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Adaptation of *Comamonas testosteroni* TA441 to utilize phenol: organization and regulation of the genes involved in phenol degradation

Hiroyuki Arai,[1] Saiko Akahira,[1] Tohru Ohishi,[1] Michihisa Maeda[2]† and Toshiaki Kudo[1,2]

Author for correspondence: Hiroyuki Arai. Tel: +81 48 467 9545. Fax: +81 48 462 4672. e-mail: harai@postman.riken.go.jp

[1] Laboratory of Microbiology, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-0198, Japan

[2] Research Development Corporation of Japan, Wako, Saitama 351-0198, Japan

***Comamonas testosteroni* TA441 was not able to grow on phenol as a sole carbon and energy source, but it gained the ability to utilize phenol after a 2–3-week incubation in a medium containing phenol. Phenol hydroxylase (PH) and catechol 2,3-dioxygenase (C23O) were highly induced by phenol in the adapted strain designated as strain P1, suggesting that phenol was degraded via the *meta*-pathway. Gene clusters for phenol degradation were isolated from both strains TA441 and P1. The structural genes encoding multi-component PH and C23O (*aphKLMNOPQB*), and a regulatory gene of the NtrC family (*aphR*), were located in a divergent transcriptional organization. The cloned *aphKLMNOPQB* genes from either strain TA441 or strain P1 produced active PH and C23O enzymes in strain TA441. No difference was found between the strains in the sequences of *aphR* and the intergenic promoter region of *aphK* and *aphR*. However, the transcriptional activities of the *aphK* and *aphR* promoters were higher in strain P1 than in strain TA441. The *aphK*-promoter activity was not observed in *aphR* mutant strains and these strains could not grow on phenol. The *aphR* mutant of strain P1 was able to grow on phenol after transformation with a recombinant *aphR* gene but strain TA441 was not, suggesting that the expression of the *aph* genes is silenced by an unidentified repressor in strain TA441 and that this repressor is modified in strain P1.**

Keywords: phenol hydroxylase, catechol 2,3-dioxygenase, adaptation, regulation, *Comamonas testosteroni*

INTRODUCTION

Many micro-organisms are able to degrade a variety of aromatic compounds. In the aerobic degradation routes, the substrates are generally converted to a limited number of dihydroxylated intermediates such as catechol and protocatechuate, followed by one of the *meta* or *ortho* ring-cleavage pathways. The enzyme systems resemble each other, although a good many different metabolic pathways have been identified to date (van der Meer *et al.*, 1992). Sequence analyses of the degradation genes have suggested that the isoenzymes of the diverse catabolic pathways are derived from some common evolutionary origins (Chakrabarty, 1996; van der Meer *et al.*, 1992). The adaptation of micro-organisms to a novel compound is thought to be achieved by assembling a set of the catabolic genes, fitting the substrate specificity of the peripheral enzymes that lead the compound to one of the central dihydroxylated intermediates, and recruiting a transcriptional control system (Chakrabarty, 1996; de Lorenzo & Pérez-Martin, 1996). Understanding the mechanism of the evolution of catabolic pathways will facilitate the development of useful bacterial strains that can degrade recalcitrant synthetic compounds.

Phenol catabolism is a suitable model for analysing the mechanism of bacterial evolution or adaptation to degrade synthetic aromatic compounds. Phenol itself is not recalcitrant, but many synthetic aromatics have substituted phenol rings (Shingler, 1996). Phenol is

†**Present address:** School of Agriculture, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki, Kanagawa 214, Japan.

Abbreviations: C23O, catechol 2,3-dioxygenase; PH, phenol hydroxylase; pHB, *p*-hydroxybenzoate.

The DDBJ/EMBL/GenBank accession numbers for the DNA sequences of *aphKLMNOPQB* and *aphR*, and the partial sequence of 16S rDNA are AB006479, AB006480, and D87101, respectively.

degraded through a variety of pathways. Under aerobic conditions, phenol is usually hydroxylated to catechol and degraded via the *meta* or *ortho* pathways. Two types of phenol hydroxylase are known: one is a multicomponent enzyme encoded by *dmpKLMNOP* from *Pseudomonas* sp. strain CF600 (Nordlund *et al.*, 1990; Shingler *et al.*, 1992), *phhKLMNOP* from *Pseudomonas putida* P35X (Ng *et al.*, 1994), *pheA1A2A3A4A5A6* from *P. putida* BH (Takeo *et al.*, 1995), *phlABCDEF* from *P. putida* H (Herrmann *et al.*, 1995) or *mopKLMNOP* from *Acinetobacter calcoaceticus* NCIB 8250 (Ehrt *et al.*, 1995); the other is a single-component monooxygenase encoded by *pheA* from *Pseudomonas* sp. strain EST1001 (Kivisaar *et al.*, 1989), *tbuD* from *Pseudomonas pickettii* PKO1 (Kukor & Olson, 1992) or *pheA* from *Bacillus stearothermophilus* BR219 (Kim & Oriel, 1995). Recently, mutations that allow *P. putida* or *Escherichia coli* to utilize phenol as a carbon source have been reported (Burchhardt *et al.*, 1997; Kasak *et al.*, 1997; Nurk *et al.*, 1993). These studies used recombinant strains transformed with exogenous genes for phenol degradation. The mutations were found in the promoter regions of the recombinant genes and created constitutively active σ^{70} promoters. In this study, we found that *Comamonas testosteroni* TA441 adapts to utilize phenol. Unlike the previous reports, the mutation was not found in the promoter region. Analyses of the transcriptional activity of the genes for phenol degradation revealed that the genes are silenced in strain TA441.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *C. testosteroni* strains were grown at 30 °C in LB medium or C-medium, which contained (per litre): 5 g $(NH_4)_2SO_4$, 2·93 g KH_2PO_4, 5·87 g K_2HPO_4, 0·3 g $MgSO_4.7H_2O$, 2 g NaCl, 0·01 g $CaCl_2$, 0·01 g $FeSO_4.7H_2O$, 0·2 g yeast extract, 20 ml of a trace elements solution, and adjusted to pH 7·0. The trace elements solution contained (per litre): 0·1 g MoO_3, 0·7 g $ZnSO_4.5H_2O$, 0·05 g $CuSO_4.5H_2O$, 0·1 g H_3BO_3, 0·1 g $MnSO_4.5H_2O$, 0·1 g $CoCl_2.6H_2O$, 0·1 g $NiSO_4.7H_2O$. Phenol or *p*-hydroxybenzoate (pHB) was added to the medium as a carbon source. *E. coli* strains were grown at 37 °C in LB medium. Concentrations of antibiotics were as follows (μg ml^{-1}): ampicillin (Ap), 100; kanamycin (Km), 30 and tetracycline (Tc), 12·5 for *E. coli*; and carbenicillin (Cb), 200; Km, 400 and Tc, 50 for *C. testosteroni*. Growth was monitored by measuring the optical density of the cultures at 550 nm. Concentration of phenol in the culture supernatant was determined by the colorimetric method (Herrmann *et al.*, 1995).

Isolation and identification of strain TA441. Strain TA441 was isolated with a biphenyl-degrading bacterium, *Rhodococcus erythropolis* TA431, in gut homogenates of the wood-feeding termite *Reticulitermes speratus*, as described previously (Chung *et al.*, 1994). The identification of strain TA441 was carried out by using the classification given in *The Prokaryotes* (Balows *et al.*, 1992) and *Bergey's Manual of Systematic Bacteriology* (Krieg & Holt, 1984). Strain TA441 was considered to belong to *Comamonas testosteroni* according to the following results of the taxonomic analyses. The strain is Gram-negative and aerobic and has motile rod-shaped cells of diameter 0·6–0·8 μm and length 2·0–4·0 μm. Spores were not formed. Oxidase and catalase reactions were positive. Pigment production and arginine dihydrolase activity were negative. The organism used adipate, benzoate, caprate, malate, pHB and testosterone as a carbon source, but did not use glucose, fructose, L-arabinose, malonate, or ethanol. The fatty acid profile was typical for *C. testosteroni*. The partial sequence of the 16S rDNA (DDBJ accession number D87101) showed 100% identity to the species.

Enzyme assay. The *in vivo* activity of phenol hydroxylase (PH) was determined by measuring the consumption of phenol in a cell suspension containing 0·1 M Tris/HCl (pH 8·0), 200 μM NADPH, 100 μM $FeSO_4$ and 100 μM phenol, as described by Herrmann *et al.* (1995). The reaction was allowed to proceed for 30 min at 30 °C. The concentration of phenol was determined by high-performance liquid chromatography (Waters 600E), with a reversed-phase PEGASIL ODS column (25 × 0·45 cm) and SSC-5200 UV-detector (Senshu Scientific, Tokyo, Japan). The flow rate was 1 ml min^{-1}. The solvent system was acetonitrile/water (40:60, v/v). Phenol was detected at 260 nm. Catechol 2,3-dioxygenase (C23O) activity was determined as described previously (Maeda *et al.*, 1995), except that the cells were suspended in a 50 mM potassium phosphate buffer (pH 7·5) containing 10% (v/v) acetone and broken by sonication. β-Galactosidase assay was performed according to the method described by Miller (1992). Protein concentration was determined by the method of Bradford (1976) with a Bio-Rad protein assay kit. Bovine serum albumin was used as a standard.

Recombinant DNA techniques. Standard recombinant DNA techniques were performed as described by Sambrook *et al.* (1989). *C. testosteroni* was transformed with broad-host-range plasmids by electroporation with a Gene Pulser (Bio-Rad). Southern and colony hybridization analyses were performed by using Hybond-N^+ nylon membrane (Amersham) and a DIG DNA labelling and detection kit (Boehringer Mannheim).

Cloning of genes involved in phenol degradation. For isolation of the C23O gene, total DNA of TA441 was partially digested with *Sau*3AI and ligated to the *Bam*HI digest of pUC18. *E. coli* JM109 was transformed with the ligation mixture. pYM20 was obtained from a transformant colony which turned yellow after being sprayed with a catechol solution. pYM20 carried a 2·6 kb fragment containing the C23O gene (see Fig. 2). pYM50 was obtained by cloning a 12 kb *Eco*RI fragment from TA441 DNA that hybridized with the 2·6 kb fragment of pYM20 into pUC18. pYM30 was obtained by cloning a 6·5 kb *Sac*I fragment of TA441 DNA that hybridized with the 1·1 kb *Eco*RI–*Xho*I fragment of pYM50 into Charomid 9-36 (Nippon gene). pYM60 was constructed by cloning a 4·2 kb *Eco*RI fragment from TA441 DNA that hybridized with a part of pYM30 into pUC19. pYM31 and pYM61 were made in the same way as pYM30 and pYM60, respectively, using total DNA from strain P1. pYM55 was made by cloning the 6 kb *Bgl*II fragment from strain P1 into the *Bam*HI site of pUC19. pYM50, pYM30, pYM60, pYM31 and pYM61 were identified by colony hybridization. The *E. coli* cell carrying pYM55 was identified by development of a yellow colour after being sprayed with a catechol solution.

DNA sequencing. The restriction fragments derived from pYM50, pYM30, pYM60, pYM31 or pYM61 were ligated into the *Hin*cII site of pUC19 after blunting the cohesive ends. The inserted fragments were deleted by a Kilo-sequence deletion kit (Takara Shuzo). A series of the deletion derivatives was used for DNA sequencing. The sequence was determined

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics*	Source/reference
Strains		
C. testosteroni		
TA441	Wild-type (JCM9806)	This study
P1	Phenol-utilizing mutant of TA441	This study
TR921	*aphR* mutant of TA441; Km^r	This study
PR921	*aphR* mutant of P1; Km^r	This study
E. coli JM109	Host strain for DNA manipulation	Toyobo
Plasmids		
pUC18	Cloning vector; Ap^r	Toyobo
pUC19	Cloning vector; Ap^r	Toyobo
Charomid 9-36	Cloning vector; Ap^r	Nippon gene
pMMB67EH	IncQ; broad-host-range expression vector; *tac* promoter; Ap^r/Cb^r	Fürste *et al.* (1986)
pRW2	IncP; *lacZ* promoter-probe vector; Tc^r	Lodge *et al.* (1990)
pYM20	2·6 kb fragment from TA441 in pUC18	This study
pYM30	6·5 kb *Sac*I fragment from TA441 in Charomid 9-36	This study
pYM31	6·5 kb *Sac*I fragment from P1 in Charomid 9-36	This study
pYM50	12 kb *Eco*RI fragment from TA441 in pUC18	This study
pYM55	6 kb *Bgl*II fragment from P1 in pUC19	This study
pYM60	4·2 kb *Eco*RI fragment from TA441 in pUC19	This study
pYM61	4·2 kb *Eco*RI fragment from P1 in pUC19	This study
pHAM911	6·3 kb *Eco*RI–*Eco*81I fragment from TA441 in pMMB67EH; *aphKLMNOPQB*	This study
pHAM912	6·3 kb *Eco*RI–*Eco*81I fragment from P1 in pMMB67EH; *aphKLMNOPQB*	This study
pHAD921	A *Hin*dIII–*Eco*RI fragment of *aphR*::Km^r in pUC19	This study
pHAM931	1·9 kb *Hin*cII–*Eco*RI fragment in pMMB67EH; *aphR*	This study
pHAW941	1·2 kb *Ava*I fragment in pRW2; *aphKL*::*lacZ* transcriptional fusion	This study
pHAW942	1·2 kb *Ava*I fragment in pRW2; *aphR*::*lacZ* transcriptional fusion	This study

* Ap^r, ampicillin-resistant; Cb^r, carbenicillin-resistant; Tc^r, tetracycline-resistant; Km^r, kanamycin-resistant.

with an ABI model 373A automated DNA sequencer with dye primer or dye terminator sequencing protocols (Perkin-Elmer). The templates for dideoxy chain-termination reactions were prepared by Wizard minipreps (Promega). The sequence was determined for both strands of DNA.

Construction of plasmids. pHAM911 was constructed by introducing a 6·3 kb *Eco*RI–*Eco*81I fragment of pYM50, which contains the complete *aphKLMNOPQB* genes from strain TA441, into the *Eco*RI and *Hin*cII sites of a broad-host-range expression vector pMMB67EH (Fürste *et al.*, 1986). The corresponding *Eco*RI–*Eco*81I fragment of strain P1 was also constructed from pYM31 and pYM55 on pMMB67EH, resulting in pHAM912.

pHAD921 is a derivative of pUC19 and is used for disruption of the *aphR* gene. It was constructed by introduction of a 2·9 kb *Eco*RI–*Hin*dIII fragment from pYM60 in pUC19 and the *aphR* gene in the inserted fragment was disrupted by introduction of an end-blunted 1·5 kb *Hin*dIII–*Sal*I fragment of pSUP5011, which contained the Km-resistance gene of Tn5 (Simon, 1984) in an *Eco*RV site.

pHAM931 was constructed by insertion of a 1·9 kb *Eco*RI–*Hin*cII fragment, which contained a *aphR* gene from pYM60, into the respective sites of pMMB67EH (Fürste *et al.*, 1986).

pHAW941 and pHAW942 are derivatives of the *lacZ* promoter-probe plasmid pRW2 (Lodge *et al.*, 1990). They carry a 1·2 kb *Ava*I fragment containing the intervening regulatory region of *aphK* and *aphR*. The *Ava*I fragment was cloned into the *Hin*cII site of pUC19 after blunting the *Ava*I ends. Each of the fragments inserted in the opposite direction was cut out by *Bam*HI and *Hin*dIII and introduced into the respective sites of pRW2. pHAW941 and pHAW942 carried the *aphKL*::*lacZ* and *aphR*::*lacZ* transcriptional fusions, respectively.

Construction of *aphR* mutant strains. The *aphR* gene of strain TA441 was disrupted by insertion of the Km-resistance gene into *aphR* as follows. Plasmid pHAD921 was introduced into strain TA441 by electroporation and the transformed cells were plated on agar plates containing Km. The Km-resistant colonies were transferred to plates containing Cb. A Cb-sensitive and Km-resistant colony, whose *aphR* gene was exchanged for the disrupted *aphR* gene by double crossover reactions, was selected and designated as strain TR921. An *aphR* mutant of strain P1 was constructed in the same way and designated as strain PR921. Insertion of the 1·5 kb fragment in *aphR* was confirmed by PCR (data not shown).

RESULTS

Adaptation of *C. testosteroni* TA441 to phenol

While analysing the degradability of various aromatic compounds by *C. testosteroni* TA441, we found the strain was able to adapt to utilizing phenol after incubation with phenol as the only carbon source for a few weeks. The growth profiles of strain TA441 in a baffled Erlenmeyer flask (500 ml) containing 200 ml C-medium supplemented with 5 mM phenol are shown in

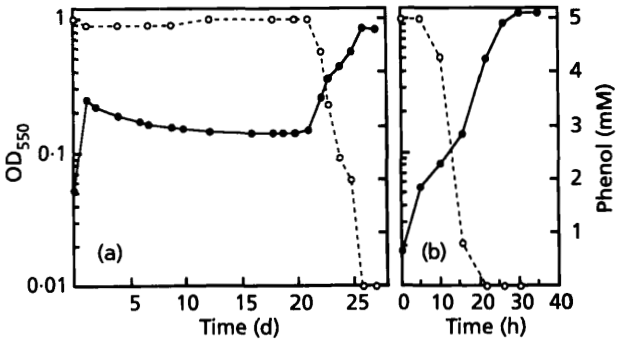

Fig. 1. Growth of *C. testosteroni* TA441 in C-medium supplemented with 5 mM phenol. (a) Strain TA441 grown in LB medium was inoculated for the first time. (b) Cells previously grown on phenol were inoculated to the same medium. Preculture was inoculated at 1% for all experiments. The data are representative of three independent cultures. ●, OD_{550}; ○, phenol.

Fig. 1. When TA441 cells grown in LB were inoculated, the strain began to grow after a long lag period of about 20 d (Fig. 1a). Phenol was consumed along with an increase in optical density. When the cells previously grown on phenol were inoculated, the long lag period was not observed (Fig. 1b). The strain also grew on phenol without the long lag phase even after the cells were grown in rich medium (data not shown), suggesting that the ability of the adapted strain to grow on phenol was stable and that some genetic changes had occurred during incubation with phenol.

To confirm the reproducibility of the adaptation, 50 colonies of strain TA441 isolated on LB plates were precultivated in LB and inoculated to 10 ml C-medium supplemented with 5 mM phenol in test tubes. All the tested cells had adapted to phenol after about 2–3 weeks (data not shown). One adapted strain, designated strain P1, was selected and used for further experiments.

Induction of enzyme activity for phenol degradation

The activities of the enzymes for phenol degradation, PH and C23O, were measured in strains TA441 and P1 (Table 2). Almost no activity was observed when the strains were grown in rich media (data not shown). The activities were very low when the strains were grown with pHB as the only carbon source, but were enhanced by adding phenol with pHB in strain P1. Strain P1 expressed the highest PH and C23O activities when phenol was used as the sole carbon source. These results indicated that the expression of these enzymes is subjected to repression by the presence of alternative carbon sources. The induction by phenol was also observed in strain TA441, but the activity was low compared with that of strain P1. The activity of catechol 1,2-dioxygenase was not induced by phenol (data not shown), suggesting that phenol is degraded by the *meta*-pathway in strain P1.

Cloning and sequencing of the genes involved in phenol degradation

The fragments of pYM20, pYM30, pYM50 and pYM60 were isolated from the total DNA of TA441 (Fig. 2). Sequencing analyses revealed that the open reading frames (ORFs) are similar to the well-characterized *dmpKLMNOPQB* genes for degradation of phenol and dimethylphenol from *Pseudomonas* sp. CF600 (Nordlund *et al.*, 1990; Shingler *et al.*, 1992) and are clustered. The order of the ORFs is conserved between the two strains. We designated the ORFs as *aphKLMNOPQB*. *aphKLMNOP* encodes the components of PH. *aphQ* and *aphB* encode a ferredoxin-like protein and C23O, respectively. An ORF, designated *aphR*, is located 306 bp upstream of the *aphK* gene in divergent transcriptional organization and encodes a transcriptional activator of the NtrC family as in the cases of other phenol degraders. Similarities in the translated sequences of the genes with the counterparts from other organisms are shown in Table 3. Sequences from TA441 were not as highly similar to those from *Pseudomonas* or *Acinetobacter* strains (34–63% amino acid identity), as the latter are to each other. In *Pseudomonas* strain CF600, the genes for the downstream *meta*-pathway enzymes (*dmpCDEFGHI*) are located just downstream of the genes for PH and C23O as an operon (Shingler *et al.*, 1992). By contrast, a putative gene encoding 2-hydroxymuconic semialdehyde dehydrogenase, corresponding to *dmpC* of strain CF600, was found about 2·5 kb downstream from

Table 2. Activities of PH and C23O of strains TA441 and P1

Cells were grown in C-medium supplemented with the carbon sources indicated. Values are means ± SD from more than two independent experiments. ND, Not determined.

Carbon source	PH (nmol min^{-1} mg^{-1})		C23O (μmol min^{-1} mg^{-1})	
	TA441	P1	TA441	P1
p-HB (5 mM)	0·05 ± 0·05	0·09 ± 0·09	0·02 ± 0·01	0·06 ± 0·04
p-HB (2·5 mM) plus phenol (2·5 mM)	0·39 ± 0·33	5·78 ± 2·21	0·21 ± 0·03	1·42 ± 0·47
Phenol (5 mM)	ND	14·91 ± 2·97	ND	6·70 ± 1·06

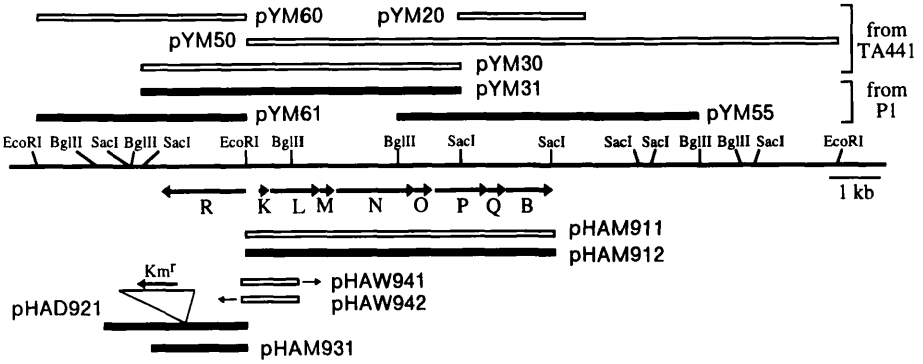

Fig. 2. Physical map of the *aph* genes from *C. testosteroni* TA441 and P1. Arrows indicate the size and direction of the *aph* genes. Open bars labelled pYM20, pYM30, pYM50 and pYM60 indicate the fragments cloned from strain TA441. Solid bars labelled pYM31, pYM55 and pYM61 indicate the fragments cloned from strain P1. pHAM911 and pHAM912 are pMMB67EH derivatives carrying the 6·3 kb *Eco*RI–*Eco*81I fragments from strain TA441 and strain P1, respectively. pHAW941 and pHAW942 are derivatives of pRW2 carrying a 1·2 kb *Ava*I fragment containing the intervening promoter region. Small arrows indicate the directions of *lacZ* on the plasmid. pHAD921 carries a fragment containing the *aphR* gene that is disrupted by insertion of the Km^r gene. pHAM931 is a derivative of pMMB67EH designed to express the *aphR* gene.

Table 3. Comparison of the *aph* genes with the corresponding genes from other phenol degraders

Gene (no. of amino acids)	Presumed properties or roles*	Corresponding gene products from other phenol degraders† (% amino acid identity with *aph* gene products)				
		dmp	*phh*	*phe*	*phl*	*mop*
aphK (63)	Assembly of active oxygenase component	35·6	35·6	35·6	35·6	34·1
aphL (330)	Hydroxylase component	44·5	44·5	44·5	44·2	37·2
aphM (97)	Optimal turnover of hydroxylase	46·5	44·2	46·5	44·2	43·7
aphN (536)	Hydroxylase component	61·8	63·1	61·8	62·6	63·0
aphO (118)	Hydroxylase component	44·5	45·4	44·5	44·5	37·5
aphP (357)	Reductase component	57·1	55·0	57·2	56·1	52·5
aphQ (121)	Ferredoxin-like protein	37·4	35·5	–	35·5	–
aphB (314)	Catechol 2,3-dioxygenase	41·6	41·0	–	42·2	–
aphR (565)	Transcriptional activator	48·4	50·5	48·4	50·7	43·3

* Speculated from the corresponding genes of *Pseudomonas* sp. CF600 (Powlowski *et al.*, 1997; Qian *et al.*, 1997; Shingler, 1996).

† *dmpKLMNOPQB* and *dmpR*, *Pseudomonas* sp. CF600 (Nordlund *et al.*, 1990; Shingler *et al.*, 1992, 1993); *phhKLMNOPQB* and *phhR*, *P. putida* P35X (Ng *et al.*, 1994, 1995); *phe*A1A2A3A4A5A6 and *pheR*, *P. putida* BH (DDBJ accession number D63814; Takeo *et al.*, 1995); *phlABCDEFGH* and *phlR*, *P. putida* H (Herrmann *et al.*, 1995; Muller *et al.*, 1996); *mopKLMNOP* and *mopR*, *A. calcoaceticus* NCIB8250 (Ehrt *et al.*, 1995; Schirmer *et al.*, 1997).

Table 4. Activities of PH and C23O of strain TA441 transformed with pHAM911 or pHAM912

The cells were grown in LB medium supplemented with 0 mM or 1 mM IPTG. Values are means ± SD from three independent cultures.

Plasmid	PH (nmol min^{-1} mg^{-1})		C23O (μmol min^{-1} mg^{-1})	
	0 mM IPTG	1 mM IPTG	0 mM IPTG	1 mM IPTG
pHAM911	0·12 ± 0·05	4·57 ± 1·09	0·03 ± 0·03	0·68 ± 0·15
pHAM912	0·66 ± 0·66	2·68 ± 1·16	0·12 ± 0·06	0·95 ± 0·40

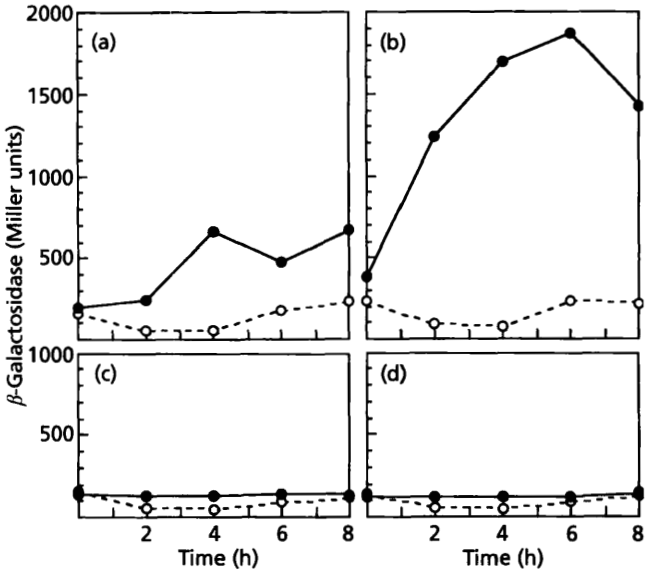

Fig. 3. Transcriptional activity of the *aphK* promoter in strains TA441 (a), P1 (b), TR921 (c) and PR921 (d). The strains were transformed with pHAW941 carrying a transcriptional fusion of *aphKL*::*lacZ*. Overnight cultures of the strains grown in C-medium with pHB as carbon source were diluted 10-fold with C-medium containing phenol (●) or pHB (○). The changes in the activity of β-galactosidase, the gene product of *lacZ*, were monitored for 8 h. The data are representative of two experiments.

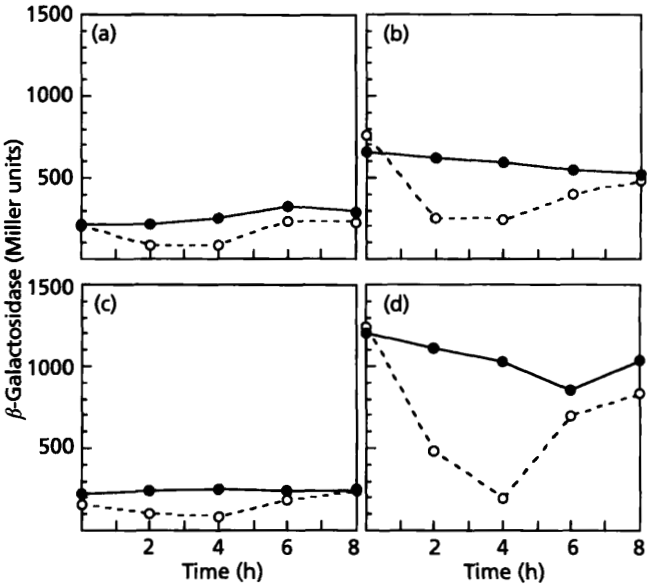

Fig. 4. Transcriptional activity of the *aphR* promoter in strains TA441 (a), P1 (b), TR921 (c) and PR921 (d). The strains were transformed with pHAW942 carrying a transcriptional fusion of *aphR*::*lacZ*. Overnight cultures of the strains grown in C-medium with pHB as carbon source were diluted 10-fold with C-medium containing phenol (●) or pHB (○). The changes in the activity of β-galactosidase, the product of *lacZ*, were monitored for 8 h. The data are representative of more than two experiments.

aphB as a different transcriptional unit in strain TA441 (data not shown).

The DNA fragments containing the *aphKLMNOPQB* or *aphR* genes were also cloned from strain P1 (pYM31, pYM55 and pYM61). The nucleotide sequence of *aphR* and the intergenic promoter region between *aphK* and *aphR* from strain P1 were also determined and compared with the corresponding sequence from strain TA441. However, no difference was found between the two strains.

Production of active PH and C23O enzymes from the recombinant *aphKLMNOPQB* genes of strains TA441 and P1

The derivatives of pMMB67EH, pHAM911 and pHAM912, which carry the 6·3 kb *Eco*RI–*Eco*81I fragments containing the *aphKLMNOPQB* genes from strains TA441 and P1, respectively, were introduced into strain TA441. Strain TA441 was able to grow on phenol following transformation with either pHAM911 or pHAM912 (data not shown). Table 4 shows the PH and C23O activities in strain TA441 carrying pHAM911 or pHAM912 grown in LB medium. The activities were increased by addition of IPTG, an inducer for the *tac* promoter of pMMB67EH, when either of the two plasmids was used, indicating that active PH and C23O enzymes were produced from the *Eco*RI–*Eco*81I fragment derived from strains TA441 and P1.

Transcriptional activity of the *aph* genes is elevated in the adapted strain P1

Strains TA441, P1, TR921 and PR921 were transformed with pHAW941, which carries a transcriptional fusion of *aphKL*::*lacZ*. The overnight culture grown in C-medium with pHB as a carbon source was diluted 10-fold with C-medium containing phenol or pHB. The changes in the activities of β-galactosidase, the gene product of *lacZ*, were monitored for 8 h (Fig. 3). The activity was strongly induced in the presence of phenol in strain P1. The induction was also observed in strain TA441, but the activity was low compared with that of strain P1. The induction by phenol was not observed in the *aphR* mutant strains TR921 and PR921, indicating that *aphR* is the phenol-sensing activator as in the case of *Pseudomonas* sp. CF600 (Shingler *et al.*, 1993).

Promoter activity for *aphR* was monitored in the same way with pHAW942, which carries a transcriptional fusion of *aphR*::*lacZ* (Fig. 4). The activity was not increased by addition of phenol. However, the transcriptional level of *aphR* was distinctly higher in strains P1 and PR921 than in strains TA441 and TR921. Transcription of the *aphR* gene was probably subjected to self-regulation because the promoter activity was higher in PR921 than in P1. When the cells were grown with pHB, a temporary decline of the transcriptional activity was observed during the exponential phase.

The *aph* genes are silenced in strain TA441

The low transcriptional level of *aphR* in strain TA441 suggested that inability of strain TA441 to grow on phenol was caused by a shortage of the AphR protein (Fig. 4). We examined the growth of the strains on phenol when the activator protein was overexpressed by pHAM931, which carries the *aphR* gene downstream of the *tac* promoter. Strain PR921, which was not able to grow on phenol, was able to grow on phenol following transformation with pHAM931, suggesting that the inability of strain PR921 to grow on phenol was caused by the absence of AphR. However, strains TA441 and TR921 were not able to grow on phenol even when the *aphR* gene was expressed under control of the *tac* promoter. A possible explanation for this result is that the expression of the genes for phenol degradation are usually repressed by the presence of an unknown repressor in the unadapted strains.

DISCUSSION

In this study, we found that *C. testosteroni* TA441 adapts to utilize phenol in a medium containing phenol as a major carbon source. An adapted strain P1 expressed the enzymes for the degradation of phenol via the *meta*-pathway. Strain TA441 has a catabolic gene cluster (*aph* genes) similar to the phenol- and dimethylphenol-degradation gene clusters encoding multicomponent phenol hydroxylase from *Pseudomonas* sp. CF600 (*dmp* genes; Nordlund *et al.*, 1990; Shingler *et al.*, 1992), *P. putida* P35X (*phh* genes; Ng *et al.*, 1994), *P. putida* BH (*phe* genes; Takeo *et al.*, 1995), *P. putida* H (*phl* genes; Herrmann *et al.*, 1995), and *A. calcoaceticus* NCIB 8250 (*mop* genes; Ehrt *et al.*, 1995). The *dmp* and *phl* genes are located on a plasmid (Herrmann *et al.*, 1995; Shingler *et al.*, 1989), whereas the *phh*, *phe* and *mop* genes are located on the chromosome (Ehrt *et al.*, 1995; Ng *et al.*, 1994; Takeo *et al.*, 1995). The *aph* genes are probably located on the chromosome because attempts to isolate an *aph*-gene-containing plasmid from strain TA441 failed (data not shown).

When the genes for PH (*aphKLMNOP*) and C23O (*aphB*) from strain TA441 were expressed by an expression vector, strain TA441 produced active enzymes (Table 4) and was able to grow on phenol (data not shown), indicating that the inability of strain TA441 to grow on phenol is not simply caused by a defect in the catabolic genes. The adaptation must be a consequence of some change in the regulatory system because a significant induction of *aphK*-promoter activity in response to phenol was observed in the adapted strain P1. Transcriptional control of the phenol-degradation genes is mediated by a divergently encoded σ^{54}-dependent positive regulator of the NtrC family in *Pseudomonas* sp. CF600 (*dmpR*; Shingler *et al.*, 1993). Corresponding genes were also found from *P. putida* P35X (*phhR*; Ng *et al.*, 1995), *P. putida* BH (*pheR*; DDBJ accession number D63814), *P. putida* H (*phlR*; Müller *et al.*, 1996), and *A. calcoaceticus* NCIB8250 (*mopR*; Schirmer *et al.*, 1997). A similar regulatory gene (*aphR*) is also located divergently from the *aphK* gene in strain TA441 (Fig. 2, Table 3). A putative −12 and −24 sequence for σ^{54} was found in the promoter region (data not shown). AphR was shown to be a phenol-sensing positive regulator for the *aphK* operon, because strain PR921, an *aphR* mutant of strain P1, does not grow on phenol and the phenol-induced *aphK*-promoter activity was not observed in the *aphR*-deficient strain (Fig. 3). The nucleotide sequence of the *aphR* gene from strain P1 was completely identical to that of strain TA441. Also no difference was found in the intergenic promoter region between *aphK* and *aphR*. The high-level expression of *aphR* in strain P1 was thought to be the reason why the *aphK* promoter was highly induced by phenol in strain P1 (Figs 3 and 4). However, strain TA441 could not grow on phenol after transformation with the recombinant *aphR* gene, whilst strain PR921 could. These results suggested that the *aph* genes were silenced by a negatively acting factor in strain TA441.

In previous studies on adaptation to utilize phenol using recombinant strains, mutations were found in the promoter region which resulted in constitutively active σ^{70}-dependent promoters (Burchhardt *et al.*, 1997; Kasak *et al.*, 1997; Nurk *et al.*, 1993). The adaptation mechanism of strain TA441 was quite different because a mutation was not found in the promoter region. Ghadi & Sangodkar (1994) reported that *Pseudomonas cepacia* AC1100, which utilizes 2,4,5-trichlorophenoxyacetic acid, adapts to utilize phenol under selective pressure. The adapted strain expressed enzymes for the *meta*-cleavage pathway. The authors suggested that the genes for phenol degradation were silent in strain AC1100 and that the activation of the silenced genes was due to the translocation of insertion elements. This phenomenon seems to be similar to the case of strain TA441. However, enzymes for phenol degradation were constitutively expressed in the adapted strain of AC1100, whereas the expression of PH and C23O was inducible by phenol in strain P1 (Table 2). No conspicuous genetic rearrangement was found around the *aph* genes for phenol degradation (data not shown).

Bacterial adaptation to a new substrate is sometimes achieved by broadened substrate specificity or overexpression of a pre-existing system for degradation of other substrates (van der Meer *et al.*, 1992). The low homology of the *aph* gene products to other phenol-degrading systems (Table 3) implies that the original role of the *aph* genes was the degradation of compounds other than phenol. However, the *aphK*-promoter activity induced by other compounds, such as methylated or chlorinated phenols or toluene, was not higher than that by phenol (data not shown). A search for the original substrates for the *aph* genes is in progress.

Most studies of bacterial adaptation so far reported have used genetically engineered strains. It should be noted that strain TA441 is not genetically engineered and we did not use phenol during its isolation. Therefore, the changes which occurred in strain TA441 must

reflect naturally occurring events. Identification of the molecular mechanism of the adaptation of strain TA441 to phenol will provide information regarding bacterial evolution or adaptation to utilize novel compounds.

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