp. strain BA-5-17 (BAA82713); TfdC/EST4002, chlorocatechol 1,2-dioxygenase of *Achromobacter denitrificans* EST4002 (AAC78501); TfdC/2A, chlorocatechol 1,2-dioxygenase of *B. cepacia* 2A (AAK81678); TfdC/TV1, catechol 1,2-dioxygenase II of *Variovorax paradoxus* TV1 (BAA88069); TfdCII/JMP134, chlorocatechol 1,2-dioxygenase of *R. eutropha* JMP134 (AAC44730); ClcA/JS705, chlorocatechol 1,2-dioxygenase of *Ralstonia* sp. strain JS705 (CAA06968); ClcA/142, chlorocatechol-1,2-dioxygenase of *P. aeruginosa* 142 (AAF00195); CatA/1CP, catechol 1,2-dioxygenase of *R. opacus* 1CP (CAA67941); CatA/K24, catechol 1,2-dioxygenase of *Acinetobacter lwoffii* K24 (AAC31767); CatA2/ANA-18, catechol 1,2-dioxygenase of *Frateuria* sp. strain ANA-18 (T48871); CatA2/TH2, catechol 1,2-dioxygenase of *Burkholderia* sp. strain TH2 (BAC16769); PheB/EST1001, catechol 1,2-dioxygenase of *Pseudomonas* sp. strain EST1001 (P31019); CatA/ADP1, catechol 1,2-dioxygenase of *Acinetobacter* sp. strain ADP1 (AAC46426).

monooxygenase activity. Both of these results suggest that reductase originating from *E. coli* could be substituted for the original reductase (probably TcpB and HadB) in order to obtain monooxygenase activity. In contrast, both NpcB and NpcA of strain SAO101 were found to be indispensable for monooxygenase activity in the crude cell extract of *E. coli*. These results indicate that no reductase originating from *E. coli* in the crude cell extract could be substituted to achieve monooxygenase activity.

In the phylogenetic tree, both the NpcB and NpcA proteins are located in branches that differ from those of the 4-NP monooxygenase protein NphA2 (reductase) and NphA1 (oxygenase) of *Rhodococcus* sp. strain PN1 (26). This might be reflective of enzyme function. NpcBA of strain SAO101 converts 4-NP to hydroxyquinol probably via 4-NCA, whereas NphA1A2 of strain PN1 simply converts 4-NP to 4-NCA. These results indicate that NpcBA of strain SAO101 and NphA1A2 of strain PN1 are in fact entirely different enzymes.

A two-component 4-NP/4-NCA monooxygenase protein from *B. sphaericus* JS905 has been characterized (11). The *npcBA* gene products of strain SAO101 and the 4-NP/4-NCA monooxygenase proteins of strain JS905 seem to have some features in common. Since the genes that encode components A and B from strain JS905 were not cloned and the structural information for these proteins was not addressed in this report, we were unable to compare the amino acid sequences and molecular sizes of the 4-NP/4-NCA monooxygenase proteins of strains JS905 and SAO101.

The *npcA* and *npcC* genes were successfully inactivated by the insertion of a kanamycin resistance gene. The resulting mutant strains, strains SDA1 and SDC1, completely lost the ability to grow on 4-NP and 4-NCA as the sole carbon source. This finding clearly indicates that *npcA* and *npcC* are essential for 4-NP mineralization in strain SAO101. When *npcA* mutant strain SDA1 was incubated in minimal medium together with 4-NP, an accumulation of 4-NCA was observed as a dead-end product. Accordingly, another 4-NP monooxygenase gene apparently played a role in the transformation of 4-NP to 4-NCA in strain SAO101. A preliminary study indicated that strain SAO101 has a *Rhodococcus* sp. strain PN1-type 4-NP monooxygenase gene (*nphA1*). A PCR experiment to amplify the partial *nphA1* was carried out, and the amplified 528-bp nucleotide sequence showed 99% identity with *nphA1* of strain PN1 (data not shown). The identified *nphA*-type gene in strain SAO101, designated $nphA_{SAO}$, might have contributed as the second 4-NP degradation gene in strain SAO101, as weak transformation of 4-NP to 4-NCA was observed in strain SDA1. Considering the 4-NP conversion velocities of the wild type and the *npcA* mutant (125 $\mu M/OD_{600}$ [1 cell/h] and 250 $\mu M/OD_{600}$ [1 cell/day], respectively), strain SAO101 seemed to convert 4-NP primarily by the *npc* gene products. In addition, strain SDA1 could not utilize 4-NP as a carbon source, and therefore *npc* genes were essential for 4-NP transformation and mineralization in strain SAO101.

The proposed 4-NP degradation pathway and corresponding genes for the enzymes of strain SAO101 are as follows. The metabolic transformation of 4-NP is initiated by monooxygenation with *npcBA*-encoded 4-NP/4-NCA monooxygenase and $nphA_{SAO}$-encoded 4-NP monooxygenase to form 4-NCA. The 4-NCA produced is converted to 2-hydroxy-1,4-benzoquinone by *npcBA*-encoded 4-NP/4-NCA monooxygenase. The generated 2-hydroxy-1,4-benzoquinone might be converted to hydroxyquinol by an unidentified quinone reductase. The hydroxyquinol is then converted to maleylacetate by *npcC*-encoded hydroxyquinol 1,2-dioxygenase. This scheme is among the most conceivable pathways. Another probable intermediate of mono-oxidized 4-NP is 4-nitroresorcinol. The potential intermediate 4-nitroresorcinol could not be used as the substrate for the NpcBA enzyme assay because it was not possible to obtain this product commercially. We have therefore not yet ruled out 4-nitroresorcinol as an intermediate.

In this study, multiple genes for the initial degradation of 4-NP were identified. Further study will reveal the entire 4-NP degradation pathway and genes of strain SAO101, including the genes for unidentified intermediates (such as 4-NCA/4-nitroresorcinol and 2-hydroxy-1,4-benzoquinone), the *npc* genes, and the $nphA_{SAO}$ genes, and the mechanism of their regulation.

ACKNOWLEDGMENTS

We thank Masao Fukuda and Hisashi Kudo for their help with the PFGE analysis.

This work was supported by a grant from the New Energy and Industrial Technology Development Organization (NEDO). W. Kitagawa was supported by a NEDO fellowship.

REFERENCES

1. **Agency for Toxic Substances and Disease Registry.** 1992. Toxicological profile for nitrophenol. Centers for Disease Control and Prevention, Atlanta, Ga.
2. **Armengaud, J., K. N. Timmis, and R. M. Wittich.** 1999. A functional 4-hydroxysalicylate/hydroxyquinol degradative pathway gene cluster is linked to the initial dibenzo-*p*-dioxin pathway genes in *Sphingomonas* sp. strain RW1. J. Bacteriol. **181:**3452–3461.
3. **Chauhan, A., A. K. Chakraborti, and R. K. Jain.** 2000. Plasmid-encoded degradation of *p*-nitrophenol and 4-nitrocatechol by *Arthrobacter protophormiae*. Biochem. Biophys. Res. Commun. **270:**733–740.
4. **Dabrock, B., M. Kesseler, B. Averhoff, and G. Gottschalk.** 1994. Identification and characterization of a transmissible linear plasmid from *Rhodococcus erythropolis* BD2 that encodes isopropylbenzene and trichloroethene catabolism. Appl. Environ. Microbiol. **60:**853–860.
5. **Daubaras, D. L., K. Saido, and A. M. Chakrabarty.** 1996. Purification of hydroxyquinol 1,2-dioxygenase and maleylacetate reductase: the lower pathway of 2,4,5-trichlorophenoxyacetic acid metabolism by *Burkholderia cepacia* AC1100. Appl. Environ. Microbiol. **62:**4276–4279.
6. **Don, R. H., and J. M. Pemberton.** 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. **145:**681–686.
7. **Duffner, F. M., U. Kirchner, M. P. Bauer, and R. Muller.** 2000. Phenol/cresol degradation by the thermophilic *Bacillus thermoglucosidasius* A7: cloning and sequence analysis of five genes involved in the pathway. Gene **256:**215–221.
8. **Hanne, L. F., L. L. Kirk, S. M. Appel, A. D. Narayan, and K. K. Bains.** 1993. Degradation and induction specificity in actinomycetes that degrade *p*-nitrophenol. Appl. Environ. Microbiol. **59:**3505–3508.
9. **Hatta, T., O. Nakano, N. Imai, N. Takizawa, and H. Kiyohara.** 1999. Cloning and sequence analysis of hydroxyquinol 1,2-dioxygenase gene in 2,4,6-trichlorophenol-degrading *Ralstonia pickettii* DTP0602 and characterization of its product. J. Biosci. Bioeng. **87:**267–272.
10. **Jain, R. K., J. H. Dreisbach, and J. C. Spain.** 1994. Biodegradation of *p*-nitrophenol via 1,2,4-benzenetriol by an *Arthrobacter* sp. Appl. Environ. Microbiol. **60:**3030–3032.
11. **Kadiyala, V., and J. C. Spain.** 1998. A two-component monooxygenase catalyzes both the hydroxylation of *p*-nitrophenol and the oxidative release of nitrite from 4-nitrocatechol in *Bacillus sphaericus* JS905. Appl. Environ. Microbiol. **64:**2479–2484.
12. **Kimura, N., and Y. Urushigawa.** 2001. Metabolism of dibenzo-*p*-dioxin and chlorinated dibenzo-*p*-dioxin by a gram-positive bacterium, Rhodococcus opacus SAO 101. J. Biosci. Bioeng. **92:**138–143.
13. **Kitagawa, W., K. Miyauchi, E. Masai, and M. Fukuda.** 2001. Cloning and characterization of benzoate catabolic genes in the gram-positive polychlorinated biphenyl degrader *Rhodococcus* sp. strain RHA1. J. Bacteriol. **183:**6598–6606.

14. **Kitagawa, W., S. Takami, K. Miyauchi, E. Masai, Y. Kamagata, J. M. Tiedje, and M. Fukuda.** 2002. Novel 2,4-dichlorophenoxyacetic acid degradation genes from oligotrophic *Bradyrhizobium* sp. strain HW13 isolated from a pristine environment. J. Bacteriol. **184:**509–518.
15. **Kosono, S., M. Maeda, F. Fuji, H. Arai, and T. Kudo.** 1997. Three of the seven *bphC* genes of *Rhodococcus erythropolis* TA421, isolated from a termite ecosystem, are located on an indigenous plasmid associated with biphenyl degradation. Appl. Environ. Microbiol. **63:**3282–3285.
16. **Louie, T. M., C. M. Webster, and L. Xun.** 2002. Genetic and biochemical characterization of a 2,4,6-trichlorophenol degradation pathway in *Ralstonia eutropha* JMP134. J. Bacteriol. **184:**3492–3500.
17. **Munnecke, D. M.** 1976. Enzymatic hydrolysis of organophosphate insecticides, a possible pesticide disposal method. Appl. Environ. Microbiol. **32:**7–13.
18. **Munnecke, D. M., and D. P. Hsieh.** 1974. Microbial decontamination of parathion and *p*-nitrophenol in aqueous media. Appl. Microbiol. **28:**212–217.
19. **Munnecke, D. M., and D. P. Hsieh.** 1976. Pathways of microbial metabolism of parathion. Appl. Environ. Microbiol. **31:**63–69.
20. **Murakami, S., T. Okuno, E. Matsumura, S. Takenaka, R. Shinke, and K. Aoki.** 1999. Cloning of a gene encoding hydroxyquinol 1,2-dioxygenase that catalyzes both intradiol and extradiol ring cleavage of catechol. Biosci. Biotechnol. Biochem. **63:**859–865.
21. **Prakash, D., A. Chauhan, and R. K. Jain.** 1996. Plasmid-encoded degradation of *p*-nitrophenol by *Pseudomonas cepacia*. Biochem. Biophys. Res. Commun. **224:**375–381.
22. **Romine, M. F., L. C. Stillwell, K. K. Wong, S. J. Thurston, E. C. Sisk, C. Sensen, T. Gaasterland, J. K. Fredrickson, and J. D. Saffer.** 1999. Complete sequence of a 184-kilobase catabolic plasmid from *Sphingomonas aromaticivorans* F199. J. Bacteriol. **181:**1585–1602.
23. **Sharmila, M., K. Ramanand, and N. Sethunathan.** 1989. Effect of yeast extract on the degradation of organophosphorus insecticides by soil enrichment and bacterial cultures. Can. J. Microbiol. **35:**1105–1110.
24. **Shimizu, S., H. Kobayashi, E. Masai, and M. Fukuda.** 2001. Characterization of the 450-kb linear plasmid in a polychlorinated biphenyl degrader, *Rhodococcus* sp. strain RHA1. Appl. Environ. Microbiol. **67:**2021–2028.
25. **Spain, J. C., and D. T. Gibson.** 1991. Pathway for biodegradation of *para*-nitrophenol in a *Moraxella* sp. Appl. Environ. Microbiol. **57:**812–819.
26. **Takeo, M., T. Yasukawa, Y. Abe, S. Niihara, Y. Maeda, and S. Negoro.** 2003. Cloning and characterization of a 4-nitrophenol hydroxylase gene cluster from *Rhodococcus* sp. PN1. J. Biosci. Bioeng. **95:**139–145.
27. **Takizawa, N., H. Yokoyama, K. Yanagihara, T. Hatta, and H. Kiyohara.** 1995. A locus of *Pseudomonas pickettii* DTP0602, *had*, that encodes 2,4,6-trichlorophenol-4-dechlorinase with hydroxylase-activity, and hydroxylation of various chlorophenols by the enzyme. J. Ferment. Bioeng. **80:**318–326.
28. **Thomas, P. M., K. F. Golly, R. A. Virginia, and J. W. Zyskind.** 1995. Cloning of *nod* gene regions from mesquite rhizobia and bradyrhizobia and nucleotide sequence of the *nodD* gene from mesquite rhizobia. Appl. Environ. Microbiol. **61:**3422–3429.
29. **van der Meer, J. R., A. R. van Neerven, E. J. de Vries, W. M. de Vos, and A. J. Zehnder.** 1991. Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzene of *Pseudomonas* sp. strain P51. J. Bacteriol. **173:**6–15.
30. **Vedler, E., V. Koiv, and A. Heinaru.** 2000. TfdR, the LysR-type transcriptional activator, is responsible for the activation of the *tfdCB* operon of *Pseudomonas putida* 2, 4-dichlorophenoxyacetic acid degradative plasmid pEST4011. Gene **245:**161–168.
31. **Worsey, M. J., F. C. Franklin, and P. A. Williams.** 1978. Regulation of the degradative pathway enzymes coded for by the TOL plasmid (pWWO) from *Pseudomonas putida* mt-2. J. Bacteriol. **134:**757–764.
32. **Zylstra, G. J., S.-W. Bang, L. M. Newman, and L. L. Perry.** 2000. Microbial degradation of mononitrophenols and mononitrobenzoates, p. 145–160. *In* J. C. Spain, J. B. Hughes, and H. J. Knackmuss (ed.), Biodegradation of nitroaromatic compounds and explosives. CRC Press, Inc., Boca Raton, Fla.