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Characterization of the *Bacillus stearothermophilus* BR219 Phenol Hydroxylase Gene

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The catabolic genes *pheA* and *pheB*, coding for the conversion of phenol to catechol and catechol to 2-hydroxymuconic semialdehyde, respectively, have been cloned from *Bacillus stearothermophilus* BR219 into *Escherichia coli*. Following its localization on the 11-kb *B. stearothermophilus* DNA insert by deletion and expression analysis, the phenol hydroxylase gene *pheA* was subcloned as a 2-kb *Hin*dIII fragment, whose transformant expressed the enzyme after phenol induction and even more strongly after *o*-, *m*-, and *p*-cresol induction. In vitro transcription-translation experiments indicated that the phenol hydroxylase and catechol 2,3-dioxygenase enzymes are constituted of single subunits with molecular weights of 44,000 and 33,000, respectively. Nucleotide sequencing of the *pheA* gene revealed a strong similarity to flavin hydroxylases from *Rhodococcus* and *Streptomyces* species. Hybridization experiments indicated that the fragment containing PheA and PheB is located on a 66-kb plasmid in the parental thermophile.

Aerobic thermophilic bacteria have remained a subject of research attention as a result of interest in their evolution, survival mechanisms, and potential for biotechnological utilization (for a review, see reference 2). Although the metabolic versatility of the ubiquitous thermophile *Bacillus stearothermophilus* is well recognized (23), little is known about the degradation of xenobiotics and other environmental contaminants by this organism. Degradation of phenol by *B. stearothermophilus* was first reported by Buswell and Twomey (5) and has subsequently been examined in our laboratory (9) and by others (1, 7). More recently, we have isolated a *B. stearothermophilus* strain with the ability to degrade a variety of aromatics including toluene and benzene (15), indicating the broad capability of this species to perform aromatic degradation. To compare the aromatic pathways of gram-positive thermophiles with those of the well-studied gram-negative mesophiles, we have initiated a more detailed study of the phenol *meta* pathway of *B. stearothermophilus* BR219 (9). In this communication, we report the cloning of *pheA* and *pheB*, coding for the first two steps of phenol degradation in *B. stearothermophilus* BR219, and some properties of the *pheA*-encoded phenol hydroxylase.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. stearothermophilus* BR219, an isolate obtained from contaminated river sediment, was maintained at 55°C in DP minimal medium containing 5 mM phenol as the major carbon source as previously described (9). *Escherichia coli* XL-1 [*recA* (*recAI lac endAI gyrA96 thi hsdR17 supE44 relA1* {F′ *proAB*, *lacI*$_q$, *lacZI*Δ*M15*, Tn*10*})] (4), used for construction and maintenance of plasmids, was cultured at 37°C on LB medium. Plasmids were introduced into *E. coli* XL-1 by electroporation (8). Ampicillin at 50 μg/ml was used for selection of plasmids. Tetracycline at 12.5 μg/ml was also incorporated during growth of the *E. coli* XL-1 host as a precaution against contamination.

Genetic procedures. Plasmid DNA was isolated from *E. coli* by alkali lysis (3). *B. stearothermophilus* BR219 DNA was prepared by the method of Saito and Miura (20). DNA fragments were isolated from agarose gels by using USBioclean (United States Biochemical Corp., Cleveland, Ohio) and electroelution (IBI, New Haven, Conn.). Restriction enzymes, DNA ligase, and alkaline phosphatase were obtained from Boehringer Mannheim Co., Indianapolis, Ind. Southern hybridizations were performed as described by Maniatis et al. (14) with Amersham H-bond nylon membrane probed with DNA labelled with [α-^{32}P]dCTP, using a Boeringer-Mannheim random-primer labeling kit. Following hybridization, blots were washed at high stringency (43°C in 0.1× SSC–0.1% sodium dodecyl sulfate [SDS]–50% formamide, where 1× SSC is 0.15 M NaCl with 0.015 M sodium citrate).

Screening for transformants carrying *pheA* and *pheB*. Transformants were spread on Luria-Bertani (LB) plates containing 1 mM phenol, 50 μg of ampicillin per ml, and 12.5 μg of tetracycline per ml and incubated overnight at 37°C. Colonies that became yellow (as a result of 2-hydroxymuconic semialdehyde) following spraying of a 0.1% of catechol solution were patched on LB plates containing 1 mM phenol. Colonies in which the yellow compound formed during growth without catechol addition were retained as putative carriers of both *pheA* and *pheB*.

Preparation of cell extracts and enzyme assays. Cells for enzyme analysis were grown in LB broth with 1 mM phenol and 50 μg of ampicillin per ml at 37°C to mid-log phase, reinoculated into fresh medium of the same composition, and grown to late-exponential phase. The cells were harvested by centrifugation at 6,000 × *g* for 10 min, washed twice in 50 mM sodium phosphate buffer (pH 7.5), disrupted sonically by six 30-s bursts with an ultrasonic homogenizer (Cole-Palmer Instrument Co., Chicago, Ill.), and centrifuged at 12,000 × *g* for 30 min. The clear supernatant was used as a crude enzyme extract. Phenol hydroxylase was assayed in the supernatant as described by Gurujeyalakshmi and Oriel (9).

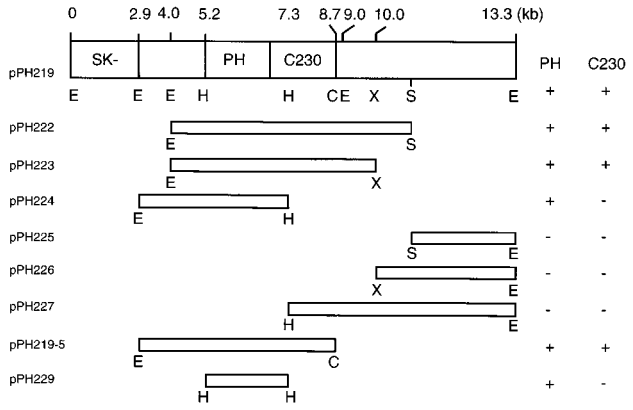

FIG. 1. Restriction enzyme map of plasmid pPH219 and its derivatives. The ability (+) or inability (−) of the plasmids to produce phenol hydroxylase (PH) and/or catechol 2,3-dioxygenase (C23O) is indicated to the right of the restriction enzyme maps. Abbreviations: E, *Eco*RI; C, *Cla*I; H, *Hin*dIII; X, *Xho*I; S, *Sal*I. Approximate positions of the phenol hydroxylase and catechol 2,3-dioxygenase genes deduced from this data are shown.

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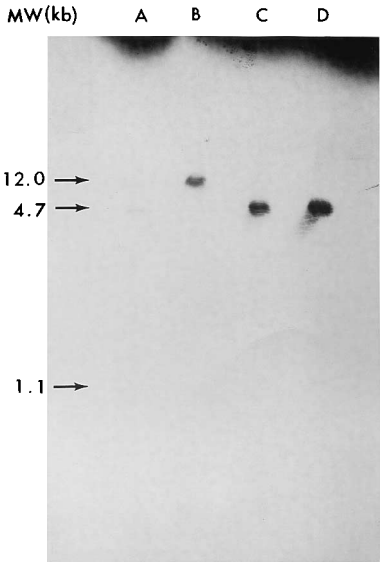

FIG. 2. Southern blot analysis. Genomic DNA and the cryptic plasmid pGGO1 were hybridized with the cloned DNA containing *pheA* and *pheB* which was labeled with [α-^{32}P]dCTP. Lanes: A, *Eco*RI-digested BR219 genomic DNA; B and C, *Bam*HI- and *Eco*RI-digested plasmid pGGO1, respectively; D, *Eco*RI-digested pPH219.

Catechol 2,3-dioxygenase was assayed as described by Nozaki (17). Protein was measured by the method of Lowry et al. (13) with bovine serum albumin for standardization. One unit of enzyme activity is defined as the amount causing the disappearance of 1 μmol of phenol per min at 55°C for phenol hydroxylase or the appearance of 1 μmol of 2-hydroxymuconic semialdehyde per min at ambient temperature for catechol 2,3-dioxygenase.

Nucleotide sequence determinations. Plasmid vector pBluescript SK− (Stratagene, La Jolla, Calif.) was used to construct the subclones for DNA sequencing. Serial deletion of subclones was made by using the exo/mung system (Stratagene). Nucleotide sequences were determined directly from plasmids by the dideoxy-chain termination method (21) with T7 DNA polymerase (Sequenase; United States Biochemical Corp.). The dITP was substituted for dGTP to eliminate band compression in GC-rich regions. Wedge gels 0.2 to 0.6 mm thick were used in electrophoresis to increase resolution. Other sequencing procedures were performed by published methods (14). Nucleotide and deduced amino acid sequences were analyzed by the GCG DNA analysis program (Genetics Computer Group, Inc., Madison, Wis.)

In vitro transcription-translation and activity staining of the SDS-polyacrylamide gel. The protein products of cloned *B. stearothermophilus* DNA inserts were identified with a DNA-directed transcription-translation system involving *E. coli* cell extracts (Amersham Life Sciences, Arlington Heights, Ill.). Proteins produced from plasmids (1 μg per reaction) were radioactively labeled with L-[^{35}S]methionine in 50-min incubation periods and a 30-min (cold) methionine chase reaction. Samples were separated on SDS–10% polyacrylamide gels, and protein bands were observed by radioautography. The catechol 2,3-dioxygenase band was located by formation of yellow 2-hydroxymuconic semialdehyde formed after gel immersion in 0.1% catechol. Prestained protein molecular size markers (GIBCO BRL, Life Technologies, Inc., Gaithersburg, Md.) were used.

Nucleotide sequence accession number. The GenBank accession number of *pheA* is U17960.

RESULTS

Cloning and expression of *pheA* and *pheB*. Mixed plasmid and chromosomal DNA was extracted from *B. stearothermophilus* BR219 and partially cleaved with *Eco*RI, and fragments ranging from 9 to 20 kb were obtained by gel electrophoresis and electroelution for ligation with pBluescriptII SK−. Transformants of *E. coli* XL-1 were grown on LB plates containing ampicillin and phenol, and those demonstrating 2-hydroxymuconic semialdehyde formation after being sprayed with 0.1% catechol were retained as putative transformants carrying the *pheB* gene. Subsequent examination of these transformants yielded EC390, a recombinant which did not grow on phenol but demonstrated production of 2-hydroxymuconic semialdehyde on LB plates containing 1 mM phenol, suggesting the presence of both *pheA* and *pheB* encoding the first two steps of the phenol pathway. The plasmid isolated from this recombinant, designated pPH219, contained a 10.3-kb *Eco*RI insert. To locate *pheA* and *pheB* within this insert, measurements of phenol hydroxylase and catechol 2,3-dioxygenase in transformants with insert deletions were carried out by using determined restriction sites. As shown in Fig. 1, both *pheA* and *pheB* were located in the 6-kb *Eco*RI-*Cla*I DNA

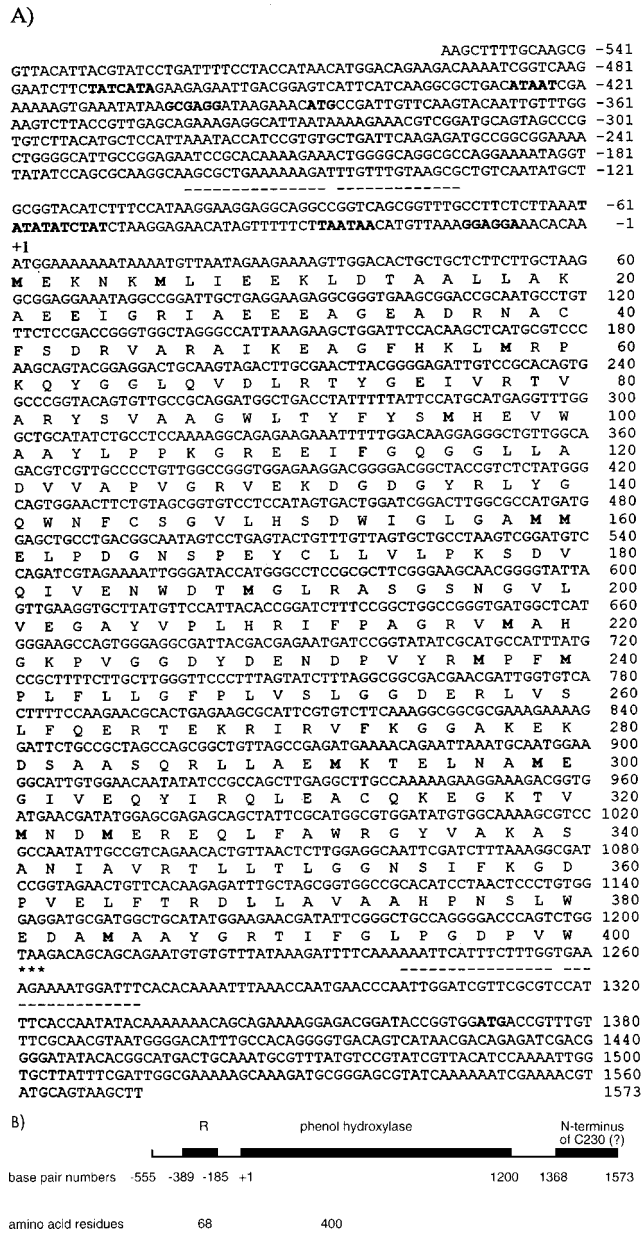

FIG. 3. (A) Nucleotide and deduced amino acid sequence of the phenol hydroxylase gene. The nucleotide sequence was numbered from the first nucleotide for translation initiation. A putative promoter (−35 and −10 region) and a probable Shine-Dalgarno sequence are indicated by boldface type. The inverted repeat sequence upstream and downstream of the coding region are marked with a dashed line. (B) Structural analysis of the 2.1-kb *Hin*dIII fragment. R designates an ORF of unknown function. C23O, catechol 2,3-dioxygenase.

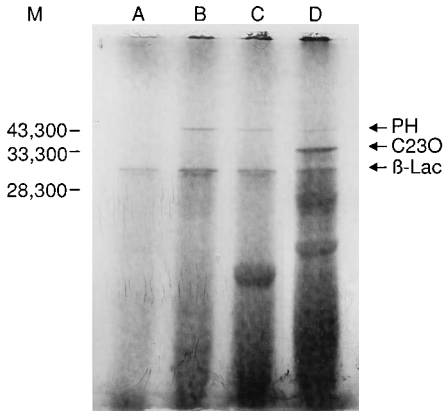

FIG. 4. Proteins synthesized by in vitro transcription-translation. Lanes: M, molecular weight markers; A, pBluescript SK− vector; B, pPH229; C, pPH224; D, pPH223. The upper, middle, and lower arrows point to the phenol hydroxylase (PH), catechol 2,3-dioxygenase (C23O), and β-lactamase (β-Lac), respectively.

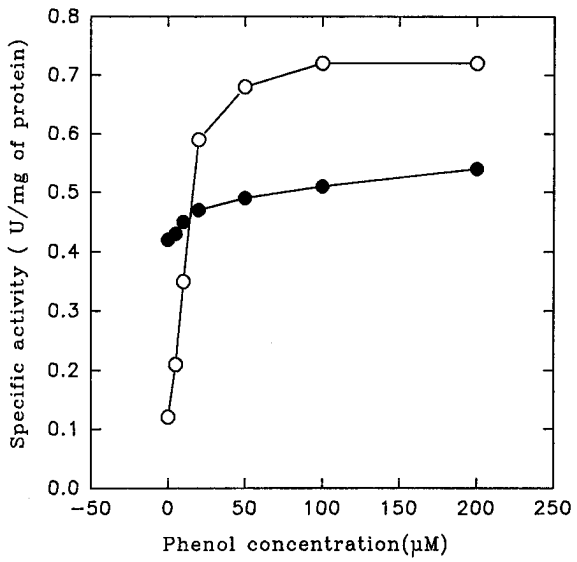

FIG. 5. Effect of phenol concentration on phenol hydroxylase induction. ○, phenol hydroxylase produced by *E. coli* containing pPH229; ●, phenol hydroxylase produced by *E. coli* containing pPH219-5.

fragment cloned in plasmid pPH219-5, and *pheA* was found in the 2.1-kb *Hin*dIII DNA fragment cloned in plasmid pPH229.

Localization of *pheA* and *pheB* in *B. stearothermophilus* BR219. *B. stearothermophilus* BR219 carries a 66-kb low-copy-number plasmid designated pGGO1 (10). With the 10.3-kb *Eco*RI insert of plasmid pPH219 as a probe, Southern hybridization analysis was carried out with *Eco*RI and *Bam*HI-digested BR219 genomic and plasmid DNA. The cloned insert carrying *pheA* and *pheB* hybridized with plasmid but not chromosomal DNA (Fig. 2), indicating that plasmid pGGO1 encodes at least part of the phenol catabolic pathway.

Structural analysis of the phenol hydroxylase gene and gene product. To determine the structure of the phenol hydroxylase gene, nucleotide sequencing of the 2.1-kb *Hin*dIII fragment (pPH229) was carried out. As shown in Fig. 3A, one open reading frame (ORF) was found encompassing 1,200 nucleotides encoding a protein of 400 amino acids. The insert also contains about 556 and 373 bp of 5′- and 3′-flanking sequences, respectively. Putative promoter regions, TATATATCTAT and TAATAA, are present from −61 to −51 and from −29 to −24, respectively, from the translational initiation site. A putative Shine-Dalgarno sequence, GGAGAA, was present at the −13 position. The sequence revealed two small additional open reading frames (Fig. 3B). The first ORF is upstream of *pheA*, starting at −389 and ending at −185, with putative promoter regions from −472 to −466 (TATCATA) and from −428 to −423 (ATAAT) and Shine-Dalgarno sequence (−414 to −409 [GCGAGG]). The function of this short ORF, which is designated R, is not known. Another ORF starts downstream of *pheA* at 1368 without termination. Since the deletion experiments indicated that *pheB* was in proximity downstream of *pheA*, it is possible that this is the N terminus of the catechol 2,3-dioxygenase gene.

Molecular mass of phenol hydroxylase and catechol 2,3-dioxygenase. Transcription-translation results (Fig. 4) indicate a protein of 43,000 Da expressed from pPH229, which encodes the *pheA* gene. This value is in excellent agreement with the 43,000 Da deduced from *pheA* sequencing data. For pPH223, which encodes both *pheA* and *pheB*, an additional 33,000-Da band is seen. Activity staining by gel immersion in 0.1% catechol, yielding yellow 2-hydroxymuconic semialdehyde, was used for identification of this protein as the *pheB* transcription-translation product (data not shown). The lower-molecular-mass band in Fig. 4, lane C, may also be the product of *pheB*, which is truncated in pPH224. Other lower-molecular-mass bands expressed in lanes C and D by subclones containing DNA regions upstream and downstream, respectively, of the sequenced insert of pPH229 have not been identified.

Expression and inducibility of phenol hydroxylase and catechol 2,3-dioxygenase genes in *E. coli*. The phenol pathway in the BR219 thermophile parent is induced by phenol (9). To determine whether the cloned insert in pPH219 contained phenol regulatory elements from parental BR219, transformants containing this plasmid and those of the deletions shown

TABLE 1. Induction of phenol hydroxylase and catechol 2,3-dioxygenase in *E. coli*

Plasmid	Sp act of phenol hydroxylase (U/mg of protein)		Sp act of catechol 2,3-dioxygenase (U/mg of protein)	
	− phenol	+ phenol	− phenol	+ phenol
SK-1	0	0	0	0
pPH219-5	0.42	0.54	0.57	0.74
pPH222	0.88	1.07	0.59	0.84
pPH223	1.11	0.83	0.67	0.97
pPH224	0.87	0.9	0	0
pPH229	0.12	0.72	0	0

TABLE 2. Induction of phenol hydroxylase by aromatic compounds

Inducer	Sp act of phenol hydroxylase (U/mg of protein)
None	0.12
Phenol	0.54
Benzoate	0.11
Toluate	0.11
o-Cresol	1.48
m-Cresol	1.6
p-Cresol	1.46
2-Chlorophenol	0.42

(A)

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ActV.   GQDRLDPEGPGMSEDTMTQERPSLTAHARRIAELAGKRAADAEQQRRLSPDVVDAVLRAG  1339
PheA.          MEKNKMLIEEKLDTAALLAKAEEIGRIAEEEAGEADRNACFSDRVARAIKEAG    53
Pig.                    MDITRTELMDRVHALVPAFAERAQKTEENRAPLDETITDLIDSG    44

ActV.   FAAHFVPVAHGGRAATFGELVEPVAVLGEACASTAWYASLTASLGRM AAYLPDEGQAEL  1398
PheA.   FHKLMRPKQYGGLQVDLRTYGEIVRTVARYSVAAGWLTYFYSMHEVW-AAYLPPKGREEI   112
Pig.    ILATLTPKEYGGLELGLDVAADIVRTISAVCPSTGWVTSFY-IGAAWRVNIFTEQAQREV   103

ActV    WSDGPDALIVGALMPLGRAEKTPGGWHVSGTWPFVSVVDHSDW----ALICAKVGEEPWF  1454
PheA    FGQG--GLLADVVAPVGRVEKDGDGYRLYGQWNFCSGVLHSDWIGLGAMMELPDGNSPEY   170
Pig.    FADKPYTLTAGTAAPLGQVQKVDGGYRITGQTAWNSGSVHAEWFTFAGVV-FEEGSAPTP   162

ActV.   --FAVPRQEYGIVDSWYPMGMRGTGSNTLVLDGVFVPDARACTRAAIAAGLGP-----DA   507
PheA.   CLLVLPKSDVQIVENWDTNGLRASGSNGVLVEGAYVPLHRIFPAGRVMAHGKPVGGDYDE   230
Pig.    LWFLVPREDVKVLDTWYIAGMSGTGSNDISVDDVFVPEYRTGPFALALAGTAP--GQLIH   220

ActV.   EAICHTVPMRAVNGLAFALPMLGAARGAAAVWTSWTAGRLAGPTGQNAVSSQDRVVYEHT  1567
PheA.   NDPVYRMPFMPLFLLGFPLVSLGGDERLVSLFQERTEKRIRVFKG--GAKEKDSAASQRL   288
Pig.    PNPMYHLPFLPFAMAEVTPVVVGALRGAADAFVQRTKDRQGTI-S--QEKASGKQAAQMR   277

ActV.   LARATGEIDAAQLLLERVAAVADAGSATGVLVGRGARDCALAAELLTAAT            1617
PheA.   LAELKTELNAMEGIVEQYIRQLEACQKEGKTVMNDMEREQLFAWRGYVAK             338
Pig.    LGRALAAADAAETLLDAFFERLTAQRPEQS---DPRDRAEMKLKAAYLAD             324
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(B)

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                  *    *** *
PheA    [211] APVGRVEKDGDGYRLYGQWN-FCSGVLHSDWIGL [229]
PheA*   [318] LQKGRVCCAGDAIHKHPPSHGLGSNTSIQDSYNL [343]
TfdB    [300] LQQGRVFCAGDAVHRHPPTNGLGSNTSIQDSFNL [325]
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FIG. 6. (A) Comparison of the deduced amino acid sequence of phenol hydroxylase with sequences of the internal region of *actVA* (6) and pigment-producing enzyme (11). Symbols: |, amino acid residue identical to that found in phenol hydroxylase; :, amino acid residue similar to that found in phenol hydroxylase. (B) Comparison of a portion of the deduced sequence of PheA with sequences of other flavoprotein hydroxylases, PheA* (18) and TdfB (19). Symbols: |, amino acid residue identical to that found in phenol hydroxylase; *, amino acid residue conserved in various phenol hydroxylases (19).

in Fig. 1 were tested for differences in phenol hydroxylase expression in cells grown at different phenol concentrations. As shown in Table 1, in *E. coli* bearing plasmid pPH219 or most of the deletions, phenol hydroxylase and catechol 2,3-dioxygenase specific activities were either unaltered or only slightly increased by growth in the presence of phenol. In pPH229, however, phenol hydroxylase expression demonstrated marked dependence on phenol. A comparison of differences in phenol hydroxylase induction for pPH229 and pPH219-5 with phenol concentration is presented in Fig. 5, which shows that pPH229 demonstrates marked phenol dependence at concentrations up to 20 μM. The level of induction of phenol hydroxylase in this pPH229 by other aromatics is shown in Table 2, which shows that enzyme levels induced by *o*-, *m*-, and *p*-cresol were even higher than that induced by phenol.

DISCUSSION

The phenol *meta* pathway of *B. stearothermophilus* BR219 is carried at least in part on the resident low-copy-number plasmid pGGO1. Although plasmid-borne *meta* aromatic pathways are commonly found in gram-negative organisms, a recent survey contains only two previous reports of catabolic plasmids in the less-well-studied gram-positive bacteria (22).

Four bacterial phenol hydroxylases, all from gram-negative bacteria, have been described previously (12, 16, 18, 19). Three are similar, being made up of a single subunit of approximately 70,000 Da and bearing a flavin adenine dinucleotide prosthetic group (12, 18, 19). The fourth is a complex of six subunits, resembling certain aromatic dioxygenases (16). The phenol hydroxylase of *B. stearothermophilus* BR219 does not resemble any of these, being significantly smaller and demonstrating an NADH rather than NADPH cofactor requirement (9). Sequence comparison of BR219 *pheA* with other GenBank protein sequences revealed no extensive homology with the above phenol hydroxylases but indicated a strong resemblance to *actV*, an antibiotic hydroxylase in *Streptomyces coelicolor* (6), and a *Rhodococcus* hydroxylase of unknown function which is capable of producing indigo and indirubin pigments from in-

dole (11) (Fig. 6A). The BR219 phenol hydroxylase is also able to catalyze formation of indigo and indirubin pigments (11a). The resemblance to hydroxylases with different functions from other gram-positive bacteria and the lack of resemblance to phenol hydroxylases of gram-negative origin suggest that either the BR219 phenol hydroxylase gene arose independently of those of gram-negative organisms or that the divergence occurred at an early time. Although identification of the prosthetic group of the BR219 phenol hydroxylase must await purification and biochemical characterization, it may not be coincidental that the BR219 phenol hydroxylase gene possesses a sequence resembling those retained in gram-negative phenol hydroxylases for flavin adenine dinucleotide binding (12) (Fig. 6B).

Expression of phenol hydroxylase in the recombinant bearing pPH229 is induced by phenol and even more strongly by cresols. Since the BR219 phenol hydroxylase is also active on cresols (11a), we speculate that degradation of cresols in the environment may be an important function of the pathway. The lack of phenol hydroxylase induction in transformants pPH219-5, pPH222, pPH223, and pPH224, which contain the catechol 2,3-dioxygenase gene and/or DNA segments upstream of the *pheA* gene, is not yet understood but suggests a complex regulatory mechanism which may involve the small ORF designated R adjacent to *pheA*. The details of phenol pathway regulation in these recombinants should prove of interest and will be the subject of further investigation.

ACKNOWLEDGMENTS

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